Experimental Models of Myasthenia Gravis: Lessons in Autoimmunity and Progress Toward Better Forms of Treatment

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The nicotinic acetylcholine receptor (AChR) is a large membrane protein found in muscle cells. It is involved in the transformation of acetylcholine packets into a membrane depolarization, which thereby leads to a muscle twitch. This large, complex molecule is the target of the autoimmune attack in myasthenia gravis, and much has been learned in the past decade about myasthenia by the induction of autoimmunity to AChR in experimental animals. Experimental autoimmune myasthenia gravis (EAMG) has been produced in a variety of animals by immunization with AChR or AChR-like material, or by the passive transfer of anti-AChR antibodies or lymphocytes from afflicted animals into normal animals. EAMG is a remarkably faithful model of human myasthenia and has provided much information about how the immune response to AChR progresses and how weakness and damage to the neuromuscular junction ensue. EAMG has also allowed the development of a number of revolutionary forms of treatment in which only the abnormal response to AChR is restrained, and other necessary immune functions are left intact. These advances in treatment are not far from being tested in human myasthenia gravis. The experience gained in applying these concepts in EAMG and human myasthenia will be helpful in developing similar forms of treatment for other autoimmune diseases.

The study of the experimental model of myasthenia, experimental autoimmune myasthenia gravis (EAMG), has been rewarding because of its great applicability to both experimental and human disease processes. EAMG has also proven invaluable because of insights gained into membrane receptor function, antibody effector mechanisms, and immunoregulation. Myasthenia gravis and EAMG are unique among autoimmune diseases in that: *(1)* the target of the autoimmune attack, the acetylcholine receptor (AChR), has been purified, characterized [1], and sequenced [2,3]; *(2)* the experimental model is remarkably faithful to the human disease [4,5]; and *(3)* a wide variety of ligands, both agonists and antagonists, for the AChR, as well as natural sources of large amounts of AChR (i.e., from electric fishes and eels), is available. Because of the special advantages of this system, experimental myasthenia has been the subject of intensive research over the past decade. Some areas in this research most applicable to the neurologist will be reviewed here.

EAMG: INDUCTION, CHARACTERIZATION, AND DEVELOPMENT

There are four ways that experimental myasthenia has been induced: immunization with acetylcholine receptor, passive transfer of poly- or monoclonal anti-AChR antibodies, passive transfer of AChR-sensitized cells, and immunization with the "internal image" of AChR (anti-AChR ligand antibodies).

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Immunization with AChR

EAMG has been induced in rats [6], guinea pigs [7], rabbits [8], monkeys [9], and mice [4,10] by immunization with AChR. Generally, EAMG induced by active immunization with AChR has been studied using AChR from various animals with electric organs, particularly *Torpedo californica, Electrophorus electricus,* and *Narke japonica;* however, immunization with mammalian AChR has also induced EAMG (e.g., immunization of rats [11] or BALB/c mice [12] with rat AChR). There is a considerable degree of variability in the manifestations of EAMG, ranging from no clinical disease even after multiple boosting immunizations (e.g., many mouse strains) to severe weakness progressing after only one immunization to death (e.g., rabbits).

EAMG has been most frequently studied in rats and mice. After immunization with 3.5 to 350 pmoles (0.8–80 micrograms) of AChR, rats developed a biphasic illness [5], with an initial weakness a few weeks after the initial immunization associated with a cellular infiltrate in the muscle. Later in the course of the disease, the rats were once again weak, but pathology only revealed a flattening of the normally frondlike postsynaptic neuromuscular junction without a cellular infiltrate. The evolution of the two phases of neuromuscular junction damage are not well understood, and a correlate of the early phase of damage has not been found in human myasthenia gravis. The early phase of cellular infiltration has not been seen in EAMG in mice. Another major difference between EAMG in rats and mice is that the severity of weakness in mice is much less than in other experimental animals studied; this is most likely due to variations in the immune responsiveness to AChR between the animals, similar to the variation in the clinical severity of EAMG in different mouse strains [13]. EAMG has been induced in rodents primarily through the use of fish AChR (i.e., the immunogen has not been the actual autoantigen, and the development of clinical weakness thus depends on the extent of cross-reaction of the immune response to fish AChR with rat or mouse AChR). In a study of Lewis rats immunized with *Torpedo* AChR [14], there was a several-week delay in the rise to a plateau of anti-rat AChR antibody compared to anti-*Torpedo* AChR antibody, and the titers of the latter were only 1 percent of the former.

A number of confirmatory tests have been used to support the postulate that EAMG resembles human myasthenia. In both diseases there is: *(i)* a marked decrease in the number of functional AChRs (as detected by bungarotoxin binding to AChR), *(ii)* a decremental response of the muscle action potential to repetitive nerve stimulation, *(iii)* a decrease in the amplitude but not frequency of miniature end-plate potentials, and *(iv)* a dramatic improvement in strength with cholinesterase inhibitors, and worsening with AChR blockers such as snake toxins and curare. The extremely high affinity of snake toxins, such as alpha-bungarotoxin from the Formosan many-banded krait, for the AChR allows the quantification of AChRs at the neuromuscular junction [15]. An electron-dense material such as horseradish peroxidase (HRP) is coupled to alpha-bungarotoxin (BTX), the HRP-BTX is applied to the muscle, carefully washed away, and the electron-dense particles can be visualized at the neuromuscular junction by electron microscopy; their density correlates with the number of AChRs. This loss of BTX binding can also be measured by incubating muscle homogenates with radiolabeled BTX, and expressing AChR content as the number of counts bound per mg of protein. Both electromyography (EMG) and the amplitude of miniature end-plate potentials (MEPPs) measure electrophysiological correlates to the loss of AChR. In

the former [16], repetitive stimulation, usually at 5–20 Hz, is applied to the motor nerve of the human or anesthetized animal, and a recording electrode in the muscle innervated by the nerve detects the response of the muscle. In normal animals a repetitive impulse can be maintained, often for minutes, without a change in the amplitude of the evoked muscle response; in MG and EAMG there is a characteristic drop, or decrement, in the amplitude with repetitive stimulation. In MEPP studies, the frequency and amplitude of the electrical response of muscle membranes in response to spontaneous release of acetylcholine packets is measured; in MG and EAMG there is a decrease in the amplitude but not frequency of the end-plate potentials. Finally, the most frequently used confirmatory test for human myasthenia is the Tensilon test, in which pyridostigmine, a short-acting cholinesterase inhibitor, is injected intravenously, and the clinical response is tested. When weakness in a patient is due to MG, there is usually a dramatic and instant positive response to Tensilon; this type of response is also seen in EAMG. In addition, both EAMG and MG clinically deteriorate by the injection of AChR antagonists, such as d-tubocurare, at doses that do not affect the disease-free subject.

One major difference between EAMG and MG is the absence of thymic abnormalities in the former. The obvious, but untested, corollary to that statement is that the initiating abnormality in MG is in the thymus, and the damage to the nicotinic AChR of muscle is secondary. "Myoid" tissue with BTX-binding AChR has been found in the thymus [17], and the induction of the anti-AChR autoimmunity of MG in the thymus has been postulated [18]. The immunological activity of the thymus in myasthenia is deranged; histology of this organ in the majority of myasthenics is abnormal, and staining of the thymus reveals that both B- and T-lymphocytes are grouped together in patterns identical to those of lymphoid follicles. These cells are able to make anti-AChR antibody *in vitro* [19]. The hypothesis that the thymus is crucial for the induction of myasthenia is attractive but remains unproven.

Induction of Experimental Myasthenia by Passive Transfer of Antibody

One of the most convincing early pieces of evidence that the pathogenesis of myasthenia gravis was antibody-mediated effects on the AChR was the production of the disease in rodents by transfer of serum from myasthenics into mice [20] and rats [21]. Since that time, the development of techniques for producing monoclonal antibodies [22] has led to the production of monoclonal anti-AChR antibodies in a number of laboratories [23,24,25,26]. These monoclonal antibodies (mcAbs) were then injected into animals; some mcAbs produced weakness, and some did not. Although it was hoped that the *in vitro* characteristics of the monoclonal antibodies (that is, affinity, determinant on the AChR to which they bound, subclass) could be correlated with the *in vivo* effect, this turned out not to be the case. Monoclonal antibodies directed against a number of different epitopes are able to induce disease; some investigators feel that there seem to be at least two types of disease induced, depending on the specificity of the mcAb used [27]. The "hyperacute" type is characterized by the development of weakness within a few hours after injection of the mcAb and is not associated with inflammatory infiltrates within the muscle. This form of passively transferred myasthenia is induced by mcAbs directed against the acetylcholine binding site of the AChR, which is very close or identical to the bungarotoxin binding site [27,28]. This group of anti-AChR antibodies is especially

interesting clinically because it is not tested for in the routinely available clinical assay (i.e., since anti-AChR antibodies are generally measured by immunoprecipitation using AChR labeled with radio-iodinated BTX, antibodies of this specificity are prevented from binding in this assay and are not measured). The other type of experimental myasthenia passively transferred with mcAbs resembles that induced with polyclonal antisera; it develops more slowly and is associated with inflammatory lesions at the endplates [26,29]. This form of myasthenia is caused by mcAbs directed against sites on the AChR other than the BTX-binding site. A problem further complicating the situation is a marked difference in sensitivity to mcAb passive transfer of EAMG among various animals. These differences cannot be predicted by *in vitro* studies. For example, the monoclonal anti-AChR antibody, 5.5, which has activity *in vitro* against both mouse and chick muscle [30], induces paralysis and death on injection into chicks [28] but has no effect at all upon mice, even with huge intravenous doses [31].

Passive Transfer of AChR-Sensitized Cells

Experimental myasthenia has also been produced by transfer of cells from animals with EAMG to naive animals [18,32,33]. These experiments are generally difficult and complicated, and the efficacy of cell transfers has been low, especially relative to antibody transfers. All experiments in which successful transfers occurred included both B cells and T cells, sometimes associated with immunization with AChR. Certainly, direct effector T-cell mediation of AChR damage seems unlikely, since highly purified anti-AChR T cells at relatively high concentrations were completely ineffective in inducing disease in rats [18]. Many objections could be raised to the conclusions, based on these studies, that there is definitely no direct effect of T cells or their products on the AChR, but certainly the major effector of pathogenicity seems to be antibody. The predominant contribution of the T-lymphocyte to this process is most likely its control of the quantity and quality of anti-AChR antibody production (see below) [34].

Induction of Experimental Myasthenia by Immunization with the "Internal Image" of AChR

Jerne's network hypothesis postulates the existence of a subset of anti-(antibody) antibodies, called anti-idiotypic antibodies, that are structurally related to the original antigen; he labeled this population the "internal image" of the antigen. In a number of receptor systems [35,36], antibodies to receptor ligands have been shown to mimic the receptor in that injection of these anti-ligand antibodies has been able to induce anti-idiotypic antibodies that bind to the receptor (i.e., mimic ligand). In a series of experiments using bisQ, a structurally constrained ligand of the AChR, anti-AChR antibodies and EAMG have been induced by immunization with anti-bisQ antibodies [37,38]. We extended this work by using a naturally occurring ligand of the AChR, cobratoxin [39]. Rabbits and mice were immunized with affinity purified anti-cobratoxin antibodies. All animals developed anti-AChR antibodies, and some developed weakness and electromyographic abnormalities characteristic of EAMG. The study of this disease will be greatly facilitated by the use of a monoclonal anti-neurotoxin antibody that is the "internal image" of the AChR [40].

EAMG: IMMUNOREGULATION

When purified *Torpedo* or *Electrophorus* AChR is injected into mice or rats, it behaves like other soluble protein antigens. The antibody it induces is thymus-dependent [41], and both AChR-specific helper-proliferative T cells [42] and suppressor T cells [43] participate in controlling the production of anti-AChR antibody. Shortly after immunization, helper-proliferative T cells appear in the lymph nodes draining the sites of immunization, along with B cells, either those with anti-AChR immunoglobulin on their surface or those which have matured into anti-AChR antibody-producing plasma cells. AChR-specific B cells *in vitro* are responsive to T-cell help, which can be provided either by the T cells themselves or by supernatants from AChR-specific helper-proliferative T-cell lines [42]. The response is dependent on the presentation of AChR on the surface of macrophages to T cells [44]. The AChR-specific helper-proliferative T-cell response can be measured in a number of ways: degree of proliferation in the presence of soluble AChR, amount of help provided for primed B cells to make anti-AChR antibody, AChR-induced lymphokine release [58], or swelling when AChR is injected into the ear or foot [41,45]. I-A antigens on the surface of macrophages are involved in the generation of the anti-AChR response, and administration of anti-I-A monoclonal antibodies decreases the amplitude of the response [46]. Suppressor cells specific for the AChR appear [43,47,48], although the stimuli for their generation and optimal function are unknown. It seems likely that the differences in EAMG among different mouse strains are related to variations in immunoregulation [13], particularly suppressor T cells.

Our current knowledge about immunoregulation in EAMG has been gained by focusing on the early events after eel (*Torpedo* or *Electrophorus*) AChR immunization. The antigen used in these *in vitro* studies has been eel AChR, which is readily available and easily purified. The *auto*immune response is of more interest, however, than that generated against mouse AChR. This response is difficult to measure accurately because purified mammalian receptor is very difficult to obtain in large enough quantities to study. Generally, the anti-rodent AChR antibody response is measured by immunoprecipitation of muscle homogenates labeled with iodinated bungarotoxin. The amount of antibodies present in the serum measured in this way is at least one hundred times lower than the antibody content directed against *Torpedo* AChR, even at the height of muscle weakness. T cells require more purified antigens for stimulation experiments, and thus, an adequate study of T-cell responses to mammalian AChR in EAMG has not been done. Recently, synthetic peptides of the AChR have been produced which represent areas of the molecule that are able to induce EAMG upon immunization [49,50]. These peptides are portions of the AChR molecule that function in snake toxin binding; their amino acid sequences are highly preserved among species. The immune responses to these peptides are probably important in the induction and propagation of myasthenia, and their study will provide valuable information about immunoregulation in EAMG and MG.

NOVEL FORMS OF TREATMENT SUCCESSFUL IN EAMG AND THEIR APPLICABILITY TO HUMAN MG

All current forms of treatment of MG are "antigen non-specific"; that is, despite the fact that it is known that the AChR is the autoantigen in this autoimmune disease, no

available therapy makes use of that knowledge. EAMG is an ideal model to test new forms of treatment, especially "AChR-specific" ones in which only the immune response to AChR is blunted, and not immunity to other antigens. The two most promising areas of work center around suppressor T cells and anti-idiotypic antibodies. (Some interesting "AChR non-specific" forms of therapy have been attempted, such as treatment with monoclonal anti-I-A antibodies [46] or monoclonal anti-helper T-cell antibodies [51].)

It has been shown that suppressor T cells suppress the immune response to the AChR *in vitro* [43,47,48]. In the only *in vivo* study to date, AChR-specific suppressor T cells, injected into mice prior to immunization with *Torpedo* AChR, diminished the anti-*Torpedo* AChR antibody titer and electromyographic evidence of disease but had no effect on the immune response to an irrelevant antigen [43]. This specificity is highly desirable, as all AChR non-specific forms of immunosuppression will suppress beneficial forms of immunity (e.g., to pathogens and tumors). Recent studies on AChR-specific suppressor T-cell hybridoma factors that are active *in vitro* raise the possibility that such purified material may be used *in vivo* [52]. Suppressor T cells and factors most probably act on the AChR-specific helper-proliferative-inducer cells and function in blocking the ability of these cells to help B cells make antibody against AChR. There are two major stumbling blocks to the rapid expansion of this work and its application to human disease. First, unlike B-cell products (i.e., antibodies), there are no rapid screening assays for T-cell products such as suppressor factors. Suppression must be tested on T-cell functions such as proliferation or *in vitro* help, assays which require five or six days to develop and only allow a limited number of products and conditions to be tested. Second, very little is understood about antigen-specific T-cell factors, especially in the immune response to complex proteins; most studies have been performed with antigens such as haptens or sheep red blood cells. These problems, however, are purely methodologic, and it is likely that T-cell suppression will prove applicable to therapy of myasthenia and other autoimmune diseases.

Anti-idiotypic antibodies are really autoantibodies in that they are directed against a "self antigen" (i.e., idiotypic determinants, present on the antigen combining portion of immunoglobulin). Anti-idiotypic antibodies in the anti-AChR response, thus, are those antibodies with specificity for the portion of anti-AChR antibodies that are involved with AChR binding. A commonly used and well-accepted assay for anti-idiotypic antibodies is blocking of binding of the idiotype to the antigen (i.e., in the AChR system, blocking of the binding of anti-AChR antibodies to AChR). A number of investigators have studied anti-idiotypic antibodies in MG and EAMG [53,54,55,56,57]; their results and conclusions vary depending on the system studied, particularly whether the anti-idiotypic antibodies were developed against a polyclonal versus a monoclonal preparation. In the only successful experiment with anti-idiotypic antibodies *in vivo,* ongoing myasthenia, induced by passive transfer of a monoclonal antibody, has been reversed by treatment with polyclonal anti-idiotypic antibodies [28]. This success in a monoclonal system augurs well for successful treatment in the polyclonal response, since anti-idiotypic antibodies to polyclonal anti-idiotypic antibodies have been produced in a number of the above-referenced studies. Again, the barriers to the application of these techniques to the human disease are purely methodological, and anti-idiotypic antibodies may well find a useful place in antigen-specific therapy for myasthenia gravis and other autoimmune diseases in the future.

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