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Myasthenia Gravis and the Tops and Bottoms of AChRs Antigenic Structure of the MIR and Specific Immunosuppression of EAMG Using AChR Cytoplasmic Domains

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Abstract

The main immunogenic region (MIR), against which half or more of the autoantibodies to acetylcholine receptors (AChRs) in myasthenia gravis (MG) or experimental autoimmune MG (EAMG) are directed, is located at the extracellular end of $\alpha 1$ subunits. Rat monoclonal antibodies (mAbs) to the MIR efficiently compete with MG patient autoantibodies for binding to human muscle AChRs. Antibodies bound to the MIR do not interfere with cholinergic ligand binding or AChR function, but target complement and trigger antigenic modulation. Rat mAbs to the MIR also bind to human ganglionic AChR $\alpha 3$ subunits, but MG patient antibodies do not. By making chimeras of $\alpha 1$ subunits with $\alpha 7$ subunits or ACh binding protein, the structure of the MIR and its functional effects are being investigated. Many mAbs to the MIR bind only to the native conformation of $\alpha 1$ subunits because they bind to sequences that are adjacent only in the native structure. The MIR epitopes recognized by these mAbs are not recognized by most patient antibodies whose epitopes must be nearby. The presence of the MIR epitopes in $\alpha 1/\alpha 7$ chimeras greatly promotes AChR expression and sensitivity to activation. EAMG can be suppressed by treatment with denatured, bacterially expressed mixtures of extracellular and cytoplasmic domains of human $\alpha 1$, $\beta 1$, γ , δ , and ϵ subunits. A mixture of only the cytoplasmic domains not only avoids the potential liability of provoking formation antibodies to pathologically significant epitopes on the extracellular surface, but also potently suppresses the development of EAMG.

Keywords

nicotinic acetylcholine receptor; AChR; myasthenia gravis; MG; antigenic structure; main immunogenic region; specific immunosuppressive therapy; experimental autoimmune myasthenia gravis; EAMG

Introduction

AChR Structure and Function

Nicotinic acetylcholine receptors (AChRs) are ACh-gated cation channels, which are part of a superfamily of neurotransmitter receptors including GABA_A, glycine, and 5HT₃ receptors.

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Conflicts of Interest

The authors declare no conflicts of interest.

¹ The postsynaptic AChRs of skeletal muscle and fish electric organs were first to be characterized.^{2,3} Subsequently, neuronal AChRs, many of which are presynaptic, were characterized.²⁻⁴ Recently AChRs have been found in many non-neuronal tissues.^{5,6}

Antibody-mediated autoimmune responses to $\alpha 1$ muscle AChRs cause myasthenia gravis (MG),^{3,7} responses to $\alpha 3$ ganglionic AChRs cause autoimmune autonomic neuropathy,⁸ and responses to $\alpha 9$ AChRs in skin contribute to pemphigus.⁹ Mutations in muscle AChR subunits cause forms of congenital myasthenia,¹⁰ and mutations in neuronal $\alpha 4\beta 2$ AChRs cause autosomal-dominant, nocturnal, frontal-lobe epilepsy.¹¹ By far the most prevalent disease associated with AChRs is addiction to tobacco, which is mediated by nicotine acting on $\alpha 4\beta 2$ and other brain AChR subtypes.¹²

AChR subtypes are defined by their subunit composition and stoichiometry. In each AChR there are five homologous subunits oriented like barrel staves around the ion channel.¹⁻⁴

Fetal muscle AChR subunits have the subunit order $\alpha 1\gamma\alpha 1\delta\beta 1$, whereas adult muscle AChR subunits are ordered $\alpha 1\epsilon\alpha 1\delta\beta 1$. ACh binding sites are formed at the interfaces of $\alpha 1$ with γ , δ , or ϵ subunits. $\beta 1$ is an accessory subunit that does not participate in forming ACh binding sites but contributes to the channel and the conformational changes associated with activation and desensitization.

Primordial proton-gated cation channels which are structurally related to AChRs have been found in bacteria.¹³ These have five identical subunits, but lack the signature disulfide-linked loop found in the AChR superfamily. They also lack a large cytoplasmic domain.

$\alpha 7$ AChRs represent the archetypical AChR.¹⁻⁴ These are composed of five identical $\alpha 7$ subunits and contain five ACh binding sites. Cobra and krait toxins are competitive antagonists of both $\alpha 1$ and $\alpha 7$ AChRs.

$\alpha 4\beta 2^*$ AChRs are the predominant subtype with high affinity for nicotine.¹⁻⁴ At least in cell culture, these are found in a sensitive stoichiometry with $\beta 2$ accessory subunits ($\alpha 4\beta 2\alpha 4\beta 2\beta 2$) and a less sensitive stoichiometry with $\alpha 4$ accessory subunits ($\alpha 4\beta 2\alpha 4\beta 2\alpha 4$).¹⁴⁻¹⁶ In brain about 20% of $\alpha 4\beta 2$ AChRs have $\alpha 5$ as an accessory subunit, and $\beta 3$ can also assemble as an accessory subunit, at least in transfected systems.^{4,16}

Autonomic ganglia postsynaptic AChRs contain $\alpha 3$ subunits, usually as $(\alpha 3)_2(\beta 4)_3$ AChRs, but also as $(\alpha 3)_2(\beta 4)_2\alpha 5$, $(\alpha 3)_2(\beta 2)_3$, $(\alpha 3)_2(\beta 2)_2\alpha 5$, and perhaps also $(\alpha 3)_2\beta 2\beta 4\alpha 5$ AChRs.²⁻⁴

Minor AChR subtypes are also important. $\alpha 6$ AChRs are found primarily in aminergic neurons.^{2-4,17} $\alpha 6$ AChRs usually contain $\beta 3$ as an accessory subunit. In addition to combining with $\beta 2$ or $\beta 4$ subunits, $\alpha 6$ can combine with $\alpha 4$ to form $\alpha 6\beta 2\alpha 4\beta 2\beta 3$ AChRs.⁴ $\alpha 9$ can form functional homomeric AChRs, but is usually found in combination with $\alpha 10$ in auditory hair cells.¹⁸ $\alpha 8$ has been found only in chickens as a homomer or in combination with $\alpha 7$.¹⁹

The overall shape and dimensions of muscle-type AChRs have been determined through electron microscopic studies of two-dimensional crystalline arrays of *Torpedo* electric organ AChRs.²⁰ Viewed from the side, a *Torpedo* AChR is about 14 nm long and 8 nm wide, with 6.5 nm on the extracellular surface, 4 nm across the lipid bilayer, and 3.5 nm below. Viewed from the top, the extracellular vestibule is a pentagonal tube with walls 2.5 nm thick and a 2 nm diameter central pore. At the bottom, the cytoplasmic domain is not well visualized because much of this structure is rather flexible, unlike the more rigid structure of the extracellular domain.

High-resolution structure of the extracellular domain showing the detailed organization of the polypeptide chains and the structure of the ACh binding site was first revealed by X-ray crystallography of ACh binding proteins (AChBP) secreted by mollusk glia.²¹ These are homopentamers with substantial homology to the extracellular domain of $\alpha 7$ AChRs. When appropriately linked to the transmembrane portion of 5HT₃ receptor subunits, AChBP forms functional AChRs.²²

The ACh binding sites at subunit interfaces are about half way up the extracellular domain and are accessible from the outer surface.²¹ In the resting state the C loop (residues $\alpha 1$ 174–209) is in an open position providing access to the ACh binding site. Binding of small agonists like ACh or nicotine causes closing of the C loop.²³ Presumably this reflects the initial conformation change initiated by agonists, which is propagated through the AChR to cause the opening of a gate located at the middle or cytoplasmic end of the channel. Binding of large antagonists like α bungarotoxin (which is used in iodinated form to label solubilized AChRs to provide an antigen for immunodiagnostic assays for MG) acts like a foot in the door to prevent closing of the C loop and activation of the AChR.²⁴

The sequences of all AChR subunits share several features. There is an N-terminal extracellular domain of about 210 amino acids.^{1–4} The N-terminal 13 amino acids of AChBP form an α helix, but most of the remainder of the extracellular domain has β structure.²¹ In molecular dynamic simulations of $\alpha 7$ AChR molecular motions, the N-terminal α helix and adjacent main immunogenic region (MIR) region (see below) located at the extracellular top of the subunit are as mobile as the tip of the C loop, by contrast with the majority of the β strand-based structure of the extracellular domain.²⁵ A disulfide-linked loop (in $\alpha 1$ between C128 and C142) with a highly conserved sequence is found in all subunits of this receptor superfamily.¹ In most subunits it contains an N-glycosylation site. This loop is at the base of the extracellular domain, where it may form part of a fulcrum interacting with the loop between the M2 and M3 transmembrane domains through which conformation changes are transmitted from the ACh binding site to the channel gate.¹ The C loop, which closes on agonist binding, is formed from β strands 9 and 10 ($\alpha 1$ 174–209).^{21,24} α subunits are identified by the presence of an adjacent pair of disulfide-linked cysteines at the tip of the C loop ($\alpha 1$ 192, 193). These were the target of an affinity label by which the first AChR $\alpha 1$ subunit was initially identified.²⁶ Three α helical transmembrane domains, M1–M3 ($\alpha 1$ 210–299), link the extracellular domain to the beginning of a large cytoplasmic domain ($\alpha 1$ 300–401) which extends to a fourth transmembrane domain, M4 ($\alpha 1$ 402–420), which leads to a short extracellular C-terminal domain ($\alpha 1$ 421–430).^{1–4} ²⁰ The large cytoplasmic domain at the bottom of the subunit is the most variable in sequence between AChR subunits.² The size of the cytoplasmic domain is usually underrepresented in crystallographic images, and only the most rigid parts of this domain are visualized because much of the large cytoplasmic domain is flexible.²⁰ This domain of muscle AChRs interacts with rapsyn, a protein that links muscle AChRs to the cytoskeleton and is critical for forming the complex architecture of the muscle postsynaptic membrane.²⁷ Rigid, amphipathic α helices that immediately precede M4 form intracellular portals that regulate access of ions to the central cation channel.^{20,28} The cytoplasmic domain contains phosphorylation sites involved in regulating desensitization and sequences involved in intracellular transport.^{29,30}

Antigenic Structure

Immunization with native muscle-type AChRs from electric organs provokes formation of antibodies primarily directed at the extracellular surface.^{31,32} About half of the antibodies to native muscle-type AChRs are directed at the MIR at the extracellular top of $\alpha 1$ subunits.^{2,3,33–35} A similarly high proportion of autoantibodies to AChR in sera from humans or dogs with MG or rats with experimental autoimmune MG (EAMG) are directed at the MIR.^{7,34,36} The MIR is defined by the ability of a rat EAMG monoclonal antibody (mAb), such as mAb 35 to

inhibit the binding to native muscle AChR of other mAbs or serum autoantibodies.³⁴ mAb 35 depends for its ability to bind on the native conformation of the $\alpha 1$ subunit. Denaturation destroys the native conformation of the MIR. Immunization with denatured muscle-type AChRs (in which the MIR conformation has been destroyed) by default predominantly makes antibodies to the cytoplasmic (bottom) surface of AChRs.³² The high variability in sequence between subunits and between species of the large cytoplasmic domain may account for its high immunogenicity, and the normally flexible structure of this region may account for the ease with which it is recognized by antibodies to denatured subunits and synthetic peptides.^{2, 20}

Pathologically significant autoantibodies must be directed at the extracellular surface of the AChR, where they can bind *in vivo*, whereas autoantibodies to the cytoplasmic domain must be pathologically insignificant because they cannot bind *in vivo*. In MG and chronic EAMG, autoantibodies bound to muscle AChRs target the postsynaptic membrane for binding of complement, which results in focal lysis that reduces the number of AChRs and disrupts the architecture of the postsynaptic membrane and its normal position next to active zones of ACh release from the presynaptic membrane.⁷ In the acute phase of active EAMG or EAMG passively transmitted with serum, binding of antibody and complement targets the postsynaptic membrane for attack by macrophages.⁷ Neuromuscular transmission is further impaired by loss of AChRs through antigenic modulation, meaning that cross-linking of AChRs by autoantibodies increases the rate of AChR internalization and destruction in lysosomes.⁷ mAbs to the MIR exhibit both of these pathological activities. mAbs to the MIR efficiently passively transfer the acute form of EAMG and cause antigenic modulation of AChRs on cells in culture.⁷ Bivalent Fab fragments of mAb 35, which can cross-link adjacent AChRs but cannot fix complement, can also passively transfer EAMG, showing that antigenic modulation alone can cause acute EAMG, though less potently than with the effects of complement.³⁷ Immunization with purified denatured *Torpedo* AChR $\alpha 1$, $\beta 1$, γ , or δ subunits can cause EAMG, but is extremely inefficient compared to native AChR.³⁸ Purified $\alpha 1$ is the most potent, as might be expected because its sequence is the most conserved, because there are two $\alpha 1$ in an AChR which permits cross-linking by antibodies, and because renaturation might provoke antibodies to the MIR.

Sequences contributing to the MIR have been identified in various ways. Mutation of $\alpha 1$ amino acids 68 or 71 prevented binding of all MIR mAbs tested.³⁹ Some mAbs to the MIR react detectably with denatured $\alpha 1$ or synthetic $\alpha 1$ peptides. Using synthetic peptides and these mAbs, sequences contributing to the MIR were mapped to $\alpha 1$ 66–76.⁴⁰ From the original discovery of the MIR, it was always evident that the MIR was a region rather than a single epitope because a variety of mutually competitive mAbs mapped to the MIR, which differed in their species-specificity and dependence on the native conformation of the AChR.^{33,40} Studies of the crystal structures of antibodies bound to proteins revealed that although one or two amino acids in an epitope could have a large influence on the affinity of antibody binding, a bound antibody obscured a large area of the antigen covering many amino acids.⁴¹ The crystal structures of two Fab fragments of rat mAbs to human AChRs have been determined.^{42,43} Both bind to the MIR. Their antigen binding sites are large and morphologically different. Both compete for binding to human AChR, but one does not bind to electric organ AChR. Thus antibodies to AChR which recognize distinct closely spaced epitopes can compete for binding to the AChR.

Visualization of binding of mAb 35 to two-dimensional crystalline arrays of electric organ AChRs revealed that mAbs bound to the tip of the extracellular end of AChRs angled out from the central axis of the AChR.⁴⁴ This was consistent with prior data showing that the MIR was oriented away from the axis of the AChR so that a single mAb could not cross-link the two $\alpha 1$ subunits within an AChR, but that mAbs to the MIR were very efficient at cross-linking

adjacent AChRs.⁴⁵ The crystal structure of the AChBP revealed that the sequence homologous to $\alpha 1$ 66–76, though different enough that it did not bind mAbs to the MIR, was a loop located at the extracellular top of the subunits and oriented away from the central axis of the AChBP, suggesting that if an antibody were directed at the tip of the loop it could not bind to another such loop of another subunit on the opposite side of the AChBP.²¹ Thus the putative MIR loop on the AChBP looked just as expected. A molecular model of the crystal structure of Fab 198 docked on the MIR of the structure of *Torpedo* AChR has been prepared.⁴⁶

The pathological significance of the MIR is explained by these structural features.⁷ The MIR is at the extracellular top of the AChR, where it is easily accessible to autoantibodies and complement. There are two MIRs in an $(\alpha 1)_2\beta 1\gamma\delta$ AChR, and they are oriented to promote cross-linking and antigenic modulation of AChRs by antibodies to the MIR. The immunogenicity of the MIR might result from its prominent location and multivalence, which might allow native AChRs to be especially effective at binding to receptor immunoglobulins on the surface of pre-B cell lymphocytes. The lack of block of function by mAbs bound to the MIR was explained by the large distances between the top of the AChR $\alpha 1$ subunits, where the mAb binds, and the sides, where the ACh binding sites were located. This separation also allowed antibodies to the MIR, or the cytoplasmic domain, and probably other areas on the extracellular domain, to bind without preventing binding of α bungarotoxin to the ACh binding site. This allowed ¹²⁵I α bungarotoxin-labeled AChR to efficiently function as antigen for immunodiagnostic assays for MG.⁴⁷

Despite these structural insights to AChRs and MG pathology, many questions remained. What produced the dependence for binding of most MIR mAbs on the native conformation of $\alpha 1$ subunits? Do rats with EAMG and humans and dogs with MG recognize the same epitopes within the MIR? What are the structures of these epitopes? At X-ray crystallographic resolution, how are antibodies bound to the MIR? Can a conformationally correct MIR alone efficiently provoke or suppress EAMG? What roles does the MIR play in AChR structure and function? Below we answer some of these questions and reveal approaches to answering the others.

Because of the basically homologous structures of all AChR subunits, some mAbs to the MIR on muscle-type AChRs react with homologous epitopes on other AChR subunits. For example, consider the rat MIR mAbs 35 and 210. mAb 35 was made to AChRs from electrophorus electric organ and is absolutely conformation dependent.⁴⁸ mAb 210 was made to fetal bovine and mouse muscle AChRs and binds to both denatured $\alpha 1$ and native AChRs with high affinity.⁴⁹ Both of these mAbs bind to human $\alpha 1$, $\alpha 3$, $\alpha 5$, and $\beta 3$ subunits. mAb 210 has proven the more useful for binding to neuronal AChR subunits.^{17,50,51} Although it works well on Western blots of $\alpha 1$ subunits, it does not work on Western blots of the neuronal AChR subunits (Kuryatov and Lindstrom, unpublished). It has lower affinity for $\alpha 5$ and $\beta 3$ than for $\alpha 3$ subunits. Although mAb 210 is a very useful probe for human $\alpha 3$ AChRs, it does not bind to rat $\alpha 3$ AChRs (Luo and Lindstrom, unpublished). Although human $\alpha 1$ and $\alpha 3$ AChRs share epitopes recognized with high affinity by mAb 210, autoantibodies from MG patient sera do not react with human $\alpha 3$ AChRs and autoantibodies from patients with autonomic autoimmune neuropathy do not react with human $\alpha 1$ AChRs.^{50,52} Thus AChRs may contain potent epitopes which are not recognized as autoantigens but which can be recognized in other species. Discovering in detail the structures of these epitopes will help to explain the specificities of these useful reagents and help to explain what might trigger an autoimmune response to AChRs in muscle in one case or AChRs in autonomic ganglia in another.

Specific Immunosuppressive Therapy of EAMG

Immunization with denatured electric organ AChR can prevent and suppress EAMG.^{53,54} Denatured AChR, such as bacterially expressed AChR subunit sequences, can induce high

concentrations of antibodies, but these are selectively directed at the cytoplasmic surface where they are pathologically irrelevant.^{32,55} This selectivity reflects loss of the highly immunogenic but conformation-dependent MIR and the stimulatory effect of the interspecies sequence differences in the large cytoplasmic domains.

Tolerance can be induced by administering antigens systemically, orally or nasally.^{56,57} Induction of EAMG (by immunization with native electric organ AChR) can be inhibited by mucosal therapy with either native electric organ AChR, denatured bacterially expressed subunit fragments, or short synthetic subunit sequences.^{58–64} It is thought that low doses of antigen induce regulatory T lymphocytes, which release suppressive lymphokines, whereas high doses are thought to cause deletion of reactive T cells.⁵⁶ Antigen therapy risks stimulating the immune response if antigen is exposed to compartments containing more helper than suppressor T cells.⁵⁸ This is a particular problem if the therapeutic antigen contains pathologically significant B cell epitopes which could provoke synthesis of pathologically significant autoantibodies. Mucosal immunosuppressive therapy with native AChR is effective at preventing induction of EAMG but not at treating ongoing EAMG.^{58,61} This is probably because native AChR contains the MIR and other pathologically significant epitopes.⁶⁵

Fuchs and co-workers have reported success at preventing EAMG and suppressing ongoing EAMG with oral therapy of rats using the bacterially expressed extracellular domain of human or rat AChR $\alpha 1$ subunits ($\alpha 1$ 1–205).^{66,67} Tolerance to AChR was produced through active T cell suppression mediated by up-regulation of anti-inflammatory cytokines and down-regulation of pro-inflammatory cytokines. The longer $\alpha 1$ sequence 1–210 expressed as a fusion protein with thioredoxin was not effective at immunosuppression because of partial renaturation of the MIR.⁶⁵

In our hands, human $\alpha 1$ 1–209 has not been as effective at suppressing EAMG as the thioredoxin-205 construct used by Fuchs and co-workers (Luo, Kuryatov, and Lindstrom, unpublished).^{55,66,67} This is probably because the $\alpha 1$ 1–209 construct we used provoked autoantibodies to the MIR, but perhaps also because of other technical differences such as our use of Titer Max adjuvant rather than the complete Freund's adjuvant which they used.

We have been able to significantly prevent and suppress EAMG using a 2:1:1:1:1 mixture of bacterially expressed human AChR $\alpha 1$, $\beta 1$, γ , δ , and ϵ subunit extracellular and cytoplasmic domains (Luo, Kuryatov, and Lindstrom, unpublished).⁵⁵ Transmembrane domain sequences were not used because they contained few MG T cell epitopes and interfered with bacterial expression.⁶⁸ Intraperitoneal injection of these constructs proved as effective as oral therapy with $\alpha 1$ extracellular domains was reported to be. For example, Maiti and colleagues treated rats orally every other day after 24 hours of food deprivation with 1 mg/dose of $\alpha 1$ extracellular domain for 4 weeks.⁶⁹ We injected 5 mg/dose once per week for 5 weeks (unpublished). Their net effect was to reduce the average clinical score of untreated EAMG from 2.4 to 1.0, compared with a reduction from scores of 2.75 to 0.8 in our case.

An ultimate goal for a specific immunosuppressive therapy of MG is that it should be effective. Therapy should also be robust, meaning that it should be applicable to many different patients, who are more heterogeneous than inbred rats and whose autoimmune responses to AChR might be induced or sustained by somewhat different mechanisms and involve slightly different epitopes. Small differences in the therapeutic antigen structure should not determine effectiveness. Most importantly, the therapy should be safe, minimizing any risk that the therapeutic antigen might provoke pathologically significant autoantibodies and thereby make the MG worse.

Data in this Paper

In order to better characterize the antigenic structure of the MIR at the top of the AChR, chimeras were constructed between human AChR $\alpha 7$ subunits and human $\alpha 1$ subunits and tested to determine which $\alpha 1$ sequences were required to confer the antigenicity of an $\alpha 1$ MIR on an $\alpha 7$ AChR. This revealed that $\alpha 1$ sequences which were not immediately contiguous were required to confer binding of conformation-dependent mAbs to the MIR, such as mAb 35. This did not confer recognition by most MG patient sera, hence the MIR, epitopes which are recognized by MG autoantibodies must be different than those recognized by rats, though close enough to the epitopes recognized by rats to permit competition for binding with mAb 35. The most antigenic MIR chimera increased assembly of chimeric AChRs well beyond that of wild-type $\alpha 7$ and increased sensitivity to activation by ACh. This suggests that the intrinsic function of the MIR may involve subunit conformational maturation or subunit assembly and also conformation changes associated with AChR activation. Chimeras of human $\alpha 1$ with AChBP behave like the same chimeras with $\alpha 7$ in their ability to bind mAbs to the MIR but not MG sera. AChBP chimeras can be prepared in large amounts as soluble proteins and should permit both high-resolution structural studies and manipulation of the autoimmune response in EAMG with just a conformation-dependent MIR antigen and immunogen.

In hopes of improving the effectiveness, robustness, and safety of specific immunosuppression of EAMG, therapy using only bacterially expressed cytoplasmic domains of human muscle AChR subunits from the bottom of the AChR was investigated. Cytoplasmic domains were as effective at suppressing ongoing EAMG as were a mixture of extracellular and cytoplasmic domains, and much more effective than the extracellular domain of $\alpha 1$ 1–209. These results suggest that effective therapy is achieved by a combination of suppressing the autoimmune response and diverting it to pathologically irrelevant epitopes while avoiding provoking autoantibody responses to pathologically significant epitopes.

Results and Discussion

Mapping the Antigenic Structure of the MIR

Figure 1 depicts a chimera of human muscle $\alpha 1$ subunit sequences highlighted on the structure of an AChBP subunit.²¹ We have constructed such chimeras between human $\alpha 1$ sequences and *Aplysia* AChBP to form water-soluble constructs, and between human $\alpha 1$ and human $\alpha 7$ AChR subunits to form functional chimeric $\alpha 7$ ACRs expressed in *Xenopus* oocytes (Luo and Lindstrom, unpublished).²⁴

Incorporating only the putative MIR loop $\alpha 1$ 66–76 into $\alpha 7$ produced a chimera which could be immune precipitated by the rat mAbs 6 and 210 to the MIR, both of which can bind both native and denatured $\alpha 1$.² This chimera was not precipitated by rat mAbs 35 or 198 to the MIR, both of which depend much more on the native conformation of $\alpha 1$ for their affinity.² Increasing the length of the $\alpha 1$ insert to 60–81 did not improve binding. Also including the N-terminal a helical sequence permitted binding of the conformation-dependent mAbs, and the longer 60–81 version of the MIR loop was especially effective. These results are summarized in Figure 2. The dependence for binding of mAb 35 and 198 on sequences that are adjacent only in the native structure explains the dependence of the binding of these mAbs to the MIR on the native conformation of the AChR.

Although the $\alpha 1$ 2–14, 60–81/ $\alpha 7$ chimera efficiently bound the conformation-dependent mAb 35, which has been used in competitive binding assays to inhibit binding of half or more of MG patient serum autoantibodies,³⁴ this chimera did not bind to any of 50 MG sera tested (Luo and Lindstrom, unpublished). Thus MG patient antibodies to the MIR must bind to a distinct nearby epitope or epitopes. These will be mapped by further studies of $\alpha 1$ chimeras.

Studies of $\alpha 1/\alpha 7$ chimeras suggest possible normal structural and function roles for the MIR. Rather than $\alpha 1/\alpha 7$ chimeras exhibiting reduced expression, as one might expect from a Frankenstein structure, the chimeras exhibited greatly increased expression. The chimera with the most native MIR structure was expressed at the highest level, a 26-fold increase over wild-type $\alpha 7$ on the cell surface, as shown in Figure 3. $\alpha 7$ AChRs are notoriously difficult to express from cloned subunits. In part this is because of a need for special chaperones like ric-3.^{71,72} However, even in the presence of ric-3 we have found that only a small fraction of the abundant $\alpha 7$ subunits synthesized assume a native conformation and properly assemble into mature pentamers capable of binding α bungarotoxin (Kuryatov and Lindstrom, unpublished). In the case of muscle AChR subunits, conformational maturation of α subunits (detected by the ability to bind mAb 35 to the MIR and α bungarotoxin to the ACh binding site) must precede assembly of subunits.⁷³ Assuming that conformational maturation of $\alpha 7$ subunits must also precede their assembly, the $\alpha 1$ sequences in the chimera may help to promote $\alpha 7$ subunit maturation. They might do this by promoting interaction between the parts of the sequences that interact to form the conformation-dependent MIR. The $\alpha 1$ sequences in the chimera do not appear to be located on interfaces between subunits where they could directly take part in assembly of subunits into pentamers. One of the normal functions of the MIR may be to help nucleate conformational maturation of unassembled subunits. In $\alpha 1$ subunits, conformational maturation of the MIR and α bungarotoxin binding site occur simultaneously 15–30 min after synthesis of the subunits and prior to assembly, which occurs 60–90 min after synthesis.⁷³

$\alpha 1/\alpha 7$ chimeras can also greatly influence the sensitivity to activation of chimeric AChRs by ACh. Rather than reducing the sensitivity to activation by ACh, as one might expect of a Frankenstein structure, most of the $\alpha 1/\alpha 7$ chimeras tested increased the sensitivity to activation by ACh, as shown in Figure 4. The same interactions between parts of the $\alpha 1$ sequences in the chimera which form the conformation-dependent, high-affinity MIR epitope may not only nucleate conformational maturation of the subunit, as suggested above, but also these interactions may influence the conformation changes in this region associated with activation.²⁵ Binding of mAb 210 to this chimera reduced the response by 70%. mAbs to the MIR do not typically block AChR responses. However, the presence of five MIR epitopes in the chimera as compared to two in a muscle AChR might cause a greater effect of mAb binding, especially if the mAb could link the epitopes on adjacent chimera subunits. In muscle AChR, the two $\alpha 1$ subunits are not adjacent, and a single mAb to the MIR cannot link them.⁴⁵

Chimeras of human AChR $\alpha 1$ subunits (1–30, 60–81) with *Aplasia* AChBP produce MIR epitopes with properties very similar to those of $\alpha 1/\alpha 7$ chimeras. The *Aplasia* AChBP clone was provided by Palmer Taylor.²⁴ The $\alpha 1$ /AChBP chimeras can be produced in large amounts as soluble proteins secreted by transfected human embryonic kidney cells and purified using a FLAG epitope tag on the chimera. Figure 5 shows that $\alpha 1$ /AChBP chimeras labeled with ¹²⁵I α bungarotoxin can be efficiently immune precipitated using rat mAbs to the MIR. By contrast, none of the sera tested from seven MG patients was able to precipitate these chimeras. Thus, both $\alpha 1$ /AChBP and $\alpha 1/\alpha 7$ chimeras efficiently exhibit the conformation-dependent rat MIR but do not exhibit an MIR recognized by MG patients.

The large amount of pure soluble $\alpha 1$ /AChBP chimera should allow testing of the rat MIR as an inducer and suppressor of EAMG and allow assay of the efficiency in passive transfer of EAMG of antisera from which all EAMG antibodies have been absorbed to determine what fraction of serum pathogenicity measured in this way depends on the MIR. Further, it should be possible to crystallize complexes of Fab fragments of MIR mAbs bound to these chimeras in order to precisely determine how the mAbs are bound. Additional chimeras will have to be constructed in order to determine the sequences which are recognized by human autoantibodies to the MIR.

Specific Immunotherapy of EAMG

Human muscle AChR subunit constructs lacking transmembrane domains were expressed in bacteria and a mixture of $\alpha 1$, $\beta 1$, γ , δ , and ϵ constructs in a 2:1:1:1:1 ratio were used to prevent and treat ongoing EAMG.⁵⁵ These subunit proteins are in denatured conformations. They were solubilized from inclusion bodies present in the bacteria using 3% sodium dodecyl sulfate (SDS) and 0.1 M dithiothreitol at 100°C. Each subunit was purified on an AcA-34 Ultrogel sizing column (Sigma, St. Louis, MO). Then the subunits were precipitated with 1.5 M NaCl at 0°C, resolubilized in saline and the majority of the SDS was removed by dialysis.

Pretreatment of Lewis rats with the subunit mixture by oral or nasal routes prevented induction of EAMG caused by immunizing the rats with native *Torpedo* AChR in adjuvant.⁵⁵ Prevention of ongoing EAMG by starting treatment on day 14 after the acute phase was ineffective by the nasal route, but beneficial by the oral route.⁵⁵

Intraperitoneal treatment with the subunit mixture was found to be more effective than oral treatment for suppressing ongoing EAMG. Figure 6 shows an experiment in which control therapies consisting of 5 mg intraperitoneal (i.p.) doses at 1-week intervals starting on day 14 of either ovalbumin, $\alpha 4$ subunits, or the extracellular domain 1–209 of $\alpha 1$ subunits produced little benefit. By contrast, Figure 7 shows that the same weekly dosage schedule of a 5 mg dose of the subunit mixture substantially reduced weakness. This dose of human subunits given to the rats decreased the titer of antibodies to rat AChR by half, from an average of 210 nM in untreated rats to 105 nM in the treated rats.

The pathogenicity of the autoantibodies in the sera of untreated EAMG rats was compared with that of the autoantibodies in the sera of rats treated with 5 mg doses of the subunit mixtures by comparing their abilities to passively transfer EAMG. Serum pool aliquots containing equal 70 pmol amounts of antibody titer to rat AChRs were used on three rat groups. All of the serum pools had the same high proportion of the complement-fixing IgG isotype IgG 2a. This amounted to 0.79 mL of serum from treated rats and 0.53 mL from untreated rats. Adjuvant control serum was used at 0.91 mL/rat. Serum from treated rats showed very little ability to passively transfer EAMG as compared to that of untreated rats, as shown in Figure 8. Successful therapy was associated with a 48% decrease in total titer of autoantibodies to rat AChR and an additional 88% decrease in pathologically significant autoantibodies as measured by passive transfer. Clearly, the mechanism by which this therapeutic approach suppresses EAMG is primarily by diversion of the autoimmune response to irrelevant epitopes rather than just by suppression of the total amount of autoantibodies. This is not surprising since the treated rats were given 714-fold more denatured AChR protein than the initial amount of native AChR which was used for immunization.

In order to further test whether diversion of the autoimmune response could efficiently treat ongoing EAMG, rats were treated with a mixture containing only the cytoplasmic domains of human AChRs in the same 2:1:1:1:1 ratio of $\alpha 1$: $\beta 1$: γ : δ : ϵ used with constructs containing both extracellular and cytoplasmic domains. In order to obtain efficient expression of soluble complexes, these were expressed as two constructs which together reflect the desired stoichiometry and the order of subunits around the AChR: $\beta 1$ 310–446- $\alpha 1$ 297–408- γ 307–451 and ϵ 309–436- $\alpha 1$ 297–408- δ 313–451.

Figure 9 shows that treatment with weekly 5 mg i.p. doses of cytoplasmic domain sequences starting at day 14 is as effective as treating with constructs also containing extracellular domains. Measurements of weight paralleled the measurements of clinical state shown in Figure 9, with the groups treated with subunit or cytoplasmic domain mixtures gaining weight at the same rate as adjuvant controls, while rats treated with only $\alpha 1$ extracellular domains lost weight and untreated rats with EAMG lost the most weight. By day 45, two-thirds of untreated

rats with EAMG were dead, while one-third of those treated with $\alpha 1$ extracellular domains died, and none of those that were treated with either the mix of cytoplasmic domains or both cytoplasmic and extracellular domains had died. Even the lowest dose of cytoplasmic domains used, 0.5 mg, prevented any deaths. In this experiment, unlike that shown in Figure 6 and others, $\alpha 1$ extracellular domain produced significant benefit, though less than the mixtures of subunits. Unlike previous experiments, in this experiment, to insure solubility of the constructs, after purification all constructs were reduced with 1 mM glutathione to promote solubility. This may have inhibited some renaturation of the MIR in the $\alpha 1$ extracellular domain construct and thereby may have prevented formation of some pathologically significant antibodies in response to this protein.

Specific immunosuppression of EAMG with bacterially expressed human AChR cytoplasmic domains seems very promising, but requires further investigations to optimize its potency and investigate its mechanisms of action. Then this approach might be suitable for testing on authentic MG, first in dogs or cats, and, if successful, in people.

There are several potential virtues to the use of AChR subunit cytoplasmic domains for specific immune suppression of MG. This part of the subunits is at least as potent as whole subunit constructs and more potent than $\alpha 1$ subunit extracellular domains. Use of a mixture of AChR subunit cytoplasmic domains should be a very robust approach to immunosuppression because it employs a wide variety of highly immunogenic AChR epitopes. Finally, this approach should be safe, much safer than the use of $\alpha 1$ extracellular domains, which might renature sufficiently to provoke formation of autoantibodies to the MIR, because cytoplasmic domains cannot provoke the formation of autoantibodies which could bind *in vivo* and thereby be pathologically significant.

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References

1. Sine S, Engle A. Recent advances in Cys-loop receptor structure and function. *Nature* 2006;440:448–454. [PubMed: 16554804]
2. Lindstrom J. The structures of neuronal nicotinic receptors, in *Neuronal Nicotinic Receptors*. In: Clementi, F.; Gotti, C.; Fornasari, D., editors. *Handbook of Experimental Pharmacology*. Vol. 144. Springer; New York, NY: 2000. p. 101-162.
3. Kalamida D, Konstantinos P, Auramopoulou V, et al. Muscle and neuronal nicotinic acetylcholine receptors: Structure, function and pathogenicity. *FEBS J* 2007;274:3799–3845. [PubMed: 17651090]
4. Gotti C, Zoli M, Clementi F. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol. Sci* 2006;27:482–491. [PubMed: 16876883]
5. Grando S. Cholinergic control of epidermal cohesion. *Exp. Dermatol* 2006;15:265–282. [PubMed: 16512874]
6. Tracey K. Physiology and immunology of the cholinergic anti-inflammatory pathway. *J. Clin. Invest* 2007;117:289–296. [PubMed: 17273548]
7. Lindstrom J. Acetylcholine receptors and myasthenia. *Muscle Nerve* 2000;23:453–477. [PubMed: 10716755]
8. Vernino S, Ermilov L, Sha L, et al. Passive transfer of autoimmune autonomic neuropathy to mice. *J. Neurosci* 2004;24:7037–7042. [PubMed: 15306637]
9. Grando S. Autoimmunity to keratinocyte acetylcholine receptors in pemphigus. *Dermatology* 2000;201:290–295. [PubMed: 11146336]
10. Engel A, Ohno K, Sine S. Sleuthing molecular targets for neurological diseases at the neuromuscular junction. *Nat. Rev. Neurosci* 2003;4:339–352. [PubMed: 12728262]

11. Klaassen A, Glykys J, Maguire J, et al. Seizures and enhanced cortical GABAergic inhibition in two mouse models of human autosomal dominant nocturnal frontal lobe epilepsy. *Proc. Natl. Acad. Sci* 2006;103:19152–19157. [PubMed: 17146052]
12. Tapper A, McKinney S, Nashmi R, et al. Nicotine activation of $\alpha 4^*$ receptors: sufficient for reward, tolerance, and sensitization. *Science* 2004;306:1029–1032. [PubMed: 15528443]
13. Bocquet N, Prado de Carvalho L, Cartand J, et al. A prokaryotic proton-gated ion channel from the nicotinic acetylcholine receptor family. *Nature* 2006;445:116–119. [PubMed: 17167423]
14. Nelson M, Kuryatov A, Choi C, et al. Alternate stoichiometries of $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *Mol. Pharmacol* 2003;63:332–341. [PubMed: 12527804]
15. Kuryatov A, Luo J, Cooper J, Lindstrom J. Nicotine acts as a pharmacological chaperone to upregulate human $\alpha 4\beta 2$ AChRs. *Mol. Pharmacol* 2005;68:1839–1851. [PubMed: 16183856]
16. Tapia L, Kuryatov A, Lindstrom J. Ca^{++} permeability of the $(\alpha 4)_3(\beta 2)_2$ stoichiometry greatly exceeds that of $(\alpha 4)_2(\beta 2)_3$ human acetylcholine receptors. *Mol. Pharmacol* 2007;71:769–776. [PubMed: 17132685]
17. Tumkosit P, Kuryatov A, Luo J, Lindstrom J. $\beta 3$ subunits promote expression and nicotine-induced up-regulation of human nicotinic $\alpha 6^*$ nicotinic acetylcholine receptors expressed in transfected cell lines. *Mol. Pharmacol* 2006;70:1358–1368. [PubMed: 16835356]
18. Elgoyhen A, Vetter D, Katz E, et al. Alpha 10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc. Natl. Acad. Sci. USA* 2001;98:3501–3506. [PubMed: 11248107]
19. Keyser K, Britto L, Schoepfer R, et al. Three subtypes of α bungarotoxin-sensitive nicotinic acetylcholine receptors are expressed in chick retina. *J. Neurosci* 1993;13:442–454. [PubMed: 8426223]
20. Unwin N. Refined structure of the nicotinic acetylcholine receptor at 4Å resolution. *J. Mol. Biol* 2005;346:967–989. [PubMed: 15701510]
21. Brejc K, van Dyk W, Klaassen R, et al. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* 2001;411:269–276. [PubMed: 11357122]
22. Bouzat C, Gumilar F, Spitzmaul G, et al. Coupling of agonist binding to channel gating of an ACh-binding protein linked to an ion channel. *Nature* 2004;430:896–900. [PubMed: 15318223]
23. Celie P, Rossum-Fikkert S, van Dyk W, et al. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron* 2004;41:907–914. [PubMed: 15046723]
24. Hansen S, Sulzenbacher G, Huxford T, et al. Structures of Aplysia AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. *EMBO J* 2005;24:3635–3646. [PubMed: 16193063]
25. Henchman R, Wang H-L, Sine S, et al. Asymmetric structural motions of the homomeric $\alpha 7$ nicotinic receptor ligand binding domain revealed by molecular dynamics simulation. *Biophys. J* 2003;85:3007–3018. [PubMed: 14581202]
26. Karlin A. Emerging structure of the nicotinic acetylcholine receptors. *Nat. Rev. Neurosci* 2002;3:102–114. [PubMed: 11836518]
27. Maimone M, Merlie J. Interaction of the 43kd postsynaptic protein with all subunits of the muscle nicotinic acetylcholine receptor. *Neuron* 1993;11:53–66. [PubMed: 8338668]
28. Hales T, Dunlop J, Deeb T, et al. Common determinants of single channel conductance within the large cytoplasmic loop of 5-hydroxytryptamine type 3 and $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *J. Biol. Chem* 2006;281:8062–8071. [PubMed: 16407231]
29. Fenster C, Beckman M, Parker J, et al. Regulation of $\alpha 4\beta 2$ nicotinic receptor desensitization by calcium and protein kinase C. *Mol. Pharmacol* 1999;55:432–443. [PubMed: 10051526]
30. Williams B, Temburni M, Levey M, et al. The long internal loop of the $\alpha 3$ subunit targets nAChRs to subdomains within individual synapses on neurons in vivo. *Nat. Neurosci* 1998;1:557–562. [PubMed: 10196562]
31. Das M, Lindstrom J. Epitope mapping of antibodies to acetylcholine receptor subunits using peptides synthesized on polypropylene pegs. *Biochemistry* 1991;30:2470–2477. [PubMed: 1705819]
32. Froehner S. Identification of exposed and buried determinants of the membrane bound acetylcholine receptor from *Torpedo californica*. *Biochemistry* 1981;20:4905–4915. [PubMed: 6170319]

33. Tzartos S, Lindstrom J. Monoclonal antibodies used to probe acetylcholine receptor structure: Localization of the main immunogenic region and detection of similarities between subunits. *Proc. Natl. Acad. Sci. USA* 1980;77:755–759. [PubMed: 6153804]
34. Tzartos S, Seybold M, Lindstrom J. Specificity of antibodies to acetylcholine receptors in sera from myasthenia gravis patients measured by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* 1982;79:188–192. [PubMed: 6948300]
35. Tzartos S, Hochschwender S, Langeberg L, Lindstrom J. Demonstration of a main immunogenic region on acetylcholine receptors from human muscle using monoclonal antibodies to human receptor. *FEBS Lett* 1983;158:116–118. [PubMed: 6862030]
36. Shelton G, Cardinet III G, Lindstrom J. Canine and human myasthenia gravis autoantibodies recognize similar regions on the acetylcholine receptor. *Neurology* 1988;38:1417–1424. [PubMed: 3412590]
37. Louttrari H, Kokla A, Tzartos S. Passive transfer of experimental myasthenia gravis via antigenic modulation of acetylcholine receptor. *Eur. J. Immunol* 1992;22:2449–2452. [PubMed: 1516631]
38. Lindstrom J, Einarson B, Merlie J. Immunization of rats with polypeptide chains from Torpedo acetylcholine receptor causes an autoimmune response to receptors in rat muscle. *Proc. Natl. Acad. Sci. USA* 1978;75:769–773. [PubMed: 273239]
39. Saedi M, Anand R, Conroy WG, Lindstrom J. Determination of amino acids critical to the main immunogenic region of intact acetylcholine receptors by in vitro mutagenesis. *FEBS Lett* 1990;267:55–59. [PubMed: 2365090]
40. Tzartos S, Barkas T, Cung M, et al. Anatomy of the antigenic structure of a large membrane autoantigen, the muscle-type nicotinic acetylcholine receptor. *Immunol. Rev* 1998;163:89–120. [PubMed: 9700504]
41. Amit A, Mariuzza R, Phillips S, Poljak R. Three dimensional structure of an antigen-antibody complex at 6 Å resolution. *Nature* 1985;313:156–158. [PubMed: 3965976]
42. Kontou M, Leonidas D, Vatzaki E, et al. The crystal structure of the Fab fragment of a rat monoclonal antibody against the main immunogenic region of the human muscle acetylcholine receptor. *Eur. J. Biochem* 2000;267:2389–2396. [PubMed: 10759865]
43. Poulas K, Elipoulas E, Vatzaki E, et al. Crystal structure of Fab 198, an efficient protector of the acetylcholine receptor against myasthenogenic antibodies. *Eur. J. Biochem* 2001;268:3685–3693. [PubMed: 11432734]
44. Beroukhim R, Unwin N. Three dimensional location of the main immunogenic region of the acetylcholine receptor. *Neuron* 1995;15:323–331. [PubMed: 7544140]
45. Conti-Tronconi B, Tzartos S, Lindstrom J. Monoclonal antibodies as probes of acetylcholine receptor structure. II: Binding to native receptor. *Biochemistry* 1981;20:2181–2191. [PubMed: 6786327]
46. Konstantakaki M, Tzartos S, Poulas K, Eliopoulous E. Molecular modeling of the complex between Torpedo acetylcholine receptor and anti-MIR Fab198. *Biochem. Biophys. Res. Commun* 2007;356:569–575. [PubMed: 17376405]
47. Lindstrom J, Seybold M, Lennon V, et al. Antibody to acetylcholine receptor in myasthenia gravis: prevalence, clinical correlates, and diagnostic value. *Neurology* 1976;26:1054–1059. [PubMed: 988512]
48. Tzartos S, Rand D, Einarson B, Lindstrom J. Mapping of surface structures on Electrophorus acetylcholine receptor using monoclonal antibodies. *J. Biol. Chem* 1981;256:8635–8645. [PubMed: 6167581]
49. Tzartos S, Hochschwender S, Vasquez P, Lindstrom J. Passive transfer of experimental autoimmune myasthenia gravis by monoclonal antibodies to the main immunogenic region of the acetylcholine receptor. *J. Neuroimmunol* 1987;15:185–194. [PubMed: 3495549]
50. Wang F, Nelson M, Kuryatov A, et al. Chronic nicotine treatment upregulates human $\alpha 3\beta 2$ but not $\alpha 3\beta 4$ AChRs stably transfected in human embryonic kidney cells. *J. Biol. Chem* 1998;273:28,721–28,732. [PubMed: 9417042]
51. Kuryatov A, Gerzanich V, Nelson M, et al. Mutation causing autosomal dominant nocturnal frontal lobe epilepsy alters Ca^{++} permeability, conductance, and gating of human $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *J. Neurosci* 1997;17:9035–9047. [PubMed: 9364050]

52. Vernino S, Adamski J, Kryzer T, et al. Neuronal nicotinic ACh receptor antibody in subacute autonomic neuropathy and cancer related syndromes. *Neurology* 1998;50:1806–1813. [PubMed: 9633732]
53. Bartfeld D, Fuchs S. Specific immunosuppression of experimental autoimmune myasthenia gravis by denatured acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* 1978;75:4006–4010. [PubMed: 279016]
54. Krolick K, Yeh T-M, Edlund S. Lewis rats given antibodies against denatured acetylcholine receptor become resistant to induction of experimental autoimmune myasthenia gravis. *Cell. Immunol* 1996;172:10–20. [PubMed: 8806801]
55. Lindstrom J, Peng X, Kuryatov A, et al. Molecular and antigenic structure of nicotinic acetylcholine receptors. *Ann. N.Y. Acad. Sci* 1998;841:71–86. [PubMed: 9668222]
56. Weiner H. Oral tolerance for the treatment of autoimmune diseases. *Ann. Rev. Med* 1997;48:341–351. [PubMed: 9046967]
57. Liblau R, Tisch R, Bercovici N, McDevitt H. Systemic antigen in the treatment of T-cell-mediated autoimmune diseases. *Immunol. Today* 1997;18:599–604. [PubMed: 9425739]
58. Xiao B-G, Link H. Mucosal tolerance: a two edged sword to prevent and treat autoimmune diseases. *Clin. Immunol. Immunopathol* 1997;85:119–128. [PubMed: 9344693]
59. Wang Z-Y, Huang J, Olsson T, et al. B cell responses to acetylcholine receptor in rats orally tolerized against experimental auto immune myasthenia gravis. *J. Neuro. Sci* 1995;128:167–174.
60. Ma C-G, Zhang G-X, Xiao B-G, et al. Suppression of experimental autoimmune myasthenia gravis by nasal administration of acetylcholine receptor. *J. Neuroimmunol* 1995;58:51–60. [PubMed: 7537280]
61. Drachman D, Okumura S, Adams R, McIntosh K. Oral tolerance in myasthenia gravis. *Ann. NY Acad. Sci* 1996;778:258–272. [PubMed: 8610979]
62. Barchan D, Asher O, Tzartos S, et al. Modulation of the anti-acetylcholine receptor response and experimental autoimmune myasthenia gravis by recombinant fragments of the acetylcholine receptor. *Eur. J. Immunol* 1998;28:616–624. [PubMed: 9521072]
63. Shi F-D, Li H, Wang H, et al. Mechanisms of nasal tolerance induction in experimental autoimmune myasthenia gravis: identification of regulatory cells. *J. Immunol* 1999;162:5757–5763. [PubMed: 10229808]
64. Karachunski P, Ostlie N, Okita D, et al. Subcutaneous administration of T-epitope sequences of the acetylcholine receptor prevents experimental myasthenia gravis. *J. Neuroimmunol* 1999;93:108–121. [PubMed: 10378874]
65. Im S-H, Barchan D, Sourojon M, Fuchs S. Role of tolerogen conformation in induction of oral tolerance in experimental autoimmune myasthenia gravis. *J. Immunol* 2000;165:3599–3605. [PubMed: 11034361]
66. Im S, Barchan D, Fuchs S, Sourojon M. Suppression of ongoing experimental myasthenia by oral treatment with an acetylcholine receptor recombinant fragment. *J. Clin. Invest* 1999;104:1723–1730. [PubMed: 10606626]
67. Im S-H, Barchan D, Fuchs S, Sourojon M. Mechanism of nasal tolerance induced by a recombinant fragment of acetylcholine receptor for treatment of experimental myasthenia gravis. *J. Neuroimmunol* 2000;111:161–168. [PubMed: 11063834]
68. Protti M, Manfredi A, Horton R, et al. Myasthenia gravis: recognition of a human autoantigen at the molecular level. *Immunol. Today* 1993;14:363–368. [PubMed: 8363727]
69. Maiti P, Teferman T, Im S-H, et al. Immunosuppression of rat myasthenia gravis by oral administration of a syngeneic acetylcholine receptor fragment. *J. Neuroimmunol* 2004;152:112–120. [PubMed: 15223243]
70. McLane K, Wu X, Lindstrom J, Conti-Tronconi B. Epitope mapping of polyclonal and monoclonal antibodies against two a bungarotoxin binding subunits from neuronal nicotinic receptors. *J. Neuroimmunol* 1992;38:115–128. [PubMed: 1374423]
71. Halevi S, McKay J, Palfreyman M, et al. The *C. elegans* nic-3 gene is required for maturation of nicotinic acetylcholine receptors. *EMBO J* 2002;21:1012–1020. [PubMed: 11867529]

72. Lansdell S, Gee V, Harkness P, et al. RIC-3 enhances functional expression of multiple nicotinic acetylcholine receptor subtypes in mammalian cells. *Mol. Pharmacol* 2005;68:1431–1438. [PubMed: 16120769]
73. Merlie J, Sebbane R, Gardner S, et al. The regulation of acetylcholine receptor expression in mammalian muscle. *Cold Spring Harbor Symposia on Quantitative Biology* 1983;48:135–146.

FIGURE 1.

Components of $\alpha 1$ chimeras highlighted on the structure of an AChBP subunit.²¹ Gray highlights sequence corresponding to $\alpha 1$ 60–81, which includes the sequence 67–76 usually associated with the MIR.⁴⁰ Darker gray highlights the N-terminal α helical sequence corresponding to $\alpha 1$ 1–14. The lightest gray highlights sequence corresponding to $\alpha 1$ 15–31, which contains glutamate 23 that is thought to be critical to the high-affinity binding of rat mAb 192 to the MIR on human muscle.^{40,42} Glutamine 111 is noted at the tip of a loop between the $\beta 5$ and $\beta 6$ strands which approaches the under side of the MIR loop.

FIGURE 2.

Immune precipitation of wild-type human $\alpha 7$ and human $\alpha 1/\alpha 7$ chimeras by mAbs to the MIR. Results were normalized to the amount of ^{125}I α bungarotoxin-labeled AChRs bound by the mouse mAb 306 made to chicken $\alpha 7$ AChRs.² The epitope recognized by mAb 306 is located in the large cytoplasmic domain of $\alpha 7$,⁷⁰ thus its binding should not be influenced by mutation of the extracellular domain. When appropriate $\alpha 1$ sequences were used in chimeras to confer binding of mAbs to the MIR, chimeric AChRs could be precipitated even more efficiently by mAbs to the MIR than by mAb 306 to chicken $\alpha 7$. mAbs 6 and 210, which work on Western blots of denatured $\alpha 1$,² were able to bind to chimeras containing only $\alpha 1$ 66–76. mAbs 35 and 198, which depend more on the native conformation of the AChR for their binding, were able to bind well only to chimeras that contained both the putative MIR loop and the N-terminal α helix region. These MIR mAbs were raised in Lewis rats to AChRs from a variety of species: mAb 6, *Torpedo californica* electric organ; mAb 210, bovine muscle; mAb 35, *Electrophorus electricus* electric organ; and mAb 198, human muscle.²

FIGURE 3.

$\alpha 1/\alpha 7$ chimeras were more efficiently expressed than wild-type $\alpha 7$. Presence of the $\alpha 1$ MIR increases assembly of mature $\alpha 1/\alpha 7$ AChRs as measured by the total amount of $\alpha 1/\alpha 7$ AChRs on the cell surface detected by binding of iodinated α bungarotoxin to intact oocytes. Only mature pentameric AChRs are expected to be expressed on the cell surface. The chimera with the most native MIR structure was expressed at the greatest level, 26-fold more than wild type. The total amount of $\alpha 7$ AChR labeled with iodinated α bungarotoxin which could be immune precipitated by mAb 306 increased in parallel with expression on the cell surface. Note also in Figure 2 that the presence of the MIR increases the total amount of AChRs which can be immunoprecipitated.

FIGURE 4.

Most $\alpha 1/\alpha 7$ chimeras exhibit increased sensitivity to activation by ACh. The chimeras were expressed in *Xenopus* oocytes and function assayed using a standard two-microelectrode voltage clamp.

FIGURE 5.

$\alpha 1(1-30, 60-81)/AChBP$ chimeras labeled with ^{125}I α bungarotoxin are efficiently immune precipitated by rat mAbs to the MIR. As a positive control, antibodies to a FLAG tag on the chimera were used to precipitate all of the labeled chimeras present. The chimera was used at a concentration of 0.5 nM and labeled with 10 nM ^{125}I α bungarotoxin. A similar experiment using 7 MG patient sera and Zyzorbin instead of goat anti-rat IgG detected no autoantibody binding.

FIGURE 6.

Intraperitoneal treatment with ovalbumin, $\alpha 4$ AChR subunits, or the extracellular domain of $\alpha 1$ subunits did not reduce signs of EAMG weakness. All rats except adjuvant controls were immunized with 35 μ g of *Torpedo californica* AChR in Titer Max adjuvant at day 0. Groups consisted of six rats. Treatments were in 5 mg doses given weekly starting on day 14. Adjuvant control rats ($\circ$), those immunized with ovalbumin instead of AChR and treated i.p. with saline ($\square$), or the subunit mixture ($\diamond$), showed no weakness. Rats with EAMG treated only with saline were the weakest ($\blacksquare$). Control therapy groups did not differ significantly ($P > 0.3$ days 30–50) from the untreated EAMG group. The control therapies of EAMG induced by immunization with AChR were treatment with ovalbumin ($\bullet$), human AChR $\alpha 4$ subunits ($\blacktriangle$), or human $\alpha 1$ extracellular domains ($\blacklozenge$). All groups showed similar acute phases peaking around day 10.

FIGURE 7.

Intraperitoneal treatment with a 5 mg dose of a 2:1:1:1:1 mixture of human $\alpha 1$, $\beta 1$, γ , δ , and ϵ subunits at weekly intervals starting on day 14 substantially reduced signs of EAMG weakness. This combines data from the six rat EAMG groups of the experiment shown in Figure 5 with six rat groups from another identical experiment giving 12 rat groups to show the consistency of the therapeutic effect. Untreated EAMG rats (■) were severely affected, while rats treated with the subunit mixture (△) showed little more weakness than adjuvant controls (●).

FIGURE 8.

Sera from successfully treated rats are much less effective at passive transfer of EAMG. Serum pools were from the rats used in Figure 6. At time 0 rats were injected i.p. with serum containing 70 pmol of titer to rat AChR. Measures of weight loss paralleled measures of clinical weakness.

FIGURE 9.

Treatment with only cytoplasmic domains was as effective in suppressing EAMG as treatment with both extracellular and cytoplasmic domains, and more effective than treatment with only extracellular domains of $\alpha 1$ subunits. (A) As in Figures 5 and 6, treatment consisted of 5 mg i.p. doses of bacterially expressed human AChR subunit constructs given weekly starting on day 14. Groups consisted of six young female Lewis rats. By contrast with the experiments in those figures, EAMG was induced by twice the dose of Torpedo AChR (70 μ g) on day 0. Also in Figure 8, unlike previously, after purification all subunit constructs were reduced to promote solubility and inhibit renaturation. (B) By day 45, 4/6 rats in the EAMG control group were dead (score = 4), 2/6 treated with $\alpha 1$ extracellular domains were dead, and 0/6 treated with either the subunit mix or cytoplasmic domain mix were dead. Note that both doses of cytoplasmic domains (0.5 and 5 mg) completely prevented mortality.