Experimental autoimmune myasthenia gravis

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SUMMARY Injection of animals with purified acetylcholine receptor in complete Freund's adjuvant causes development of antibodies which crossreact with receptors in muscle. The crossreacting antibodies impair neuromuscular transmission. Animals with experimental autoimmune myasthenia gravis (EAMG) are excellent models for studying the complex mechanisms by which the autoimmune response to receptor in myasthenia gravis causes muscle weakness. This review first briefly describes the discovery of EAMG. Then, to provide the necessary perspective, receptor structure and function and properties of anti-receptor antibodies are discussed, followed by a brief review of the pathological mechanisms in EAMG.

Introduction

In 1960 John Simpson wrote a paper which argued that myasthenia gravis (MG) was caused by antibodies to acetylcholine receptors (AChR) that impaired neuromuscular transmission by competitively inhibiting AChR. In 1968 my graduate adviser, Ed Lennox, gave me a copy to read. My thesis project was to purify AChR. Many years before, David Nachmanson had introduced the idea of using fish electric organs as a rich source of AChR, but no suitably specific method had been devised for biochemically labelling AChR or assaying their activity, much less purifying them. We were investigating affinity labelling as an approach to identifying AChR biochemically, but with considerably less success than Arthur Karlin was enjoying at that time, and in ignorance of the really critical observation by C Y Lee that labelled snake venom toxins could specifically label AChR. The use of fish electric organs as a source of AChR and labelled snake venom toxin to identify AChR would ultimately permit both the purification and characterisation of AChR and the understanding of the pathological mechanisms impairing neuromuscular transmission in MG. But at the time I first read Dr Simpson's article, my interest was in using anti-AChR antibodies from MG patients, if they existed, to identify AChR. In the natural course of things, we ended up much later using labelled AChR to identify antibodies in MG patients.

It had initially been thought that snake toxins bound very specifically, but irreversibly to the acetylcholine binding site of AChR. Thus, although they would provide a wonderfully specific method for localising and quantitating AChR, they seemed unusable as affinity adsorbents for purifying AChR. In fact, though toxins bind with high affinity to AChR, they can be competitively eluted. Using this observation, Jim Patrick and I purified AChR from the electric organs of Electrophorus electricus by solubilising the membrane proteins in Triton X-100, absorbing AChR to an affinity column of toxin-agarose, washing off contaminants, and competitively eluting AChR with the high affinity antagonist benzoquinonium. We identified AChR as a single component on sucrose gradients which bound toxin and cholineric ligands. But this left several unanswered questions. The most important was, 'was this the physiologically significant AChR or some other binding component?' We had no biochemical assay for the ion channel regulated by acetylcholine binding, so we could identify only the binding site, not the functional activity of the molecule, as one would with an enzyme. Had we left the ion channel in the membrane? We observed two polypeptide chains in the purified material. Were these AChR subunits involved in ligand binding and ion channel function or was one or both a contaminant? Were there other subunits we had missed?

We decided that the best way to test whether our purified AChR contained any part of the...
physiologically significant AChR was to make antibodies against it and see if they blocked the function of AChR in intact electric organ cells. The antibodies did block. But more interestingly, the immunised rabbits became sick and died. This immediately brought Dr Simpon's theory that MG was caused by an autoimmune response to AChR.

In the intervening years it has become obvious that experimental autoimmune myasthenia gravis (EAMG) and MG are both caused by an autoimmune response to AChR. The effects of antibodies have turned out to be much more complex than simple competitive antagonism of AChR. The excellent group of co-workers with which I have had the good fortune to be associated has done much to provide a fundamental understanding of both EAMG and MG. These investigators have included Vanda Lennon, Marge Seybold, Ed Lambert, Andy Engel, Steve Heinemann and many others. Not only do AChR and toxins been useful probes for studying EAMG and MG, but antibodies to AChR and its subunits have proven very useful for answering many of the initial questions about AChR structure and function.

In the following sections I will briefly outline the current state of our knowledge about the AChR molecule and its role in EAMG.

The AChR molecule

Structure of the AChR molecule has been studied most carefully using AChR purified from marine elasmobranch electric organs. AChR synthesis and destruction has been best studied using chicken and rodent muscle cells in tissue culture. AChR function has been best studied using intact amphibian and mammalian muscle tissue. It seems increasingly reasonable to hope that the structure, metabolism and function of the AChR is sufficiently universal that species specific variations are relatively small, and information from all these sources can be integrated to provide a reasonable view of the AChR molecule.

AChR from the electric organ of the marine elasmobranch Torpedo californica contains four strongly associated subunits in the mole ratio $\alpha_2\beta_2\delta$. The apparent molecular weights of these acidic glycoproteins are 38, 50, 57 and $64 \times 10^6$ for $\alpha$, $\beta$, $\gamma$ and $\delta$, respectively. There are two acetylcholine binding sites per AChR monomer, located on the $\alpha$ subunits. Torpedo AChR normally exist as dimers joined by disulfide bonds between $\delta$ subunits.

Although the four subunits have unique peptide maps, and are basically immunochemically distinct, there are some structural similarities. A monoclonal antibody to the $\delta$ subunit also reacts with $\gamma$, but with low affinity. Similarly, a monoclonal antibody to the $\beta$ subunit also reacts with the $\alpha$ subunit, but with low affinity. The functions of $\beta$, $\gamma$ and $\delta$ are unknown. Because AChR monomers purified under conditions which prevent denaturation of their ion channels retain agonist stimulated cation permeability when reconstituted into model membrane vesicles, it is known that the ion channel is an integral component of the AChR monomer. Thus, some of the AChR subunits must be involved in the structure and regulation of this channel.

AChR from the electric organ of the fresh water teleost Electrophorus electricus initially appeared to differ in subunit structure from torpedo AChR. Now techniques have been devised for preserving four subunits in eel AChR as well. These correspond immunochemically to the four subunits of torpedo AChR.

AChR is present at much lower concentration in muscle than in electric organs, and its structure is corresponding less certain. Until recently, there had been disagreement over the structure even of electric organ AChR. Some reports claimed that it was composed only of $\alpha$ subunits, but these erroneous results probably resulted from selective proteolysis of the higher molecular weight subunits. Antigenic determinants corresponding to the four subunits of torpedo AChR are observed in both bovine and human muscle. $\alpha$-like subunits can be affinity labelled with an acetylcholine binding site directed reagent using AChR from torpedo, eel, or muscle. However, in rat muscle, but not bovine muscle, two sizes of chains were labelled. AChR purified from rat muscle contains subunits similar in molecular weight to $\beta$, $\gamma$ and $\delta$, but these have not yet been tested to determine whether they correspond immunochemically to the subunits of torpedo AChR.

AChR metabolism has been most extensively studied by Fambrough, who has recently reviewed this field. Muscle cells in culture, like denervated or foetal muscle tissue, rapidly turn over their AChR. AChR at normal, mature synapses are turned over much more slowly, but it is presumed that the same basic mechanisms are involved. The "junctional" AChR present at neuromuscular junctions are suspected to differ from "extrajunctional" AChR of denervated muscle by a structural modification that alters their ligand
affinity, isoelectric point,22 and immunochemical properties,27 33 but the basis of this structural difference has not yet been discovered.21 In cultured cells,20 31 AChR are assembled to the point where they can bind 125I toxin in less than 15 minutes. AChR are first observed in the Golgi apparatus, oriented with their toxin binding sites toward the interior of the membrane vesicles. Completion of AChR assembly requires glycosylation. Two to four hours are required before AChR are incorporated in the surface membrane, presumably by fusion of vesicles of newly synthesised AChR with the surface so that they are oriented right side out. The half time for destruction of AChR in cell culture is of the order of 20 hours, whereas at neuromuscular junctions the half time is at least 150 hours. The degradation process appears to randomly select AChR. It is an energy-requiring process which probably involves endocytosis and certainly involves secondary lysozomes, where AChR are degraded to their component amino acids. Degradation can be easily measured by pre-labeling AChR with 125I toxin and then monitoring release of 125I tyrosine from the cells due to 125I toxin which is degraded along with the AChR to which it is attached. About 90 minutes are required for release of 125I tyrosine after the AChR first disappear from the surface. The rate of AChR synthesis or destruction can be influenced by a number of factors, including muscle activity,34 hypophysectomy,35 and binding of anti-AChR antibodies.36-44

In conclusion, the AChR molecule in muscle probably is an integral membrane glycoprotein composed of four subunits intimately associated in an α2βγδ complex which includes both the acetylcholine binding site and the cation-specific channel it regulates. Antibodies can be used to demonstrate quite similar basic subunit structures in AChR from different species, despite the fact that these antibodies are so specific in their interaction with AChR that differences in the amino acid sequence of these subunits may result in only 1% immunological crossreaction with AChR from a species different from that of the immunogen. AChR synthesis, localisation, and destruction is a complex process, potentially subject to regulation at many points even in the normal neuromuscular junction. Thus one might expect that the in vivo immune response to AChR which occurs in EAMG and MG might have complex effects on AChR metabolism, localisation, and function. In addition, of course, one might expect that an immune response to AChR would upset the normal trophic interactions between nerve and muscle, thereby perhaps affecting acetylcholine metabolism and release (for which there is some evidence in MG and EAMG65), nerve ending sprouting (for which there is some evidence64), and perhaps other presynaptic parameters.

Immunisation with AChR

EAMG has been induced in all species tested by immunisation with AChR purified from fish electric organs. The species tested have included rabbits,12 45-50 rats,51 52 mice,53-57 guinea pigs,51 58 goats,59 60 monkeys,61 and frogs.92 Detailed features of the muscle weakness characteristic of EAMG vary, depending on the species immunised. For example, monkeys with EAMG exhibit the drooping eyelids characteristic of human MG,91 while rodents do not.45-58 Most rabbits become nearly moribund 25-30 days after immunisation,12 while frogs require immunisation prolonged over 4 to 6 months.92 Genetically defined mouse strains differ in their response.53 54 Presumably these differences in response reflect primarily relatively small quantitative differences in degree of crossreaction of AChR, relative effectiveness of various components of the immune response, relative effectiveness of adaptive responses at the neuromuscular synapse, and anatomical differences rather than fundamental large qualitative differences in the component processes triggered by immunisation with AChR.

EAMG has been most thoroughly studied in young, female Lewis rats. EAMG has been induced in these rats by immunisation with AChR purified from syngenic rats,65 foetal calf muscle, (unpublished) electric eels,51 52 63 torpedoes,23 27 66 and the purified subunits of torpedo AChR.23 27 Native torpedo AChR is a very potent immunogen. Immunisation with as little as 1 microgram in complete Freund’s adjuvant produces measurable antibody and loss of muscle AChR.66 In order to achieve relatively high concentrations of antibody crossreacting with muscle AChR, single or multiple doses of 15-30 micrograms are usually used. The degree of crossreaction of electric organ AChR with muscle AChR is usually much less than 5%.66 However, high antibody concentrations against electric organ AChR are readily achieved, and the amount of muscle AChR is very small. Thus, a rat whose serum can bind 5×10^-8 moles of 125I toxin labelled torpedo AChR per litre may only cross-react 1% with rat muscle AChR, but even at 5×10^-8M, anti-muscle antibody still exceeds tenfold the amount of AChR in the rat’s muscle.
Specificities of the antibodies to AChR produced by immunisation of rats have been studied in some detail. The most important antigenic determinants depend on the native structure of the molecule. About 50% of the antibodies directed at the native molecule bind at or near a determinant on the α subunit which is not the acetylcholine binding site. Antibody to native AChR cross-react detectably, but not well with SDS denatured subunits. Interspecies cross-reaction of antiserum to native AChR is greatest with denatured α subunits. Torpedo AChR subunits are less immunogenic and myasthenogenic than the native molecule by several hundred-fold, but immunisation with any of the subunits can induce EAMG. Antibodies to the denatured subunits react well with native AChR, but recognise antigenic determinants different from the conformationally dependent determinants which dominate the immunogenicity of the native molecule. Because both native AChR and each of its four denatured subunits can cause EAMG, it is clear that there is no single “myasthenogenic” antigen critical for inducing EAMG which is shared by all AChR.

**Acute, chronic and passive EAMG**

Three forms of EAMG have been distinguished in Lewis rats: acute, chronic and passive. After a single immunisation with AChR in complete Freund’s adjuvant, plus injection of pertussis vaccine at other sites as additional adjuvant, Lewis rats undergo two phases of muscular weakness. An acute phase lasting 2 to 3 days occurs 8 to 11 days after immunisation. If pertussis is not used as additional adjuvant, an acute phase is not observed. In any case, after 28 to 30 days chronic muscular weakness begins. This may be progressive until death, especially at higher AChR doses, or diminish as the response to immunogen diminishes. Antisera from a rat with chronic EAMG can be used to passively transfer EAMG to a normal rat very efficiently. The recipient rat begins to exhibit weakness within 12 to 24 hours, but if the weakness is not fatal, this weakness, like that in acute EAMG, passes within a few days. EAMG can also be transferred with lymphocytes, but not very efficiently, and the weakness is delayed by many days, presumably due to the time required for these lymphocytes to synthesise sufficient antibody to produce an effect.

Both acute and passive EAMG are characterised by extensive phagocytic invasion of the endplates. These cells are presumably attracted by chemotactic fragments released by activated complement, and their attack on the postsynaptic membrane is triggered by binding to antibody and complement deposited on the membrane. This is shown by the observation that the phagocytic invasion which normally occurs after injection of anti-AChR into a normal rat is prevented if the rat is first depleted of the C3 component of complement. The phagocytes destroy the postsynaptic membrane producing functional denervation in many fibres. The phagocytes amplify the effect of the small amount of antibody which is actually bound to AChR during acute or passive EAMG. Large loss of muscle AChR is observed during the phagocytic invasion. After two or three days, when the phagocytic invasion is diminishing, the muscle AChR content transiently increases to more than normal, presumably due to formation of extrajunctional AChR in response to the transient denervation caused by phagocytic attack. What triggers and terminates the phagocytic response is not known. During chronic EAMG antibody and complement are present on the postsynaptic membrane in even larger amounts than observed during acute or passive EAMG, but phagocytes are not attracted or activated. It may be that chronic EAMG represents a steady state in which much of the bound complement is inactivated by endogenous processes, but that passive and acute EAMG are nonequilibrium states caused by the sudden deposition of antibody and hence, complement, and the release of chemotactic fragments occurs in significant amounts before it is terminated by a feedback mechanism. There is no obvious equivalent of acute EAMG in human MG, perhaps because the development of anti-AChR antibody is slow and insidious. There is a human parallel of passive EAMG. Infants born to mothers with MG passively acquire a significant fraction of the maternal anti-AChR concentration over the course of gestation, and this is associated with muscular weakness, in some cases, that diminishes over a few weeks after birth as the maternal immunoglobulins are removed. It might be expected that babies should be less severely affected than their mothers, because their AChR metabolism might be operating at more nearly the high foetal rate, permitting them to adapt better to the immune assault.

Chronic EAMG closely resembles human MG. Morphologically, chronic EAMG is characterised...
by the presence of a postsynaptic membrane with reduced numbers and sizes of folds. Phagocytes are not observed. Both antibody and complement are bound to the postsynaptic membrane and to fragments in the intersynaptic space evidently shed in the course of focal lysis. The AChR content of the muscle is reduced to about one-third normal, and most of the AChR which remain have antibodies bound. A decrementing electromyogram typical of MG is observed. Microelectrophysiological studies show normal numbers of quanta released but reduced sensitivity of the postsynaptic membrane, which accounts for a reduction in the size of miniature endplate potentials to about one-third normal.

The muscular weakness characteristic of chronic EAMG can readily be explained by a decrease in amount of active AChR. Simple competitive inhibition of AChR with toxin produces very similar clinical and electrophysiological signs. But this is not the way antibody to AChR acts. Few or none of the antibodies are directed at the acetylcholine binding site. Antisera can directly inhibit AChR function but in general the effects of antibody on AChR function are small. Muscle cells in cultures exposed to anti-AChR and then subjected to acetylcholine noise analysis showed approximately a 23% decrease in AChR channel opening time and a 15% decrease in AChR conductance when open. Because the safety factor for neuromuscular transmission is large, this net 38% decrease in AChR conductance (if all AChR at a normal junction were antibody bound) would not inhibit transmission. This is shown by the observation that normal rats, depleted of the C3 component of complement by treatment with cobra venom factor and then injected with anti-AChR antibodies, have normal neuromuscular transmission despite having at least 67% of their muscle AChR bound with antibody.

Complement-mediated destruction of the postsynaptic membrane subsequent to anti-AChR antibody binding appears to impair transmission in two ways. Focal lysis of the postsynaptic membrane releases membrane fragments containing AChR, antibody, and complement. Thus, lysis contributes directly to loss of AChR. The postsynaptic membrane must reseal very effectively after such lytic attacks, because the resting membrane potential in chronic EAMG is not significantly reduced. Complement-mediated lysis of the postsynaptic membrane is probably also responsible for the destruction of its normal complex folded structure. The folded structure concentrates AChR at the tips of the folds op-posite to specialised sites of acetylcholine release in the presynaptic membrane and away from the acetylcholinesterase concentrated on the basement membrane between the folds. Disruption of this spatial relationship no doubt further impairs transmission, but it is difficult to quantify the effects of focal lysis on neuromuscular transmission.

Cross-linking of AChR by antibody causes their aggregation and internalisation, where they are degraded in lysosomes. In cell culture the internalisation process is energy-temperature and cytoplasmic filament dependent, and requires about an hour and a half before amino acid residues are released from the cell. Lysozomal protease inhibitors can prevent destruction of the internalised AChR. In all these respects, except for the antibody-induced cross-linking and aggregation, the mechanism of antigenic modulation resembles the normal mechanism of AChR destruction. Antigenic modulation accelerates the rate of AChR destruction by two to threefold in both cell culture and organ culture.

Muscle from rats with EAMG does, in fact, destroy its AChR at the accelerated rate, using lysozomal enzymes, as expected of antigenic modulation. Endocytosis may be the rate-limiting step of antigenic modulation. A threefold increase in the rate of AChR destruction would be sufficient to account for the observation that AChR content in the muscles of rats with EAMG decreases to a minimum of about one-third of normal. However, it is unknown whether in vivo there is an adaptive increase in the rate of AChR synthesis from the very low rate characteristic of normal junctional AChR toward the much higher rate characteristic of extrajunctional AChR. Thus, although it is clear that antigenic modulation is a very important mechanism for causing AChR loss and thereby impairing transmission, and quantitatively it might be the most important mechanism, it is not yet possible to quantify its effects with respect to those of antibody dependent, complement-mediated focal lysis.

Concluding remarks

No special anti-muscle AChR antibody specificity appears to be required to cause EAMG. As previously discussed, antibodies against the acetylcholine binding site are not required. Cross-linking of AChR by antibody to trigger antigenic modulation or simple binding of antibody to target fixation of complement should not depend on the part of the AChR molecule to which the antibody binds. In fact, immunisation with any of the four de-
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natured AChR subunits causes EAMG. However, differences in anti-AChR subclass could affect the ability to bind complement. And, in theory, some effects of antibody specificity should exist. For example, antibodies to determinants on the interior of the cell membrane would not be able to bind in vivo, antibodies to determinants in the centre of the molecule might not be able to reach to cross-link AChR, and antibodies to determinants represented twice on the molecule might not effectively cross-link AChR because both antibody binding sites could bind within the monomer. On the other hand, antibodies to the acetylcholine binding site or ion channel opening might be very effective at inhibiting AChR function. Such effects have yet to be demonstrated with monoclonal populations of anti-AChR antibodies, but if they are, should tell us as much about the AChR molecule as about the pathological mechanisms of EAMG.

The discovery of EAMG validated the hypothesis that the weakness characteristic of MG could arise from an autoimmune response to AChR. Further, studies of EAMG provided methods for assay of human AChR, anti-AChR antibody from MG patients, and for development of other techniques which could be applied to human material for the study of MG. It is now evident that MG is in fact caused by an autoimmune response to AChR. Studies of EAMG have proven very valuable in discovering the mechanisms by which the autoimmune response to AChR impairs transmission in MG. EAMG is probably not an especially useful model for studying what causes the auto-immune response to AChR in MG. And EAMG may be of only limited utility in studying therapy of MG because, unlike MG, EAMG does not result from an endogenous self-sustaining process. But EAMG can be a very useful model of autoimmunity for immunological studies. And EAMG, along with MG, can provide an archetype for recognising and studying other auto-immune anti-receptor diseases. Finally, the anti-AChR antibodies produced in EAMG are proving increasingly valuable as probes of AChR structure, function, and metabolism.

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