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ACETYLCHOLINE NICOTINIC RECEPTOR: A KEY MOLECULE FOR THE PATHOGENESIS OF MYASTHENIA GRAVIS

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Summary. - *In this review we will describe in detail the structure of the nicotinic acetylcholine receptor, its composition and the functional role of its subunits, the probable site of the acetylcholine binding and the models of the ion channel. The immune response against the receptor, both in the experimental animals and in humans, will be reported and the biological and clinical significance of antibodies against the acetylcholine receptor in the experimental and human myasthenia gravis is discussed. Finally some possible mechanisms by which the autoimmune response against the receptor is maintained in animals and in humans is reviewed.*

Riassunto (Il recettore nicotinico per l'acetilcolina: una molecola chiave per la patogenesi della Miastenia Grave). - *In questo articolo descriviamo in dettaglio la struttura del recettore nicotinico per l'acetilcolina, la sua composizione e la funzione delle sue diverse subunità, il probabile sito di legame per l'acetilcolina e i modelli presunti del canale ionico. Viene descritta la risposta immunitaria verso questo recettore, sia in modelli animali sperimentali, sia nell'uomo e vengono discussi il significato biologico e clinico degli anticorpi diretti contro il recettore nicotinico. Infine vengono descritti alcuni possibili meccanismi mediante i quali la risposta autoimmunitaria contro il recettore nicotinico viene mantenuta sia nell'animale che nell'uomo.*

Introduction

The history of the nicotinic acetylcholine receptor (AChR) is fascinating both for the implications in cellular and molecular biology of membrane receptors and for unraveling the pathogenesis of Myasthenia gravis and, in general, of autoimmune receptor diseases. Myasthenia gravis (MG) is an autoimmune disease, characterized by a progressive failure of the neuromuscular transmission, which induces a paralysis of several muscles and finally of the diaphragm. It is a rare disease that in Italy affects about 3,000 patients. As it often happens in science, favourable circumstances cooperated for the beginning and the rapid development of this story. First of all the tools, as the electric lobes of fishes constituting a large source of receptor (AChR), the α-Bungarotoxin (αBgtx) with its high affinity for AChR, the technique of affinity chromatography, protein chemistry, and later the techniques of aminoacid sequencing and recombinant DNA; secondly the clinical understanding and careful analysis of the symptoms of MG and, finally, the serendipidy and profound cultural background of two researchers, Patrick and Lindstrom, who correlated their serendipitous findings with the clinical reality of MG.

The methodological approaches used to purify and analyze the structure and function of AChR were after applied with minor modifications to the study of several other membrane receptors. And, furthermore, the rationale linking AChR to MG was used for understanding other autoimmune diseases correlated with receptor pathology such as autoimmune diabetes, thyroiditis and so on.

In this review we will describe briefly the structure and molecular biology of the nicotinic receptor, the experimental myasthenia gravis (EMG) and the autoimmune aspects of MG. We will correlate the presence of antibodies (Abs) against AChR with the muscular weakness and with the loss of AChR observed in MG and EMG; we will finally try to clarify the mechanisms of action of Abs against AChR.

Nicotinic receptor structure

The nicotinic receptor is a transmembrane protein which is present on skeletal muscle cells and several neurons. Its activation by acetylcholine (ACh) released from nervous terminals, induces an influx of cations and depolarization of the cell [1]. In the case of neuromuscular junction (Fig. 1) the depolarization induced by the interaction of ACh with AChR triggers an action potential that spreads over the muscle membrane and ultimately causes muscle contraction. The receptor, thus, does contain a binding site for ACh and a channel

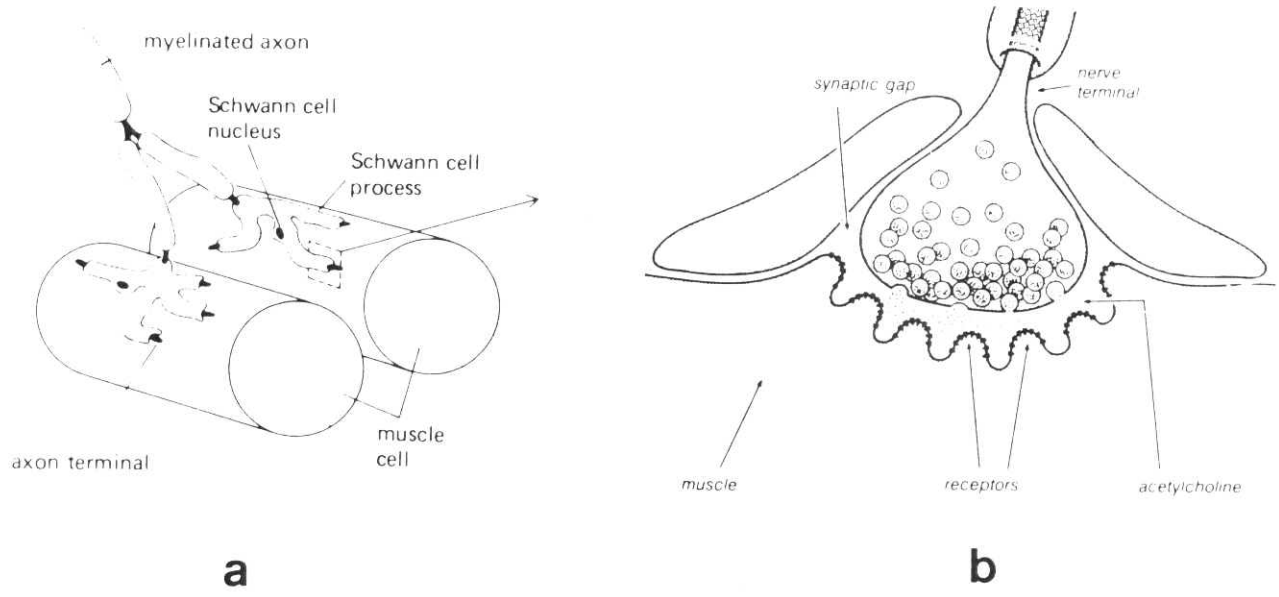

Fig. 1. - Neuromuscular junction: a) each incoming nerve fiber contacts its target muscle cell at multiple sites called "synapses"; b) from each activated nerve terminal acetylcholine is released and interacts with specific postsynaptic receptors (AChRs).

through which ions can pass. The opening of the latter is modulated by ACh binding. AChR is the first receptor characterized as a molecular entity, isolated in its active form [2], and that, inserted in an artificial membrane system, maintains completely its original functionality [3]. Studies on this receptor were possible because of the presence in nature of two important tools: electric fishes, as torpedo or electrophorus, and elapid snake venoms. The electric organs of these fishes consist of modified muscle cells and have a very high concentration of AChR (10,000/μm^2), which are very similar to those present on mammalian muscles [4, 5]. On the other hand, toxins, purified from the venom of bungarus or naja snakes, have high-affinity and high-specificity for AChR [6]. The combination of these two tools allowed receptor localization, quantification and purification.

AChR isolated from torpedo and mammalian muscle is a pentameric protein composed of four subunits, two α, one β, one γ and one δ (Fig. 2). Each subunit spans the membrane and joins the others to form a central channel. The receptor pentamer extends approximately 70 Å into the synaptic cleft and approximately 30 Å into the cytoplasm. The AChR is 140 Å long and 80 Å in diameter; the pore orifice is 30 Å in diameter at the synaptic end, while narrows in the cytoplasmic part [7, 8]. At the neuromuscular junction the AChR is concentrated at the tips of postsynaptic infoldings and organized in a paracrystalline way [2].

Physical properties of the receptor

The physical characteristics of AChR purified both from the electric organ or from the muscle are very similar. AChR is an acidic protein with an isoelectric point (IP) around 5 [2]. In the case of AChR from muscle and electrophorus two types of receptors with slightly different IP were described and, on the basis of several experimental evidences, it was suggested that the more acidic represents the junctional, while the less acidic the extrajunctional receptor [9, 10].

Aminoacid composition of receptors obtained from various tissues is remarkably similar [2]; all subunits contain carbohydrates [11].

AChR can be solubilized from torpedo electric organs in a monomeric form (sedimentation coefficient 9S) and in a dimeric form (sedimentation coefficient 13S). In the

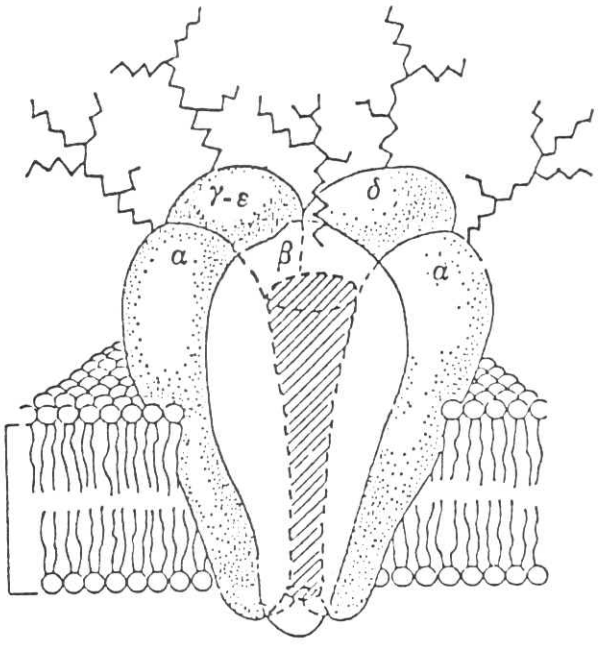

Fig. 2. - Three-dimensional model of AChR from Torpedo. The subunit locations around the channel are tentative assignments.

case of the muscle the receptor is present almost exclusively in the monomeric form [12]. The significance of the presence of both monomeric and dimeric form of AChR in torpedo membrane is unknown since some workers have shown that monomers and dimers are functionally identical [13, 14] while others have found that they are not [15, 16].

The molecular weight of AChR, determined by different physical and chemical methods, as X-ray diffraction, sedimentation at equilibrium, gel filtration and ligand cross linking, is around 250,000 daltons for the monomer and 500,000 daltons for the dimer [2, 11].

Receptor subunits

Torpedo AChR purified by affinity chromatography was shown to be composed of four polypeptides (α, β, γ and δ) with a molecular weight determined by SDS-PAGE of 40,000, 50,000, 60,000 and 65,000 daltons, respectively. AChR from mammalian muscle has the same number of subunits with approximately the same molecular weight [2, 11, 12, 17-19]. Using NH_2 aminoacid sequence analysis it was demonstrated that the α, β, γ, δ subunits are present in AChR in a stoichiometry of 2:1:1:1 and that the aminoacid sequence of the 4 subunits has a very high degree of homology having in the case of torpedo AChR 11, identical aminoacids out of the first 54 aminoacids, while in many other positions either 2 or 3 of the residues are identical or related [20]. Knowledge of the partial aminoacid sequence of each subunit enabled cloning and assaying of cDNA produced by reverse transcription of mRNAs isolated from torpedo electroplax and mammalian muscle. In this way the complete aminoacid sequence of torpedo and muscle AChR subunits was determined [12, 21-26]. From this study it turned out that the sequence omology between α chains of human and calf muscle was 97% and 80% between human and torpedo α chains.

These experiments demostrate that AChR is constituted by subunits which are different but highly homologous and that this molecule appeared very early in the phylogenesis and was conserved in its essential structure until now. This suggests that the cholinergic system was one of the first mechanisms of cell communication and that from its beginning the AChR molecule was so accurately designed for its work that remained practically unmodified during evolution [27].

The molecular weight of the subunits as determined by their aminoacid sequence is: α 53649, β 56060, γ 58053, δ 59792 daltons. The difference between the molecular weights obtained from the aminoacid sequence and those predicted from the isolated subunits may be due to post translational processing or damage of subunits during the purification process. Using the cDNA approach it was also cloned a DNA complementary to muscle mRNA encoding a novel polypeptide, called ε subunit, which shows higher sequence homology with the γ subunit than with the other subunits [28].

The α subunit which is present in two copies in each receptor is the one which binds ACh, competitive antagonists and α-Bungarotoxin. For receptor activation it is necessary that ACh binds to both subunits. The site of binding of cholinergic agents is not yet clearly defined in the aminoacid sequence, but it has been known from several years that covalent modifications of reduced subunits by affinity reagents permanently blocks agonist and some antagonist binding sites [29]. Recently the target cysteine residues of the reduction have been identified as Cys 192-193 of the α subunit [30], and the sequence flanking these cysteines is unique to the α subunit [12]. The non competitive blockers bind to a variety of sites on different subunits [26], a number of which are allosterically coupled to the ACh binding site [31, 32]. On the α subunit in its extracellular domain but distinct from the region where ACh binds or sugars are bound, there is a region called MIR (main immunogenic region) against which is directed the majority of antibodies raised in animals or present in myasthenic patient [33, 34].

During the last years the synthesis of subunits of the AChR was also studied in cell free systems. The results of these studies demonstrated that receptor subunits have a signal sequence, are synthetized on membrane bound ribosomes and are cotranslationally glycosylated. *In vivo* only about 30% of the α subunits synthetized are incorporated into mature receptors and the remainders are rapidly destroyed. Blockade of glycosylation inhibits the assembly of receptor subunits. The subunits are assembled to form mature receptors in 60 min after the synthesis and another 30-60 min are required for the transport of mature receptor through the Golgi apparatus to the surface and for the insertion in the membrane [35-38]. The ability of α subunit to bind αBgtx or curare with high affinity is not acquired until the subunits are assembled into their mature $\alpha 2\beta\gamma\delta$ stoichiometry.

Molecular biology of AChR

The knowledge of the aminoacid sequence of all AChR subunits allowed the possibility to explain how subunits are organized in the membrane and which is the relationship between their structure and function. Although each subunit has a distinct aminoacid sequence and is coded by a separate gene, all the subunits share the same basic structure. The amino terminal of each subunit is extracellular and the carboxy terminal, according to some authors, passes five times across the membrane. Four of these membrane spanning segments are hydrophobic (denoted M1 to M4) and one (MA) is amphipatic; according to these authors [39, 40], each of the 5 subunits contributes an amphipatic helix to form the charge-lined walls of a water filled pore through which ions pass. The hydrophobic helices are packed around this pore to stabilize the structure (Fig. 3, model A). Other workers using immunochemical and electron microscopic methods clearly demonstrated that the C terminal portion of the subunits is cytoplasmatic; they also found that the segments spanning the membrane are 7, 4 of them are hydrophobic helices embedded in the

membrane, the other 3 segments are amphipatic domains lining the pore [41] (Fig. 3, model B).

In order to better understand the relationships between the structure and the function of the AChR, mutations of the DNA clones of the different subunits have been made and the function of this altered AChR studied using as a cellular translation system the Xenopus oocytes. When injected into the oocyte, mRNAs derived from a set of AChR clones, are correctly translated and the protein correctly assembled and inserted into the membrane. The physiological and pharmacological properties of the AChR can be then investigated using conventional techniques. Mishina *et al.* [42] used this approach to generate and characterize AChR mutants derived from cDNA clones altered by "site directed mutagenesis" of the subunits. They showed that certain regions of the sequence are very important for different functions. Substitution of cysteine residues 130 or 144 for serine eliminated ACh induced ion flux and reduced

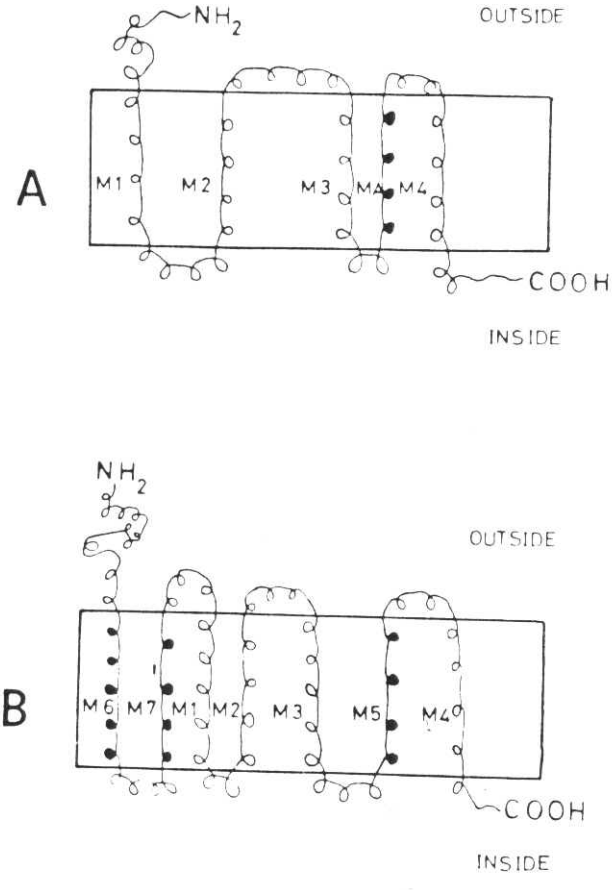

Fig. 3. - Diagrammatic representation of two different schemes for the organization of the α subunit of the nicotinic AChR in the membrane. In scheme A there are four transmembrane alpha helices, M1-M4, and a charged chain MA traverses the membrane on the pore face of the subunit [39, 40]. Scheme B has seven membrane spanning segments, segments M1-M4 are alpha helices embedded in the membrane, segments M5-M7 are amphipathyc domains that line the pore [41]. In both schemes the N terminus of the subunit is extracellular and the C terminus is in the intracellular space.

αBgtx binding. Replacement of cysteines by serine at position 192-193, destroyed ACh induced ion flux sensitivity. Substitutions of 5-10 aminoacids in M1, M2, M3 resulted in no detectable ACh sensitivity and had a profound effect on αBgtx binding, possibly by affecting assembly of the AChR complex. Almost all the substitutions in the region of the amphipatic helix MA, which may contribute to ion channel formation, eliminated completely ACh sensitivity, while reducing αBgtx binding by only 56-70%. Omission of the mRNA for the α subunit prevented αBgtx binding and omission of any subunit prevented the formation of the functional channel [43].

An other approach to studying the functional roles of the individual subunits in ion transport and gating of AChR, is to construct, using cloned cDNA, hybrid AChR molecules with subunits from different species and comparing their channel properties with those of the parental AChR. Using this technique it was demonstrated that the α and δ subunits govern the channel open time; the authors postulate that the δ subunit controls the rate of channel closing and that the α subunit exerts its effect via some other mechanism, perhaps by controlling ACh dissociation [44].

As we told before, one of the most brilliant recent result of the molecular genetic approach to the study of AChR was the discovery of a new type of subunit, called the ε subunit. There are evidences that this subunit is expressed differently during differentiation. For example, different types of AChRs are present during muscle development. AChRs on newly formed skeletal myotubes are diffusely distributed and have a rapid turnover, while AChRs at mature endplates are highly localized and metabolically stable. These two receptor types differ also in conductance and gating properties [45]. It was demonstrated by using cDNA recombinant techniques that in adult muscle there is a high expression of the ε subunit which has a high homology with the γ subunit [28]; the mRNA for this ε subunit is present only postnatally while during the gestation time only mRNA for the γ subunit is present. These results suggested that the embryonic and adult receptors are two distinct molecules, the embryonic AChR, with an $\alpha 2\beta\gamma\delta$ subunit composition, adult AChR with an $\alpha 2\beta\varepsilon\delta$ subunit structure [46].

With experiments of microinjection in Xenopus oocytes of various combination of the ε or γ subunit specific mRNAs it was clearly demonstrated that the change in conductance and gating properties of AChR is a result of a molecular shift in the expression of the two subunits [47] and that, during muscle development the γ subunit is replaced by the ε subunit and the resulting receptors are molecularly and functionally different.

Experimental myasthenia gravis

The first experimental support to the clinical suggestion that immunity against AChR could be involved in the pathogenesis of myasthenia gravis came from the classical experiments of Patrick and Lindstrom in 1973 [48]. These authors immunized rabbits against AChR

purified from eel electric organs and observed, in those animals that synthetized Abs against AChR, a flaccid paralysis that was exacerbated by exercise and could be reversed by anticholinesterase agents. After the first report of Patrick and Lindstrom, several authors have reproduced this pathology in different animals using AChR from different sources, and from those reports a complete description of EMG emerges [49-56].

Symptoms

Muscle weakness affects preferentially the neck and anterior arms, and progressively abdominal muscles and those of posterior legs; eventually the animals exhibit extreme respiratory distress and die. If the animal survives (because of the pharmacological treatment with anticholinesterase or because of the less severity of the respiratory distress) it recovers, and appears normal [49]. The electrophysiological recordings from muscles of the diseased animals show a decremental electromyogram in nearly all the fibers examined and an increased sensitivity to curare. In all the animals affected it is possible to measure anti-AChR antibodies and their titer roughly correlates with the severity of the disease.

A pathology similar to that described in rats has been induced in several animal species as rat, mouse, chicken, frog, guinea pig, sheep, goat, and monkeys, using both torpedo, eel or mammalian purified AChR, or crude fragments of electric organs of fishes particularly enriched in AChR. In all these species the simptomatology and the clinical course of the disease is similar albeit with minor differences which do not contradict the general picture.

In all animal species, but especially in rats and rabbits, if the animals survive to the acute phases, they go in a remission phase which can last for several days and, after, they enter in a chronic progressive EMG that can last for weeks, with recurring exacerbations and sometimes remissions [49, 56, 57]. This second phase of the disease is the most similar to human myasthenia gravis.

Neuromuscular junction

The two most characteristic findings in the experimental immune myasthenia gravis (EMG), both in the acute and the chronic phase, are 1) the decreased sensitivity to AChR of the affected muscles [50] with a decreased number of AChRs at the end plate [58-60], while the presynaptic part of the neuromuscular junction seems to work in the proper way [61]; this strongly suggests that the main reason for the neuromuscular failure resides in the postsynaptic membrane, and 2) the presence of anti-AChR Abs with a correlation between the amount of anti-AChR Abs and the severity of the disease.

The ultrastructural aspect of the end plate well correlates with the decreased number of AChR. In the acute phase a general derangement of the endplate region occurs with a large infiltration of macrophages and lymphocytes, a decreased number of postsynaptic folds, a decreased area of postsynaptic membrane occupied by AChR and an increase of cellular debris in the synaptic cleft [58]. In the chronic phase the inflammatory response is not present as a relevant feature but still the endplate have a reduced area and very simplified postsynaptic folds [49, 59]. In this phase, as in human MG, a complex process of denervation and reinnervation occurs [49], but no biochemical or functional markers of denervation such as an increase of extrajunctional AChR and membrane depolarization appear [50].

Receptor turnover

The reduced number of AChRs in the endplate could be due to a decreased synthesis and/or membrane insertion of AChR, or to an increased degradation of AChR, or both.

In chronic EMG in rats it has been found that the rate of synthesis of AChR in the diaphragm and in the leg muscle is decreased [62]; however, in the other animal models and *in vitro* models of EMG this finding has not been clearly seen [63, 64]. On the contrary what is dramatically increased is the rate of AChR degradation both in the endplate and in extrajunctional membrane [65, 66]. The mechanism of AChR removal from the synaptic membrane has been extensively studied both *in vivo* [67, 68] and *in vitro* [63], and the rate of removal is in good correlation with the titer of antibodies against AChR. It has been calculated that only the increased rate of degradation may be sufficient to explain the decreased amount of AChR in the neuromuscular junction.

Humoral immunity

In all animals that show signs of EMG antibodies against AChR are present, first of the IgM type and, later, predominantly of the IgG class [69]. The immune response is thymus dependent; nude mice do not develop myasthenic symptoms after AChR immunization [56, 69, 70], thymectomy delays the appearance of EMG, and reconstitution experiment showed that T cells are important for the development of EMG [69]. The immune response is, in the acute phase, mainly against the AChR used for the immunization and the severity of the disease is, in general, proportional to the titer of antibodies and to their crossreactivity with syngenic AChR. Furthermore the kinetics of autoantibody response correlates well with that of AChR loss. With the progression of the disease the amount of Abs crossreacting with syngenic AChR increases and, inversely, Abs reacting with the original antigen used for the immunization decrease [71]. This period of EMG is very important and peculiar because we observe the transition from a classical immune response against a foreign antigen, to a real autoimmune disease. This phase of EMG, that now can be named experimental autoimmune myasthenia gravis, is particularly similar to the human MG. The relationship between disease and antibodies is further

stressed by the following experimental data: a) passive transfer experiments: it is possible to induce an acute form of MG in several animal species injecting IgG from animals with EMG or from patients affected by MG [72, 73]. The injected IgGs reach the neuromuscular junction and bind to the postsynaptic membrane [74], the degree of muscular weakness induced by injected Abs is proportional to the dose of transferred IgG [75] and the transferred disease is very similar to the acute phase of EMG [72]; b) neonatal myasthenia, both in humans and in animals, that is a case of natural transfer of EMG. In rats IgG are transferred to the pup transplacentally and through colostrum and milk. Anti-AChR Abs and the muscular weakness in newborn rats disappear shortly after weaning with a similar kinetics [76]. Similar data have been found in newborn from myasthenic mothers [77, 78].

All the mentioned results support the hypothesis that anti-AChR Abs are related with the pathogenesis of myasthenic symptoms, and thus with the decreased number of AChR at the neuromuscular junction. How Abs interfere with the function and turnover of AChR will be discussed in another section of this review.

Cellular immunity

We have mentioned that the humoral response against AChR is a thymus dependent response and that T cells are necessary for its development. Peripheral T lymphocytes responsive to AChR have been shown in myasthenic guinea pigs, rats, mice and monkeys [54, 79-81]. The cellular response after immunization have a time course similar to that of muscle weakness. Furthermore delayed type hypersensitivity against AChR has been reported by several authors in myasthenic rats [69]. EMG can be transferred in recipient healthy animals by lymphnode cells of animals immunized with AChR [69]. All these data suggest that T cells play a role in the development of pathologic lesions in EMG and this is again another similarity between EMG and human MG.

Genetic control

The immune response against AChR and the ability to develop EMG is strictly genetically controlled, at least in mice where this process has been particularly studied. The genes that control this immune response are localized in the I-A subregion of the H2 [81], although some experiments suggest that the ability to form Abs against AChR is also controlled by a gene locus separated from H2 [82]. Furthermore mice and rats can be divided in "high" and "low" responder strains as far as the possibility to express the symptoms of clinical disease [82, 83]. This genetic control, both of the immune response and the susceptibility to the disease, is also found in humans where there is a correlation between the presence of MG and specific loci of the MHC [84].

Myasthenia gravis

Until the sixties, different theories have been put forward to explain the pathogenesis of myasthenia gravis. Simpson, on the basis of clinical and comparative studies, proposed in 1960 that MG could be an autoimmune disease, caused by an antibody against an "end plate" protein [85]. Meanwhile, autoimmune pathogenesis was being (not easily) accepted for an increasing number of diseases, many of which had been also described in association with MG, as mixedema, Hashimoto disease, sarcoidosis, vitiligo, hemolitic anemia, nephritis, hepatitis, pemfigo and systemic lupus erythematosous. Simpson theory remained only an assumption until 1973 when as we have discussed above, the studies of Patrick and Lindstrom draw again the attention to the immunological model.

Patrick and Lindstrom [48] wanted to study the α-Bungarotoxin binding protein of fish electric organs and injected this protein into rabbits to obtain antibodies. They had two important results: on one hand these antibodies were able to block the action of ACh on the electroplaque; on the other hand the injected animals, few weeks after the injection, developed muscular weakness with the characteristics of MG.

As we have discussed before, this first report was the beginning of a large number of works on experimental autoimmune MG, but stimulated also studies on the immunological mechanisms involved in human MG. In particular the experiments that gave a substantial contribution to the autoimmune pathogenesis were the detection of anti-AChR Abs in myasthenic patients, the passive transfer experiments, the role of thymus and T cells in the disease and, lastly, the therapeutic effect of immunosuppressive therapies.

Humoral immunity

In 1976 it was reported that patients had a remarkable remission after drainage of lymph from lymphatic duct, and relapsed promptly if their cell-free lymph was reinjected [86].

In 1977 Toika *et al.* [73] described the passive transfer of MG from man to mouse by injecting i.v. in healthy mice human myasthenic serum. The disease provoked was similar to the acute phase of EMG and presented some electrophysiological and morphological characteristics present in the exacerbations of MG.

The first assays developed to directly measure the antibody in myasthenic serum was based on the inhibition of α-Bungarotoxin binding to rat extrajunctional [87] or to human junctional [88] AChRs. A complement fixation assay was also developed [89], but all the above assays had a very low sensitivity. Only in 1976 Lindstrom *et al.* described an immunoprecipitation method which allowed the quantitation of antireceptor antibodies in myasthenic sera [90]. This method had some drawbacks (for example it did not measure antibodies directed against the αBgtx binding site, and it measured also

antibodies against the cytoplasmic domain of the receptor which are probably not pathogenetic), but it has been, in any case, the basis for the big deal of research on humoral immunity in MG. Recently it has been proposed to substiute human AChR with AChR extracted from fetal calf muscles with very reproducible results [91].

Anti-AChR antibodies are present in 90% of myasthenic patients [92] while are never found in healthy people or in other pathologies, with very few exceptions. The percentage of positive responses varies depending on the source of the antigen, but can become almost 100% if suitable methods are used which can detect the antibodies against the αBgtx binding site (occupied by the toxin in the standard assay), or by using antigen purified from the type of muscles which are more affected (i.e. the AChR from ocular muscles in pure ocular myasthenic patients) [92].

Most of the authors find that the correlation between the amount of anti-AChR Abs and clinical severity is rather poor [93] although recent reports on a very large number of patients have shown a significative correlation between Abs titer and severity of the disease. In any case in the single patient the detection of these antibodies has an important diagnostic and prognostic value. In fact it is not the absolute value of Abs titer which is important, since we can have severe affected patients with a low titer and viceversa patients with a high titer and a modest pathology (for the reasons that will later be shown) but the modification of the relative titer of Abs, since in all patients this is followed by a modification of the clinical status. Thus Abs are a good marker for following the clinical status of the single patient but cannot define *per se* the clinical status of a myasthenic patient. The lack of correlation probably comes from the difficulty we still have for a correct classification of myasthenic patients, from the individual capacity in counteracting antibodies effects at the muscle end plate, from the different ability to increase AChR synthesis, and mainly in the demonstrated heterogeneity of anti-AChR antibodies, differing in crossreactivity with AChR from different species [55], affinity for the AChR [94], and proportion of the component directed against the αBgtx binding site [34]. Efforts to study in the single patient the specificity of anti-AChR antibodies and try to correlate it with clinical severity or clinical type can bring new information on the different mechanisms eventually involved in inducing myasthenic symptoms. Recent papers have tried to identify different patient populations with respect to their antibody specificity, using the ability of monoclonal antibodies to compete with the binding of myasthenic antibodies to different regions of the AChR. While some authors found no correlation between antibody specificity and different clinical parameters [34], more encouraging results were recently obtained by Whiting *et al.* [95] using anti-human AChR monoclonal antibodies to identify different specificities in myasthenic sera. They actually found that some specificities are present in some subclasses of patient more than in others, although more works has to be done to demonstrate the pathological importance of these findings. The difficulty to found a direct link between clinical severity and the titer of anti-AChR Abs may perhaps indicate that antibodies are only an epiphenomenon instead of having a pathogenetic role. We have three lines of evidences that clearly show that anti-AChR antibodies have a fundamental role in MG: a) the passive transfer of IgG from myasthenic patients to mice induces the same kind of morphological alteration at the end plates and the same pattern of faticability of MG [73]. A natural occurring passive transfer of MG is responsible of the transient myasthenic symptoms developed in a 15% of babies born from myasthenic women [78]; b) in the single patient there is a good correlation between anti-AChR Abs titer and the clinical status, in particular when the Abs are reduced, whatever is the mean used for reducing them (plasmapheresis, immunosuppression, thymectomy [92]; c) the demonstration by electron microscopy of IgG and complement within the end plates of affected muscles [74].

Mechanism of action of anti-AChR antibodies

While there is a general agreement that myasthenic symptoms are due to a reduction in end plate AChRs and that this reduction is due to antibody binding, much less defined are the mechanisms by which antibodies do actually induce the reduction of functional receptors.

At least three mechanisms have been hypothesized and studied: 1) curare-like effect [96]; 2) complement activation [97]; 3) antibody-induced internalization and degradation of AChRs [62, 98-100], this last mechanism being probably the most relevant. All these mechanisms have been demonstrated at least in same *in vivo* or *in vitro* model of EMG, but none is, alone, probably sufficient to account for the complexity and variability of myasthenic symptoms. At the moment we think that a combination of the different antibodies effects (probably acting in different proportion in single patients) is at the basis of receptor loss and myasthenic symptoms. In fact, we and others have found a correlation between the rate of AChR degradation, induced *in vitro* by the serum of myasthenic patients, and their clinical status [68], but the best correlation is found when the clinical status of the patients is correlated to a formal index accounting for more than one of the mechanisms involved [67].

Cellular immunity

The thymus. - Since the beginning of the century it has been recognized that thymus abnormalities are common in MG patients [101]. The two most common abnormalities are an increase of thymic germinal centers in 68% of the myasthenic population and thymomas in about 10% of the patients [102, 103]. Germinal centers are found also in 70% of normal thymuses [104] although their number is probably not so high as in MG

thymuses [105] or thymuses from patients with other autoimmune disorders [106]. Hyperplastic MG thymus contain more B cells than normal, especially in the germinal centers [107]. In about 50% of MG patients, thymic lymphocytes can synthesize *in vitro* anti-AChR antibodies [108], and anti-AChR antibodies have been found in about 70% of MG thymus extracts [109]. It was also reported an increased number of Ia+ cells (antigen presenting cells) in thymuses from MG patients [110]. Less data are available on the presence and function of T lymphocytes in hyperplastic MG thymuses. T cells are present in similar amount as in normal thymuses and responds normally to mitogens, although some discrepancy between PHA and ConA induced proliferation of T lymphocytes with respect to normal thymuses was reported [111].

Thymoma, on the other hand, is present in 10% of MG patients [112] and conversely MG occurs in about 65% of patients with thymoma [103]. There is a general agreement that B cells are not present in thymomas [112, 113], and it was shown that cells originating from thymomas do not synthetize anti-AChR antibodies [108]. T cells are present in the same proportion as in normal thymus [112, 113] but seem to be more mature cells, as confirmed by their high response to mitogens (PHA, ConA, PWM) to which normal thymocytes respond poorly [114]. An increased number of Tu lymphocytes was reported in thymoma and this could correspond to an increase in helper cells at the periphery and may be one of the reasons of the increased titer of circulating antibodies in thymoma-bearing patients [115].

It was also recently shown that MG thymic cells (probably T helper lymphocytes), are able to enhance *in vitro* anti-AChR production by peripheral lymphocytes of myasthenic patients [116].

The presence of thymus pathology in MG patients stimulated studies to discover if AChR could be present in thymus cells. AChR has been found in epithelial cells of human thymuses [117] and it was shown that 60% of lymphocytes of MG thymuses stain specifically with antisera against AChR of Torpedo [111]. While these data suggest that AChR-like molecules may be present on the surface of thymic cells and that a primitive lymphocyte sensitization against AChR can start in the thymus, much more work has to be done on the characterization of these thymic receptors before any conclusion could be drawn.

Peripheral lymphocytes

For understanding the altered cellular immune mechanism in MG, several studies on the variations in the total number of peripheral lymphocytes have been performed, however they are not of great relevance since the peripheral population is a mixture of different types of lymphocytes with different functions, and also the studies on total B or T cells gave very inconsistent data [84]. More interesting are the studies on subpopulations of T lymphocytes in the periphery. There are evidences that the T suppressor cells are decreased, while T helpers are normal or increased [118-120]. It was also demonstrated in children with MG [121], a failure in expressing antigen-dependent T suppressor cell functions, and a marked decrease of a particular subset of T suppressor cells. This decrease can be due to the binding of anti-AChR antibodies to a surface receptor these lymphocytes express. The same kind of failure can be induced by these antibodies in a population of lymphocytes derived from normal people. Thus it seems that a particular type of suppressor T cells, bearing AChR on their surface may regulate the response to AChR, and that a high titer of anti-AChR IgG, as usually found in MG patients, can impair the function of these suppressor lymphocytes and maintain the abnormal response to AChR. In addition, it was shown that all T-suppressor cells express surface AChR [122] and so we can think that anti-AChR antibodies present in MG patients may cause a general impairment of T suppressor functions. This global disfunction of the immune system could explain the high incidence of other autoimmune diseases which is found in MG patients [123].

Lymphocyte response to AChR

MG patients' lymphocytes are stimulate to proliferate *in vitro*, when incubated in the presence of purified AChR. We [124] and others [125, 126] have studied, in a large population of MG patients, this *in vitro* response trying to correlate it, if possible, with different clinical parameters and also with the patients' response to different immunosuppresive treatments.

Lymphocyte stimulation by AChR is found in 60% of MG patients. There are not specific clinical characteristics in the two classes of responders and non responders patients except for the higher percentage of males in the former class. The response is, in any case, specific since lymphocytes from normal subjects or from patients affected by other neurological or muscular diseases do not respond to AChR [127]. There are some discrepancies in the literature about the correlation between the *in vitro* stimulation of lymphocytes by AChR and the clinical course and/or severity of the disease. For example Richman *et al.* found that patients over 50 years responded better than younger patients [126]. On the contrary we found a higher response in patients whose disease begun before 30 years while the older group had a response quite similar to the control group [123].

Both Abramsky *et al.* [128] and our group [129] found that the cellular response to AChR was reduced after corticosteroid treatment, and we found a rapid and dramatic decrease of this *in vitro* response also after thymectomy [129]. In both cases the reduction was specific, since the response to mitogens was not significantly changed.

As we discussed for humoral immunity in MG, also in this case some discrepancies found in the literature are due to different factors as the source and method of preparation of AChR used for the assays and to the differences in classifying clinically myasthenic patients.

Further studies are now in progress in our and other laboratories trying to better clarify the network of cellular interactions leading to the uncontrolled production of anti-AChR antibodies. This is done mainly by developing antigen-specific T cell lines, derived from both suppressor or helper T lymphocytes, isolated from sensitized animals or myasthenic patients [130, 131] and grown continuously by virtue of viral transformation [130] or stimulation with growth factors.

Conclusions

The fascinating story of AChR and myasthenia gravis is now nearly completed. Details are to be worked out but the fundamental plan is outlined. We have learned about the structure and the function of one of the most important membrane proteins; one of the first protein synthesized for the control of the cell ionic milieu and for cell to cell communication. But on the other hand we have also understood the pathogenesis of one very intriguing pathology, MG, and, even perhaps more important, we have learned, from this basic studies, a new way to treat myasthenic patients, so that now MG is a well controlled, long lasting disease and myasthenic patient face a life more comfortable and worthy to be lived.

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