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The Main Immunogenic Region (MIR) of the Nicotinic Acetylcholine Receptor and the Anti-MIR Antibodies

S. J. Tzartos,*[1] M. T. Cung,[2] P. Demange,[2] H. Loutrari,[1] A. Mamalaki,[1] M. Marraud,[2] I. Papadouli,[1] C. Sakarellos,[3] and V. Tsikaris[3]

[1]*Department of Biochemistry, Hellenic Pasteur Institute, 127, Vas. Sofias Ave., Athens 11521, Greece;* [2]*Laboratoire de Chimie-Physique Macromoleculaire, CNRS-UA-494, ENSIC-INPL, BP 451, 54001 Nancy, France; and* [3]*Department of Chemistry, University of Ioannina, Box 1186, 45110 Ioannina, Greece*

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*Author to whom all correspondence and reprint requests should be addressed.

Abstract

Myasthenia gravis (MG) is caused by autoantibodies against the nicotinic acetylcholine receptor (AChR) of the neuromuscular junction. The anti-AChR antibodies are heterogeneous. However, a small region on the extracellular part of the AChR α subunit, called the main immunogenic region (MIR), seems to be the major target of the anti-AChR antibodies, but not of the specific T-cells, in experimental animals and possibly in MG patients. The major loop of the overlapping epitopes for all testable anti-MIR monoclonal antibodies (MAbs) was localized within residues 67–76 (WNPADYGGIK for *Torpedo* and WNP*D*DYGG*V*K for human AChR) of the α subunit. The N-terminal half of α67–76 is the most critical, Asn68 and Asp71 being indispensable for binding. Yet anti-MIR antibodies are functionally and structurally quite heterogeneous. Anti-MIR MAbs do not affect channel gating, but they are very potent in mediating acceleration of AChR degradation (antigenic modulation) in cell cultures and in transferring experimental MG in animals. Fab fragments of anti-MIR MAbs bound to the AChR prevent the majority of the MG patients' antibodies from binding to and causing loss of the AChR. Whether this inhibition means that most MG antibodies bind on the same small region or is a result of broad steric/allosteric effects is under current investigation.

Index Entries: Nicotinic acetylcholine receptor; myasthenia gravis; monoclonal antibodies; main immunogenic region; epitope; synthetic peptide; antigenic modulation; antibody competition.

Introduction

Myasthenia gravis (MG) is a model autoimmune disease, mainly thanks to the well-defined autoantigen involved in this disease, the nicotinic acetylcholine receptor (AChR) (Oosterhuis, 1984; Drachman, 1987; Lindstrom et al., 1988). Progress in understanding MG has paralleled progress in AChR research. Often, studies aiming simply at understanding the AChR resulted in valuable information on MG. In fact it was the availability of purified AChR that led to the discovery that anti-AChR antibodies induce MG symptoms (Patrick and Lindstrom, 1973). The availability of a panel of rat monoclonal antibodies (MAbs) against intact AChR uncovered an immunologically hot spot on the AChR α subunit, the main immunogenic region (MIR) (Tzartos and Lindstrom, 1980).

This review presents our current knowledge of the structure of the MIR and its antigenic characteristics, and of the structure and function of the specific anti-MIR antibodies. At present, one could consider two views of the MIR: (1) The strictly defined target of the anti-MIR MAbs; the studies conducted so far leave little doubt of its existence and location; this region is on and around residues 67–76 of the AChR α subunit. (2) The as-yet vaguely defined MIR as target of the majority of the human MG antibodies; its verification and characterization needs further experimentation. There are insufficient data both in favor of and against a critical role for the MIR in MG. Nevertheless, it seems more likely that the area on or around the strictly defined MIR will be proved the target of a major fraction of the human anti-AChR antibodies. In any case it is clear that single anti-MIR MAbs (or their Fab fragments) can inhibit the majority of human MG antibodies from binding to the human AChR; this fact should prove useful in therapeutic approaches to the disease. Before describing the MIR and the anti-MIR antibodies an overview of MG and its autoantigen will be presented.

Myasthenia Gravis

MG is characterized by weakness and fatigability of the skeletal muscles. Symptoms usually begin with drooping of the eyelids and double vision, followed in most cases by impairment of speech and swallowing. At subsequent stages the muscles of the limbs and trunk are usually affected, and respiration may eventually be impaired. The

disease occurs in about one out of 20,000 persons, with a higher degree of incidence in women of child-bearing age (Oosterhuis, 1984). Young-onset MG is associated with HLA-A1, -B8, and -DR3 antigens, whereas old-onset MG is associated with HLA-A3, -B7, and -DR2 (Engel, 1984; Willcox and Vincent, 1988).

MG is diagnosed by evaluation of muscle weakness and fatigability, by evaluation of relief of muscle weakness after the administration of short-acting inhibitors of acetylcholinesterase, and by a decrementing electromyogram response that can be controlled by inhibitors of acetylcholinesterase (Oosterhuis, 1984). Objective and quantitative diagnosis is achieved by the detection and measurement of anti-AChR antibodies in the sera of suspected MG patients (Lindstrom et al., 1976b; Vincent and Newsom-Davis, 1985).

Antibodies against the AChR are detected in about 85% of MG patients. Such antibodies are essentially absent in healthy humans (Lindstrom et al., 1988; Willcox and Vincent, 1988), though with a few characteristic exceptions:

1. Most newborn infants of MG mothers do not show MG symptoms, although they carry their mothers' antibodies (Keesey et al., 1977; Morel et al., 1988a);
2. MG patients in remission usually continue having anti-AChR antibodies; and
3. A high (Lefvert et al., 1985) or low (Vincent and Newsom-Davis, 1985) percentage of relatives of MG patients have been reported to have anti-AChR antibodies.

Other autoantibodies against muscle or non-muscle body constituents are also frequent in MG, and the incidence of other autoimmune diseases in MG patients is elevated (Gilhus et al., 1983; Williams and Lennon, 1986; Connor et al., 1990). These observations suggest a major autoimmune abnormality in MG patients, but it is primarily the action of the anti-AChR antibodies that causes the MG symptoms (Lindstrom et al., 1988).

Variations in antibody titer to AChR in a particular patient's sera correlate well with clinical improvement. In contrast, studies of populations of MG patients show only a weak correlation between absolute anti-AChR antibody concentration and severity of the disease (Lindstrom et al., 1976b). It is uncertain whether some antibody specificities are more potent than others in causing impairment of neuromuscular transmission (Drachman et al., 1982; Tzartos et al., 1986b; Eymard et al., 1988).

MG patients have decreased numbers of AChR molecules at their neuromuscular junctions (Fambrough et al., 1973). This decrease is caused by the anti-AChR antibodies and seems to be the main effect of these antibodies in MG. AChR loss impairs neuromuscular transmission. At least two antibody-mediated mechanisms account for the loss of AChRs: (1) Bivalent and polyvalent antibodies crosslink membrane-bound AChRs, resulting in an increase in their internalization and degradation rate (antigenic modulation; Heinemann et al., 1977; Appel et al., 1977). (2) Complement binds to the AChR antibodies immobilized on the membrane and mediates lysis of these membranes (Engel, 1984). In addition to AChR loss, direct blockage of the function of the remaining AChRs by antibodies to the acetylcholine binding site might be a significant factor in impairment of the neuromuscular transmission (Harvey et al., 1978; Drachman et al., 1982; Gomez and Richman, 1983; Morel et al., 1988b). Anti-AChR antibody production is controlled by autoimmune AChR-specific helper T-cells (Lindstrom et al., 1988; Newsom-Davis et al., 1989).

Knowledge of MG has dramatically profited from a number of MG models:

1. Experimental autoimmune MG (EAMG) has been induced in all species tested (rats, mice, rabbits, chickens, monkeys) by the injection of purified heterologous or homologous AChR (with or without adjuvant), AChR subunits or their fragments, or anti-AChR polyclonal or monoclonal antibodies (reviewed in Lindstrom et al., 1988).
2. In addition to occurring in humans, idiopathic autoimmune MG occurs also in dogs (Dau et al., 1979). The anti-AChR antibody pattern of the antibodies in canine MG has been found to be very similar to that of human MG patients (Shelton et al., 1988). This

observation suggests that the mechanism of MG induction in dogs may be similar to that in human patients. Therefore, an extensive study of canine MG should prove valuable in understanding and treating idiopathic human MG.

3. A small percentage of patients with rheumatoid arthritis or Wilson's disease who receive D-penicillamine develop symptoms of MG (Bucknall, 1977) and their sera contain anti-AChR antibodies (Russell and Lindstrom, 1978; Vincent and Newsom-Davis, 1982). Both the symptoms and the antibodies remit after drug cessation. The anti-AChR antibody patterns of these patients seem to be similar to those of idiopathic MG patients (Tzartos et al., 1988a). Thus the drug may mimic the unknown trigger of the idiopathic MG.
4. All newborn infants of MG mothers have anti-AChR antibodies that have been passively transferred from their mothers (Keesey et al., 1977). About 10–15% of these infants develop myasthenic symptoms for about 3 wk after birth. Neonatal transient MG does not seem to be related to the severity of the mother's disease (Morel et al., 1988a) or to the specific antibody repertoire in mothers' or infants' sera (Tzartos et al., 1990a). Though it has not yet been completely elucidated, it seems that antibody titer in the infants' sera is the major determinant of MG symptoms (Morel et al., 1988a). This experiment of nature also deserves detailed study with the expectation of casting light on the mechanisms of idiopathic MG.

The role of the thymus in MG is undoubtedly very critical, though the mechanism(s) of its involvement are not yet well understood. Most MG patients have thymic hyperplasia, whereas about 10% have thymomas. Thymic lymphocytes from MG patients spontaneously produce anti-AChR antibodies (Scadding et al., 1981) and stimulate antibody production by peripheral blood cells. AChRs on the surface of myoid cells of the thymus (Wekerle et al., 1978; Schluep et al., 1987) is a possible candidate as trigger of the autoimmune response in human MG (Wekerle et al., 1978). Other proteins detected in epitheliomas of MG thymomas (but not of non-MG thymomas) with certain common epitope(s) with the AChR may also induce the initiation of the immune response against the AChR (Kirchner et al., 1988; Hohlfeld, 1990; Marx et al., 1990).

Current MG treatments are far from ideal. Nevertheless, significant progress has been achieved so that the majority of patients can now live a tolerable life, whereas deaths from MG are not frequent any more (Oosterhuis, 1984). There are four major current treatments of MG:

1. Anticholinesterase drugs (e.g., neostigmine and pyridostigmine) inhibit hydrolysis of the acetylcholine released at the neuromuscular junction, resulting in a prolonged interaction of the acetylcholine with the remaining AChRs.
2. Thymectomy is generally recommended as soon as possible after the onset of the disease. Overall, one-third of the thymectomized patients achieve remission and another one-third achieve relative improvement.
3. Immunosuppressive drugs (e.g., corticosteroids, azathioprine, and so on) are given to those patients who do not respond to the previous treatments; however, these drugs have serious side effects that limit their use.
4. Plasmapheresis results in immediate decrease of antibody titer accompanied by clinical improvement. This treatment is very expensive and its effect is only temporary; therefore, it is reserved only for severe cases, in which its use is invaluable.

The Nicotinic Acetylcholine Receptor of the Neuromuscular Junction and Its Antigenic Structure

AChR Structure and Function

The AChR of the neuromuscular junction (and of the electric organs of electric fish) is the best-known member of the nicotinic-receptor family and of the larger ligand-gated ion channel gene superfamily, which includes the γ-aminobutyric acid (GABA) and glycine receptors (Barnard et

al., 1987; Stroud et al., 1990). At the other end, the muscarinic AChR is a completely different molecule, a member of the superfamily of receptors that are coupled via G proteins to enzyme effectors (Strange, 1988). The nicotinic AChR gene family contains electric-organ, skeletal-muscle, ganglionic, and brain AChRs (Lindstrom et al., 1987; Changeux, 1990; Luetje et al., 1990; Lukas and Bencherif, 1991). The muscle and electric-organ AChR is a glycoprotein composed of five homologous subunits with the stoichiometry $\alpha_2\beta\gamma\delta$ and a total mol wt of about 290,000 (Maelicke, 1988; Changeux, 1990). In adult muscle the embryonic γ subunit is replaced by the homologous ε subunit (Numa, 1987). The molecule, approx 110 Å long and 80 Å wide, extends on both sides of the membrane (Brisson and Unwin, 1985; Mitra et al., 1989). A model of the AChR molecule is shown as part of Fig 1.

Extensive molecular studies on the AChR have been performed by many research groups. The complete amino acid sequences of *Torpedo* electric-organ and mammalian muscle AChR subunits are known. The amino acid sequence of the *Torpedo* α subunit is shown in Fig. 2. The four subunits within the same species have approx 40% homology among themselves. The α subunits of *Torpedo* electric-organ and human muscle AChR are about 80% homologous, whereas homology in other subunits, between *Torpedo* and human, is about 55%. The prevailing transmembrane model for the AChR subunits predicts that each subunit spans the membrane four times via four hydrophobic α-helices, M1–M4 (reviewed in Numa, 1987; Claudio, 1989; and Changeux, 1990).

The N-terminus of each subunit is located extracellularly, but there are conflicting models and data concerning the location of the C-terminal end (McCarthy et al., 1986; Dipaola et al., 1989). According to the four-transmembrane-helices model, the C-terminal end must be extracellular. Evidence for extracellular location of the C-terminal end has been provided for at least the δ subunit (Dipaola et al., 1989).

Acetylcholine released from the nerve terminal binds to the AChR and causes opening of the cation channel, which allows Na^+ to enter the muscle cell or the electroplaque. Acetylcholine binds to at least two binding sites per AChR molecule, one on each α subunit. Cysteines at α192 and α193 play a critical role in acetylcholine binding and function (Kao and Karlin, 1986). Also, residues Tyr93, Trpl49, Tyr190, and possibly Tyr151 and Tyr198, seem to be implicated in acetylcholine binding (Changeux, 1990).

Several agonists and antagonists of acetylcholine and other blockers of AChR function have been extensively studied. Snake venom α-toxins, a group of small proteins of approx mol wt 7500–8000, such as α-bungarotoxin, bind with high affinity to the two α subunits (K_d approx $2 \times 10^{-11} M$) at or near the acetylcholine binding sites, competitively blocking AChR function (reviewed in Maelicke, 1988; Changeux, 1990). Peptides within α170–200 are capable of binding α-bungarotoxin (Neumann et al., 1986; Aronheim et al., 1988; Wilson and Lentz, 1988; Gotti et al., 1988). The smallest α-bungarotoxin-binding peptide seems to be the α189–195 (Tzartos and Remoundos, 1990); however, the S—S bond at α192–193 does not seem to be required for binding α-bungarotoxin (Tzartos and Remoundos, 1990; Griesmann et al., 1990). Another group of molecules, including local anesthetics, block AChR function noncompetitively; these blockers have been shown to bind within the ion channel (Oberthur et al., 1986; Giraudat et al., 1987; Changeux, 1990).

The cation channel is an integral part of the AChR molecule. All four kinds of subunits are required for the assembly of a fully functional AChR (Mishina et al., 1984). The subunits are organized around the channel, the most likely order being $\alpha\beta\alpha\gamma\delta$ (Kubalek et al., 1987). However, alternative subunit orders cannot yet be excluded (Karlin et al., 1986). Site-directed mutations and binding of noncompetitive blockers have clearly shown that M2 helices from each subunit contribute to the formation of the cation channel (Hucho et al., 1986; Giraudat et al., 1987; Imoto et al., 1988; Charnet et al., 1990; Villarroel et al., 1991). The diameter of the entry well nar-

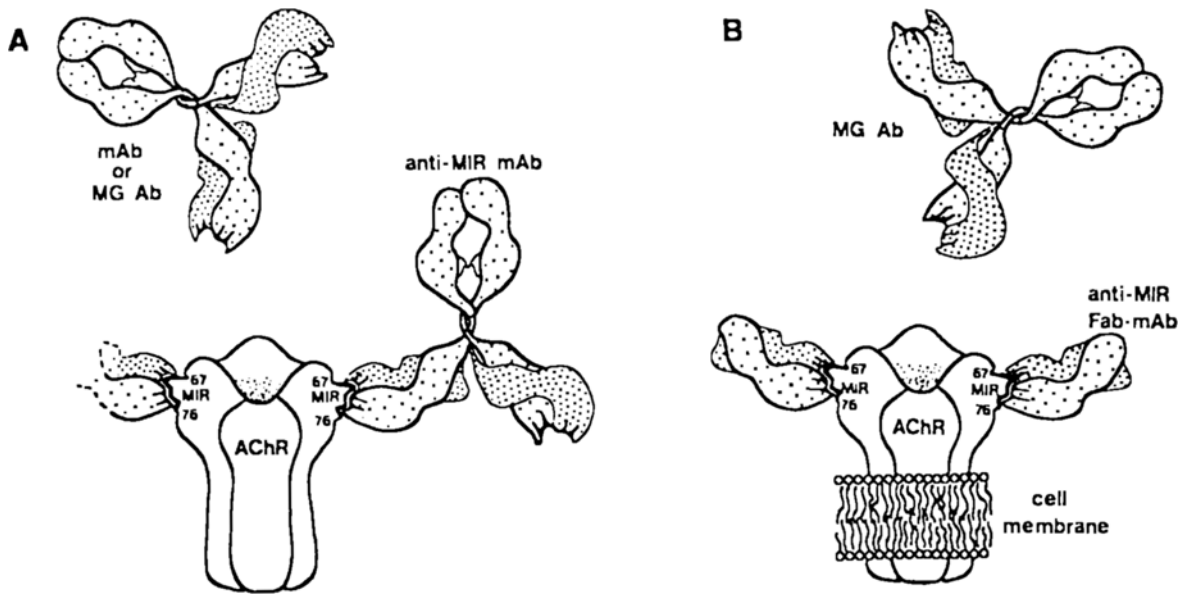

Fig. 1. Two schemes depicting the principles of antibody competition experiments for binding to the AChR (**A**) and for protecting the AChR against antigenic modulation (**B**). The AChR model has been loosely based on electron-microscopy studies (Brisson and Unwin, 1985; Mitra et al., 1989). The shown conformation of the α67–76 MIR segment is a miniature of that in Fig. 4, deduced from NMR studies, drawn approximately to scale. **A**: Antibody competition for binding to the AChR. The molecule is preincubated with an anti-MIR MAb (intact or Fab) that binds on the MIR. A subsequently added second MAb will either bind or not, depending on its epitope. Addition of an MG serum (instead of the second MAb) will be partially inhibited from binding, depending on the percentage of its anti-MIR antibodies. **B**: Protection of AChR against the activity of anti-AChR antibodies on muscle-cell cultures. Fab of an anti-MIR MAb is allowed to bind on the membrane AChR and shield the MIR. The monovalent Fab cannot crosslink; thus it cannot induce antigenic modulation of the AChR. Subsequently, a test myasthenic serum is added. Usually a large fraction of the serum antibodies are inhibited from binding and therefore from modulating the AChR. Thus the Fab effectively protects the AChR. This protection strategy may prove applicable in therapeutic approaches.

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SEHETRLVANLLENYNKVIRPVEHHTHFVDITVGLQLIQLISVDEVNQIVETNVRLRQQW  60

IDVRLRWNPADYGGIKKIRLPSDDVWLPDLVLYNNADGDFAIVHMTKLLLDYTGKIMWTP 120
      MIR

PAIFKSYCEIIVTHFPFDQQNCTMKLGIWTYDGTKVSISPESDRPDLSTFMESGEWVMKD 180

YRGWKHWVYYYTCCPDTPYLDITYHFIMQRIPLYFVVNVIIPCLLFSFLTGLVFYLPTDSG 240
       Toxin site                                  M1

EKMTLSISVLLSLTVFLLVIVELIPSTSSAVPLIGKYMLFTMIFVISSIIITVVVINTHH 300
      M2                                          M3

RSPSTHTMPQWVRKIFIDTIPNVMFFSTMKRASKEKQENKIFADDIDISDISGKQVTGEV 360

IFQTPLIKNPDVKSAIEGVKYIAEHMKSDEESSNAAEEWKYVAMVIDHILLGVFMLICII 420
                                                  M4

GTYSVFAGRLIELSQEG 437
```

Fig. 2. Amino acid sequence of the *Torpedo californica* AChR α subunit deduced from cDNA sequencing (Numa, 1987). Underlined segments mark the putative locations of the MIR, of the most critical part of the α-bungarotoxin binding site (toxin site), and of the four transmembrane helices M1–M4.

rows from 25 Å at the extracellular entry to about 7–8 Å at the transbilayer region (Stroud et al., 1990).

Antigenic Structure of the AChR in EAMG

AChR is a very potent immunogen. Animals of various species immunized with intact or fragmented AChR respond by the production of antibodies to various sites on the molecule. A single injection of <1 μg of purified AChR per rat is sufficient to induce an immune response to the AChR (Lindstrom et al., 1976c). Generally, when intact AChR is used as immunogen most of the antibodies are directed against extracellular parts of the AChR. In contrast, when SDS-denatured AChR or its isolated subunits are used as immunogens, the majority of the antibodies are directed against cytoplasmic sites of the AChR (Froehner, 1981; Sargent et al., 1984).

The majority of rat antibodies (polyclonal and monoclonal) against intact solubilized AChR from *Torpedo* (Tzartos and Lindstrom, 1980) or *Electrophorus* (Tzartos et al., 1981) electric organs and from human muscles (Tzartos et al., 1983) compete for binding to a specific region on each of the two α subunits, the main immunogenic region (MIR). Immunization with fetal calf muscle AChR resulted in MAbs against the MIR but also against additional extracellular sites on α, β, and γ subunits (Tzartos et al., 1986a). MAbs derived from mice immunized with intact human-muscle AChR were found to bind on overlapping regions, at or near the MIR or near the toxin binding sites, apparently all on the extracellular side of the AChR (Whiting et al., 1986; Heidenreich et al., 1988a).

Polyclonal and monoclonal antibodies against the acetylcholine and/or the α-bungarotoxin binding sites have been obtained by several groups (Mochly-Rosen and Fuchs, 1981; Watters and Maelicke, 1983; Mihovilovic and Richman, 1984; Fels et al., 1986; Heidenreich et al., 1988a). Such MAbs could cause acute myasthenic symptoms within an hour after their injection into experimental chickens (Gomez and Richman, 1983). Such an immediate response, however, is unusual when whole MG antisera are injected into experimental animals (Lindstrom et al., 1976a); this supports the notion that antisite antibodies do not play the major role in MG.

Synthetic peptides corresponding to segments of the AChR have been used in order to determine the epitopes of the serum anti-AChR antibodies. Using sera from animals immunized with intact AChR, antibody binding to several sequences was detected (Ralston et al., 1987; Mulac-Jericevic et al., 1987). This mapping, however, although it identifies immunogenic regions, should not be taken as representative of the whole antibody repertoire in the sera studied. Since the major fraction of the serum anti-AChR antibodies either do not bind or bind with very low affinity to the denatured peptides, peptide mapping may easily lead in misleading conclusions. In contrast, when denatured subunits are used as immunogens, epitope determination is easier and probably reliable, since the majority of the antibodies produced are expected to bind to synthetic peptides. Under such conditions, certain regions toward the C-terminal end of each subunit seem to be very antigenic (Ratnam et al., 1986a; Souroujon et al., 1986; Ralston et al., 1987; Tzartos et al., 1988b). Epitopes for AChR-specific T-cells derived from animals immunized with AChR have been detected on various parts of the α subunit, but not on the MIR segment α67–76 (Fujii and Lindstrom, 1988; Zhang et al., 1990a).

Antigenic Structure of the AChR in Human MG

In contrast to experimental MG, in which the trigger of the anti-AChR antibodies is the injected AChR, in human MG it has not yet been proved unequivocally that the AChR, rather than a cross-reactive antigen, is the immunogen. Therefore, the antibody repertoire in human MG could be very different from that in experimental MG. However, it was found that the repertoire of the anti-AChR antibodies in human MG patients is very similar to that of the anti-AChR antibodies in experimental rats (Tzartos et al., 1982) as well as in spontaneously autoimmune MG dogs

(Shelton et al., 1988). Considerable heterogeneity exists in the fine antigenic specificities of anti-AChR antibodies among MG patients (Tzartos et al., 1982, 1985; Heidenreich et al., 1988a,b). Within a single patient the antibody repertoire seems quite stable with time, despite various treatments (including thymectomy) and variations in the anti-AChR titer (Tzartos et al., 1982; Heidenreich et al., 1988b). All the above studies were performed by competition experiments between MAbs and MG sera.

Antibodies to or around the acetylcholine and α-bungarotoxin binding sites also occur in MG (Drachman et al., 1982; Schuetze et al., 1985; Morel et al., 1988b), but the extent of their contribution to the symptoms of the average MG patient is yet uncertain. Drachman et al. (1982) observed a significant correlation between the capacity of MG sera to block toxin binding to human-muscle AChR and the clinical status of the patients. Blocking antibodies were found to be significantly more abundant in sera from MG mothers who transfer the disease to their infants than in sera from those who do not transfer it (Morel et al., 1988b). These data suggest a significant pathogenic role for blocking antibodies in MG. In human MG, as in EAMG, epitopes for AChR-specific T-cells have been detected mainly on several parts of the α subunit, other than the MIR (Hohlfeld et al., 1987; Newsom-Davis et al., 1989; Protti et al., 1990; Zhang et al., 1990b).

The MIR as Defined by Anti-MIR Monoclonal Antibodies

Localization of the MIR has been studied by several approaches, using either intact AChR or its fragments. Since none of these techniques is ideal, use of multiple approaches has been necessary both for verifying and supplementing the provided data. So far little is known of the structure of the anti-MIR antibodies; this is currently studied by means of molecular biology approaches. Their function has been studied by the use of experimental animals and muscle-cell cultures.

The MIR on the Intact AChR

Identification of the MIR by Antibody Competition Experiments

Because of the large size of the AChR molecule it was expected that the antibody repertoire of animals immunized with AChR would be directed to multiple sites; thus, several antibodies would be able to bind simultaneously on the AChR. However, when a significant number of anti-AChR MAbs became available, competition experiments among them for binding on the intact AChR showed that most of these MAbs competed with each other for binding to, apparently, a single region, the MIR (Tzartos and Lindstrom, 1980; Tzartos et al., 1981, 1983). The principle of the antibody-competition experiments is depicted in Fig. 1A. Of 66 MAbs to intact AChR, 41 (62%) efficiently competed with one (MAb 35) or more MAbs for binding to the homologous AChR. Competition between Fab fragments of MAb 35, which are one-third the size of the intact molecule, and several anti-MIR MAbs was also as effective as with the intact MAb (Tzartos et al., 1986a).

More than half of the anti-MIR MAbs could bind detectably to the denatured AChR α subunit; the rest were unable to bind to any denatured subunit. It was thus concluded that the MIR is on the α subunit of the AChR (Tzartos and Lindstrom, 1980; Tzartos et al., 1981). Since each AChR molecule has two α subunits, it should have two MIRs as well. In fact the presence of two MIRs per molecule was subsequently confirmed by the study of the molecular weights of all possible complexes formed when incubating AChR with an anti-MIR MAb (Conti-Tronconi et al., 1981). Among the anti-MIR MAbs, No. 35 (now available from the American Type Culture Collection, Rockville, MD) exhibited very good crossreactivity with AChRs from almost all tested species and thus became the reference anti-MIR MAb.

Inhibition of binding of various MAbs by a single reference MAb (i.e., MAb 35) could simply mean that this MAb binds at the center of a large area, inhibiting by steric hindrance other

MAbs binding around it. Furthermore, this specific MAb could cause multiple allosteric effects, which would be responsible for most observed inhibitions. More detailed studies were necessary in order to determine whether the MIR is a small or a very broad area. Extensive cross-competitions performed among these anti-MIR MAbs (each MAb was tested against almost each one of the others) strongly suggested that the region to which the anti-MIR MAbs bind is a very concrete and small region (Kordossi and Tzartos, 1989).

The sensitivity and reliability of the MAb-competition experiments could be tested with another group of MAbs that bind to known sequential epitopes on the cytoplasmic side of the AChR. Knowledge of the exact epitopes of these MAbs, several of which were overlapping (Ratnam et al., 1986a,b), allowed a detailed investigation of the sensitivity and limitations of the competition technique (Kordossi and Tzartos, 1987). It was shown that MAbs to epitopes separated by only about seven residues did not inhibit each other, and MAbs to actually overlapping epitopes exhibited differential competition with other MAbs. Therefore, despite the large size of the antibody molecule, the competition technique seems to be very sensitive.

Unfortunately, the epitopes of only few of the MAbs against intact AChR obtained by other laboratories have been characterized in relation to the MIR. At least two other groups have raised anti-AChR MAbs in rats. Lennon and Lambert (1980,1981) produced five rat anti-AChR MAbs. Using antiidiotypic antisera, the four MAbs were shown to share the same idiotype. Since one of these MAbs was tested with MAb 35 and they were found to inhibit each other practically completely from binding to the AChR (Lennon and Griesmann, 1989), it can be concluded that four of the five MAbs bind to the MIR. Despite the extensive use of the other group's rat anti-AChR MAbs, we know very little yet about their relation with the MIR (Gomez and Richman, 1983; Mihovilovic and Richman, 1986; Xu et al., 1988).

Mouse MAbs against intact AChR seem to be less MIR-directed than rat MAbs (Whiting et al., 1986; Heidenreich et al., 1988a). Three out of 10 mouse anti-human AChR MAbs bound at or near the MIR, as defined by antibody-competition experiments among MAbs. Five regions were identified by these MAbs. Region 4 (one MAb) is equivalent to the MIR, and region 3 (two MAbs) overlaps with region 4; each of these two regions seems to be on both α subunits of the AChR molecule. Regions 1 and 2 seem to be near one of the two α-bungarotoxin binding sites and region 5 near the second α-bungarotoxin binding site. Partial competition between the anti-MIR MAb and the MAbs directed to region 5 was also observed. In another group of five mouse MAbs against *Torpedo* AChR, two MAbs completely competed with MAb 35 (Chase et al., 1987).

The anti-MIR MAbs were also tested in similar competition experiments with whole anti-AChR sera from immunized animals, rather than with other MAbs. It was shown that each anti-MIR MAb could inhibit binding of about two-thirds of the total anti-AChR serum antibodies of rats immunized with intact *Torpedo, Electrophorus*, calf, or human AChR from binding to the homologous AChR (Tzartos and Lindstrom, 1980; Tzartos et al., 1981,1982). Such a percentage was also observed with human MG serum antibodies (*see below*) and idiopathic canine MG antibodies (Shelton et al., 1988), but the same MAbs inhibited only one-third of the mouse anti-AChR antibodies from binding (Tzartos and Lindstrom, unpublished). The latter is in agreement with the observed lower occurrence of mouse anti-MIR MAbs, as described above.

Localization of the MIR on the Extracellular Side of the AChR

Use of Animals, Cell Cultures, and Electron Microscopy. Studies of the MAbs with muscle-cell cultures and experimental animals were performed mainly in order to investigate the pathogenic potential of these antibodies, as described below. However, at the same time, these studies uncovered the location of the MIR relative to the cell membrane. Since anti-MIR MAbs were able to bind to intact cells in culture and modulate their

AChR (Conti-Tronconi et al., 1981; Tzartos et al., 1985) and, furthermore, were capable of causing AChR loss in experimental rats (Tzartos et al., 1987), it was evident that the MIR is located extracellularly.

Despite the fact that electron microscopy does not allow the determination of the exact epitope because of its limited sensitivity, it is nevertheless valuable, as it allows the approximate but unequivocal localization of the bound MAb on the antigen. Indeed, the transmembrane orientation et al. (1984) and Ratnam et al. (1986b) labeled α-bungarotoxin, anti-MIR MAbs, and MAbs to other AChR sites with gold particles of various sizes and studied by electron microscopy their localization on neuromuscular junction sections or *Torpedo* membranes. The anti-MIR antibodies were always located at the same side as the α-bungarotoxin, across the plasma membrane, i.e., extracellularly. Kubalek et al. (1987) performed a finer localization of the MIR by electron image analysis of two-dimensional AChR crystals with bound Fab MAb 35, and compared its binding site with the binding site of a Fab MAb to the cytoplasmic side as well as with the binding site of α-bungarotoxin, with a resolution of approx 17 Å. They found that the two MIR of the two α subunits are located at or close to the side of the α subunits, between the synaptic end and the membrane surface. Such a location would facilitate crosslinking of the AChRs by the anti-MIR antibodies, a function critical for the pathogenicity of the anti-AChR antibodies.

Localization of the MIR by Genetic Engineering Approaches

Site-directed mutagenesis on intact AChR was also employed for the localization of the MIR. Saedi et al. (1990) expressed mutants of *Torpedo* AChR in *Xenopus* oocytes by coinjecting the cDNAs of *Torpedo* β,γ, and δ subunits with a mutated cDNA *Torpedo* α subunit. They replaced Asn68 by Asp and/or Asp71 by Lys. These substitutions were selected: (1) because it was known that anti-MIR MAbs bind to synthetic peptides corresponding to α67–76, and (2) because the α67–76 sequence of *Xenopus* muscle AChR, the only known muscle AChR to which anti-MIR MAbs cannot bind (Sargent et al., 1984), has the above two substitutions on α68 and α71. Indeed, none of the seven anti-MIR MAbs tested bound to the mutant AChRs, whereas binding of a MAb to the cytoplasmic side of the AChR was not affected. Although these data are very interesting, further verification of the role of α68 and α71 by using several mutants (or other approaches) and more MAbs (to various sites) is necessary, since the two tested point mutations may have caused major conformational changes in broader areas of the molecule.

The MIR and the Ion-Channel Mechanism

The physiological role of the MIR remains unknown. Several lines of evidence have shown that the MIR is not involved in the primary AChR function, i.e., in the control of the ion channel. Anti-MIR MAbs do not significantly inhibit binding of ^{125}I-α-bungarotoxin to the intact AChR (although partial competition with MAbs that inhibit toxin binding has been reported: Heidenreich et al., 1988a; Xu et al., 1988), nor does α-bungarotoxin inhibit anti-MIR MAb binding. On the primary structure of the α subunit, the main loop of the epitopes, for at least some anti-MIR MAbs (α67–76) (Tzartos et al., 1988b), is distant from the major binding site for α-bungarotoxin and acetylcholine (located approx within α170–200) (Wilson et al., 1985; Barkas et al., 1987; Aronheim et al., 1988; Changeux, 1990) although the peptide α55-74 was reported to bind weakly ^{125}I-α-bungarotoxin (Conti-Tronconi et al., 1990). On the other hand, none of the tested anti-MIR MAbs interfered with cation-channel function (Lindstrom et al., 1981; Wan and Lindstrom, 1985). Also, induction of EAMG symptoms by injecting anti-MIR MAbs into rats requires at least several hours (Tzartos et al., 1987), in contrast to <1 h required for MG symptoms when MAbs against the acetylcholine binding site are injected

(Gomez and Richman, 1983); i.e., the MAbs act by causing AChR loss, rather than blocking AChR function. Finally electron-microscopy studies showed that the binding sites for α-bungarotoxin and MAb 35 on the intact AChR are independent of each other (Kubalek et al., 1987).

MIR Characterization Using AChR Fragments

Localization of the MIR Within the α-Subunit

Binding of anti-MIR MAbs is clearly conformation-dependent. Nevertheless, the very low but detectable affinity for binding to denatured α subunit and to its fragments has gradually allowed the localization of parts of the MIR epitopes on the α-subunit sequence. Proteolytic α-subunit peptides, fusion proteins, many conventional synthetic peptides, and hundreds of peptides synthesized by the parallel solid phase synthesis technique (PEPSCAN) have been used for the characterization of the MIR.

Initial localization of the MIR within part of the α-subunit was first achieved by a combination of α subunit proteolytic fragments, anti-MIR MAbs, and animal antisera to certain synthetic peptides of the α subunit. Binding of MAb 35 was restricted between residues 1–168 of the α subunit (α1–168) (Barkas et al., 1986). Using similar techniques, Ratnam et al. (1986a) further restricted binding of another anti-MIR MAb (No. 210) within α46–120. By a series of recombinant proteins containing segments of the mouse α subunit, binding of six anti-MIR MAbs was localized between residues α6 and 85, whereas binding of three of them was further localized between α37 and 85 (Barkas et al., 1987). By another set of overlapping fusion proteins, binding of four anti-MIR MAbs was indirectly localized between α61 and 76 of *Torpedo*, mouse, and chicken AChR. This was directly confirmed by the synthesis of a *Torpedo* α61-76 decahexapeptide (Barkas et al., 1988). However, MAb 35 did not significantly bind to α61–76.

Twenty-six overlapping synthetic peptides (14 to 20 residues long), which covered 83% of the human AChR α-subunit sequence, were subsequently screened with many anti-AChR MAbs (Tzartos et al., 1988b). Six anti-MIR MAbs bound almost exclusively to the α63–80 peptide and another three MAbs bound mainly to this segment. Some other anti-MIR MAbs could not be mapped, but none bound predominantly to any peptide apart from α63–80. Some MAbs, especially those of IgM class, were apparently sticky, binding weakly to several peptides. Several non-MIR MAbs bound to other α-subunit peptides. A group of smaller peptides within α63–80 further localized binding of at least eight MAbs within the decapeptide α67–76 (WNPDDYGGVK) of human AChR (Tzartos et al., 1988b). Human α67–76 is identical to mouse α67–76, while differs only by one nonconservative (Asp instead of Ala70) and one conservative (Val instead of Ile75) substitution from the equivalent *Torpedo* decapeptide (Fig. 3).

Thirty-one synthetic peptides covering the whole *Torpedo* AChR α subunit were then synthesized and tested for anti-AChR MAb binding. Binding of 11 anti-MIR MAbs was localized within α67–76. Their pattern of binding to an overlapping peptide (α55–74) suggested that residues α75 and α76 may play a role for binding of only a few of the anti-MIR MAbs (Tzartos et al., 1990b).

Das and Lindstrom (1989), using the anti-MIR MAb 210, confirmed the MIR localization on *Torpedo* α66–76. Further, by the PEPSCAN systematic peptide-synthesis technique they synthesized peptides of increasing length by single residues, from the pentapeptide α72–76 to the nonapeptide α68–76. They detected MAb binding only to α68–76.

Detailed MAb mapping by all possible continuously overlapping hexapeptides within *Torpedo* and human α40–91, produced and tested by the PEPSCAN technique, restricted MIR localization to the *Torpedo* and human hexapeptides α66–71, α67–72, and α68–73 (Papadouli et al., 1991). This suggested that α68–71 is the critical binding segment. The shortest unit required for anti-MIR

AChR source	Subunit	67–76 sequence	Anti-MIR mAb binding to synthetic peptide	Anti-MIR mAb binding to intact AChR
Torpedo	α	WNPADYGGIK	+	+
Mammalian muscle	α	---D----V-	+	+
Chicken muscle	α	---D----V-	+	+
Xenopus muscle	$\alpha 1_a$	-D-E------	-	[-]
	$\alpha 1_b$	-D--K---V-		
Chicken brain	α2	---E-FDNVT	+	[+]
	α3	---V----AE		
	α4	-D-QE-ENVT		
	α5	---E--A--T		
Chick brain αBgt binding protein	α1	--VSE-P-VK		[-]
	α2	-DQYE-P-VQ		
Rat brain	α2	-D--EF-NVT	-	[-]
	α3	-K-S--Q-VE		
	α4	-D-G--ENVT		
	α5	---D------		
Torpedo	β	-D--A-E---	-	
	γ	--TSE-E--D	-	
	δ	--ASE-SDIS	-	

Fig. 3. Amino acid sequences of the 67–76 segments of various AChR subunits and anti-MIR MAb binding to peptides and corresponding AChRs. Black and variously dotted rectangles above the *Torpedo* α67–76 represent indispensable and less critical residues, respectively. Only the substituted residues are shown. +, –, or no symbol represent positive, negative, or not tested MAb binding. It is shown that (a) overall anti-MIR MAbs bind only to peptides and AChRs with unchanged both N68 and D71 and (b) there is an excellent correlation between binding of MAb to synthetic peptides and to the corresponding intact AChR.

MAb binding was then directly determined by the synthesis of peptides with lengths increasing by single residues, from the tetrapeptide Tα68–71 to the decapeptide Tα62–71. Tα68–71 bound the anti-MIR MAbs rather weakly, but addition of α67 resulted in a binding activity of the same level as that of the longer peptides. Therefore, the pentapeptide Tα67–71 is the minimum peptide with satisfactory anti-MIR binding capacity (Papadouli, 1991). However, the conventional soluble pentapeptide Tα67–71, immobilized on plastic, did not bind the MAbs, unlike the decapeptide α67–76, which did bind them (Papadouli, Tzartos, Tsikaris, and Sakarellos, unpublished). Probably immobilization of the small pentapeptide resulted in masking of some critical subsites. The epitope for a mouse anti-MIR MAb (D6, region 4) was also localized within the segment α65–78 of human AChR (Wood et al., 1989).

Antigenic Role of Single Residues Within α67–76

The contribution of each residue to the antigenicity of the MIR has been extensively studied by the use of analogs of *Torpedo* and human MIR peptides, in which single amino acid residues were substituted by other amino acids. Thirteen analogs of *Torpedo* and human α67–76 were initially synthesized (Tzartos et al., 1989; Papadouli et al., 1990). Nine analogs had one residue of the *Torpedo* decapeptide replaced by L-Alanine, three had an intermediate sequence between the *Tor-*

pedo and human α67–76, and one had D-Alanine in position 73. Binding studies employing six anti-MIR MAbs and the above peptides revealed that Ala-substitutions of either Asn68 or Asp71 resulted in practically complete loss of binding of all tested MAbs. Ala-substitutions at residues α67, α69, α70, and α72 reduced binding of only some MAbs. Overall, substitutions in the area α68–71 were the most critical. It was also suggested that fish electric-organ and human MIR form two distinct groups of strongly overlapping epitopes (Tzartos et al., 1989; Papadouli et al., 1990).

Bellone et al. (1989) used mostly Gly-analogs (Ala on Gly73 and on Gly74) on the human eicosapeptide Hα$(67–76)_2$. Despite several differences between these and the above studies (Gly vs Ala substitutions, decapeptide dimer vs monomer, human vs *Torpedo* original peptides, differences in the ionic strength of the binding solutions, and so on), overall their results were not very different from the above: they showed that Gly substitutions at α68 and α69 dramatically diminished binding of most MAbs and that substitutions at α71 and α72 also decreased binding of several MAbs.

A systematic screening of multiple peptide analogs was then performed for further understanding the MIR structure. Each residue within the segments α67–74 and α67–76 of both *Torpedo* and human AChRs was substituted by various amino acids (seven for each *Torpedo* residue and three for each human residue). Residues Asn68 and Asp71 were found indispensable for anti-MIR MAb binding, whereas Pro69 and Ala/Asp70 (for *Torpedo* and human, respectively) were less important but still significant (Papadouli et al., 1991).

Conformation of the MIR—Predictions and 2D-NMR Studies

The sequences α63–80 and α67–76 of both *Torpedo* and human AChR were analyzed for the possibility of forming secondary structure and models were constructed (Bellone et al., 1989; Conti-Tronconi et al., 1989; Tzartos et al., 1990b). Structural predictions indicated a high sequential mobility for the sequence α70–77 and a very strong turn potential centered around residues α68-71. The model of α63-80 predicted α β-sheet followed by a type I β-turn centered around residues 68–71 and another β-sheet. According to this model, the tertiary structure that the segment might acquire is a hairpin loop whose two arms are β-sheets; α67–76 could form a very similar structure. The apex of the loop (α68–71) is charged and hydrophilic, thus justifying its important role in antibody-binding.

Two-dimensional NMR experiments were performed with the α67–76 peptide and several of its analogs, both free or bound on the anti-MIR MAbs, for the elucidation of the MIR conformation (Cung et al., 1989,1991a,b). Free decapeptides in aqueous solution did not exhibit any Nuclear Overhauser Enhancement (NOE) connectivity. However, when solubilized in the aprotic solvent DMSO, in which the intramolecular hydrogen bonds and the folded conformation are generally less perturbed, strong and multiple short- and long-range NOEs in the *Torpedo* α67–76 were observed, which argued in favor of a rigid folded conformation. The temperature-dependence measurements indicated the existence of three interactions involving the Asp71, Gly74, and Lys76 amide protons. Any Ala substitution, except that on α75, significantly affected the conformation of the decapeptide (Cung et al., 1989).

The conformation of α67–76 and its Ala76 analog (which binds to anti-MIR antibodies more efficiently than the native decapeptide) was subsequently elucidated by analyzing the NMR data by molecular dynamics programs. Since α67–76 presented the most numerous and strongest NOE connectivities, it was less flexible than the Ala76 analog. The N-terminal heptapeptide in both sequences assumed two very similar folded conformations, whereas the Ala76 analog induced conformational flexibility in the C-terminal tripeptide (Cung et al., 1991a). The conformation of α67–76 in DMSO is shown in Fig. 4.

The *Torpedo* decapeptide and four analogs with different MAb-binding potencies were

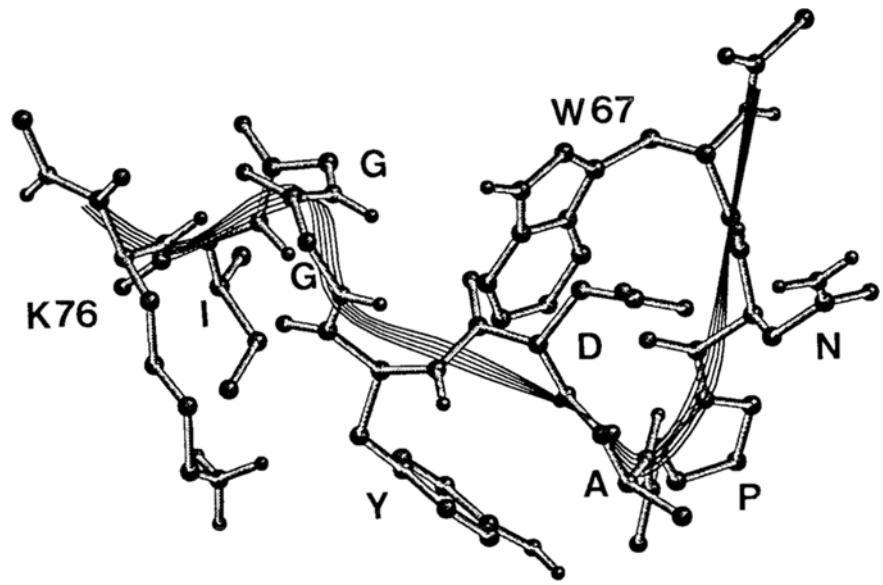

Fig. 4. Conformation of the *Torpedo* α67–76 MIR decapeptide (WNPADYGGIK) in the aprotic solvent DMSO as revealed by 2D-NMR experiments and molecular dynamics analysis. From Cung et al., 1991a.

also examined in presence of the anti-MIR MAbs in aqueous solutions; the intensities of the observed NOE effects correlated well with the MAb-binding potencies of the corresponding peptides. The results suggested that an adequate structuration of the N-terminal sequence is required for full molecular recognition, whereas intrinsic nonstructuration of the C-terminal part by Ala-substitution is not of primary importance for molecular recognition by the anti-MIR MAbs (Cung et al., 1991b).

Is α67–76 Actually Part of the MIR on the Intact AChR?

Although results from five laboratories confirm that anti-MIR MAbs bind to synthetic peptides containing or within α67–76, these data may not unequivocally prove that the MAbs bind to the same segment on the intact AChR as well. Binding of an antibody to a synthetic peptide does not necessarily mean that the antibody will bind primarily to the same sequence segment on the intact molecule. The fact that the anti-MIR MAbs bind to the synthetic peptides with very low affinity, compared with their binding to the intact AChR, further weakens the validity of conclusions. The site-directed mutagenesis experiments of Saedi et al. (1990), which showed that mutations on α68 and/or α71 cause complete loss of anti-MIR MAb binding (*see above*), are in favor of a critical role of α67–76; yet a major conformational effect could have been induced by these substitutions that would be potentially responsible for the observed effects.

Comparison of MAb binding to AChRs from different species and to the corresponding α67–76 peptides proved that peptide-mapping experiments indeed depicted the actual location of the MIR on the intact AChR. Testing 11 anti-MIR MAbs of various degrees of crossreactivity with *Torpedo* and human intact AChR showed that their relative binding to the *Torpedo* vs human α67–76 peptide correlated very well with their relative binding to the *Torpedo* vs human intact AChR (Tzartos et al., 1990b). Recently, Papadouli et al. (1991) tested binding of MAb to the α67–76 peptides corresponding to the α subunits of AChRs from five different sources: *Torpedo* electric organ, human muscle, *Xenopus* muscle, chicken brain, and rat brain. Binding of MAb to

the peptides correlated very well with their binding to the corresponding intact AChRs. The critical role of α68 and α71 was further confirmed (Fig. 3).

We believe that the above fine correlations between MAb binding to the intact AChRs and to the corresponding peptides prove that α67–76, or segments within it, constitute a critical part of the actual MIR on the intact AChR. However, it is most likely that other residues, from other parts of the primary AChR structure, contribute to the whole MIR epitopes on the intact AChR. This is inferred from the following observations:

1. All protein epitopes, in other antibody–antigen systems, that have been studied by X-ray diffraction analysis, consist of about 14–20 discontinuous amino acids (Laver et al., 1990; Bentley et al., 1990).
2. The affinity of the MAbs for the synthetic α67–76 peptide is much lower than that for the intact AChR.
3. Although α67–76 is identical among all studied nammalian muscle AChRs, some anti-MIR MAbs bind with dramatically different efficiency to the various mammalian AChRs.

Is the MAb-Defined MIR a Single Epitope?

Although all testable anti-MIR MAbs bind to α67–76, and to smaller peptides within it, the MIR is not a single epitope, or at least the anti-MIR MAb binding sites are clearly heterogeneous. The studies with continuously overlapping peptides suggest that the MIR epitopes have, overall, almost the same limits on the α subunit sequence, but with some variations that suggest that these epitopes are not absolutely identical. Furthermore, we do not know whether the putative parts of the MIR outside α67–76 are also common among the epitopes for the different anti-MIR MAbs.

Even if the MIR is essentially a single defined epitope, it is clear that the anti-MIR antibodies and their antigen binding sites are very heterogeneous. Such heterogeneity may be expressed by variations in the overall antibody–antigen fitness, residue-to-residue complementarity (hydrogen bonds, charge, hydrophobicity, and so on), antibody orientation, and the like. Several data support the heterogeneity of the anti-MIR MAb binding sites:

1. Crossreactivity of the anti-MIR antibodies among AChRs from different species varies a lot, from absolutely species-specific to well-crossreacting with AChRs from almost any tested species (Tzartos et al., 1981,1986a; Lindstrom et al., 1987).
2. Most anti-MIR MAb molecules are capable of crosslinking two AChR molecules, i.e., each of the two antibody arms binds to an α subunit of a different AChR molecule. However, some other anti-MIR MAbs form complexes of only one AChR molecule plus one MAb molecule; in this case, each antibody crosslinks the two α subunits of the same AChR molecule intramolecularly (Conti-Tronconi et al., 1981; Tzartos et al., 1986a). This heterogeneity requires that at least the orientation of the bound MAb differs between the two groups of MAbs.
3. Only approximately half of the anti-MIR MAbs bind to the denatured α subunit, whereas binding of the rest appears to be completely conformation-dependent (Tzartos and Lindstrom, 1980; Tzartos et al., 1981,1983).
4. Antiidiotypic sera against the antigen binding sites (paratopes) of anti-MIR MAbs showed little crossreaction with the antigen-binding sites of other anti-MIR MAbs. Among antiidiotypic sera to four anti-MIR MAbs, only antibodies against one MAb partially crossreacted with paratope-related idiotopes of the other anti-MIR MAbs (Verschuuren et al., 1991). In another study, the overall crossreactivity was very weak (Killen et al., 1985). This is unfortunate, since it renders distant the possibility of treating MG by the use of monoclonal antiidiotypes against anti-MIR antibodies.
5. The use of peptide analogs showed clearly that the role of each single residue to the MIR peptide–MAb interaction, despite showing an overall common trend, varied significantly from MAb to MAb (Tzartos et al., 1989; Bellone et al., 1989; Papadouli et al., 1990,1991).
6. Finally, as described below, cDNA sequencing of four anti-MIR MAbs (Mamalaki,

Trakas, and Tzartos, unpublished) showed explicitly that the antigen-binding sites of these molecules are not identical.

Molecular Characteristics of the Anti-MIR MAbs

Study of the immunoglobulin class and subclass of the anti-MIR MAbs showed that, of the 39 tested anti-MIR MAbs, three are of IgM class, 15 are IgG1, 12 are IgG2a, and nine are IgG2b. This distribution is rather similar to that of the rat anti-AChR MAbs (Tzartos et al., 1981,1983, 1986a). Therefore, no trend of homogeneity among the anti-MIR MAbs could be noticed at this level.

The VH (variable domains of heavy immunoglobulin chains) gene families to which three anti-MIR MAbs belong were studied (Verschuuren, 1989). Dot-blot hybridization of RNAs extracted from hybridomas producing the anti-MIR MAbs were performed. RNAs were hybridized with nine mouse VH probes representative of the nine VH families. MAbs 6 and 35 were both recognized by the probe VH Q52, but the third MAb (No. 65) was recognized by a different probe, the one from the PC7183 VH family.

The cDNA sequence of the VL (variable domains of light immunoglobulin chains) domains from four anti-MIR MAbs was recently obtained (Mamalaki, Trakas, and Tzartos, unpublished) and compared with the VL domain from an MAb against the cytoplasmic side of the AChR and with the known sequence of the VL domain from an irrelevant MAb of rat origin (Riechmann et al., 1988). The overall VL sequences of the anti-MIR MAbs were very similar to each other (on average, 91% homology within each pair), whereas they were quite different from those of the other two rat MAbs (on average, 67% homology within each heterologous pair). Complementarity-determining regions (CDRs) were also very similar among the anti-MIR MAbs. On average, only 3.2 of the 26 amino acid residues of the CDRs differed between anti-MIR MAbs (88% homology), whereas they differed, on average, by approx 16 residues from the CDRs of the control MAbs (38% homology). Of the CDR residues, 76% were conserved in all four anti-MIR MAbs. In conclusion, the VL domains of the anti-MIR MAbs have quite similar, but not identical, amino acid sequences. This weak heterogeneity probably accounts for both the practically common epitope of the anti-MIR MAbs, on the one hand, and the significant heterogeneity in their binding characteristics, on the other hand.

Function of the Anti-MIR MAbs

The potential functional role of the anti-MIR MAbs has been investigated on both experimental animals and muscle-cell cultures. Use of experimental animals provides the advantage of possibly testing the whole mechanism leading to MG. Use of muscle-cell cultures, on the other hand, provides the advantage of analyzing the MG mechanism and the ability to use human material, i.e., to test antibody function on human AChR.

All rats receiving any of five tested anti-MIR MAbs (of either IgG1 or IgG2a subclass) exhibited unambiguous symptoms of MG within 1–3 d. Most of them showed severe MG symptoms and lost approx half of their muscle AChR (Tzartos and Lindstrom, 1980; Tzartos et al., 1987). Similarly, rats and guinea pigs injected by two apparently anti-MIR MAbs (Lennon and Griesmann, 1989) also exhibited MG symptoms and loss of muscle AChR (Lennon and Lambert, 1980). In contrast, four MAbs to non-MIR sites (one against the extracellular part of the β-subunit and four probably against cytoplasmic sites) did not cause MG symptoms nor AChR loss (Tzartos et al., 1987).

AChR loss via antigenic modulation can be tested independently of MG mechanisms in muscle-cell and tissue cultures (Heinemann et al., 1977). The techniques that have been used for the evaluation of antigenic modulation of animal and human AChR were somewhat different. In animal-cell cultures, AChRs present at a given moment on the cell surface were

labeled by ^{125}I-α-bungarotoxin before the test MAb was added. The toxin–AChR–MAb complex is internalized faster than the toxin–AChR complex. The complexes are subsequently degraded within the phagolysosomes, and the degradation products, including ^{125}I-Tyr, are released in the culture medium. Released ^{125}I is considered a measure of the internalized AChR, and therefore of the induced antigenic modulation of the AChR. In human-cell cultures, where ^{125}I-α-bungarotoxin is quickly dissociated from the AChR, a modification of the technique was applied. In this, the amount of the remaining surface AChRs was measured (by ^{125}I-α-bungarotoxin-labeling at the end of the incubation with the test MAb), rather than the degraded ones (Tzartos et al., 1986b).

The modulating capacity of the anti-MIR MAbs was tested on fetal calf muscle cells (Conti-Tronconi et al., 1981), on the mouse muscle cell line BC3H1 (Tzartos et al., 1985), on human muscle cells (Tzartos and Starzinski-Powitz, 1986), and on the human rhabdomyosarcoma cell line TE671 (Sophianos and Tzartos, 1989). All tested anti-MIR MAbs, crossreactive with the solubilized AChR of the corresponding species under study, were very potent modulators of the AChR. They accelerated AChR degradation by 2–3 times, as compared with its degradation rate in absence of antibody, i.e., they produced an effect very similar to that of the MG serum antibodies (Conti-Tronconi et al., 1981; Tzartos et al., 1985; Tzartos and Starzinski-Powitz, 1986). Three MAbs to other regions of the extracellular side of the AChR (on α, β, and γ subunits) were either noneffective (when using mouse muscle cell cultures) or significantly less effective (when using cell cultures of human origin) than the anti-MIR MAbs. Five other MAbs, which are probably directed against the cytoplasmic side of the AChR, did not cause antigenic modulation (Tzartos et al., 1985; Tzartos and Starzinski-Powitz, 1986; Sophianos and Tzartos, 1989). Although these data could be explained by the particular epitope of each of these MAbs, it is nevertheless interesting that among the MAbs tested for AChR antigenic modulation, the anti-MIR MAbs were the most effective.

Antigenic modulation of AChR alone can be also studied in vivo by the injection of the bivalent F(ab)$_2$ fragments of the antibodies. F(ab)$_2$s are as capable of crosslinking the AChRs as the intact antibodies; thus they must be equally potent in causing antigenic modulation. However, since they lack the complement-binding Fc portion of the immunoglobulin they cannot cause AChR loss through the complement-mediated mechanism. F(ab)$_2$ fragments of anti-MIR MAb 35 injected into rats caused approx 30-40% AChR loss, compared with the controls, and clear symptoms of MG within 24–46 h (Loutrari, Kokla, and Tzartos, unpublished). This observation showed that anti-MIR antibodies are capable of causing MG even when only the antigenic modulation mechanism is active.

The MIR and ANTI-MIR Antibodies in Human MG

Although there is no longer much doubt that a large fraction of the rat MAbs raised against intact AChR bind to a small segment of the AChR (which includes α67–76), there is still considerable uncertainty concerning the anti-AChR antibody repertoire in MG patient sera. The situation is far less clear than with the MAbs, since until now significant data have been derived only from antibody competition experiments. Several arguments worth considering exist.

Indications in Favor of a Major Role of the MIR in Human MG

It has been repeatedly shown that preincubating the AChR with an anti-MIR MAb (intact or Fab fragment) not only inhibits binding of other MAbs, but also excludes binding of a large fraction of the polyclonal anti-AChR antibodies in human MG sera (Tzartos et al., 1982; Heidenreich et al., 1988b; Lennon and Griesmann, 1989). In a study of sera from 86 patients from

the USA, the proportion of serum antibodies inhibited by an anti-MIR MAb varied from patient to patient (from approx 10 to 100%), but in most sera it was more than 50%. On average, the anti-MIR MAb 35 inhibited approx 65% of the human MG anti-AChR antibodies from binding to the human AChR (Tzartos et al., 1982). Studies on MG sera from Greek (Tzartos et al., 1985; Sophianos and Tzartos, 1989), German (Hohlfeld et al., 1987), and French (Tzartos et al., 1990a) patients gave similar percentages of inhibition. Use of Fab fragments of the MAbs, rather than the bulky intact MAbs, had a similar protective effect (Tzartos et al., 1985; Sophianos and Tzartos, 1989). These observations suggest that about two-thirds of the human MG antibodies bind to the MIR.

Monoclonal antibodies to some extracellular regions other than the MIR also inhibited large (though smaller than the anti-MIR) fractions of the anti-AChR antibodies from binding to human AChR (Tzartos et al., 1982). Nevertheless, the epitopes of these MAbs, although located on the β and γ subunits, seemed to be near the MIR on the intact AChR, since these MAbs partially compete with the anti-MIR antibodies (Tzartos et al., 1986a).

Similar antibody-competition experiments were also used by other groups, which obtained results rather similar to those above. Whiting et al. (1986) and Heidenreich et al. (1988a,b) used mouse anti-human AChR MAbs and MG sera in order to study the antigenic specificities in various groups of MG patients. The MAbs used were classified, as mentioned previously, into five specificities, including the MIR. MAbs to several regions were capable of inhibiting large fractions of the serum antibodies from binding to the human AChR. The above authors observed major variations among patients in the proportions of serum antibodies inhibited by each MAb; nevertheless the anti-MIR MAb (D6) was usually the most potent inhibitor, with an average inhibition of serum antibody binding above 60%. Similarly, Lennon and Griesmann (1989) also found that a rat anti-MIR MAb from their MAb library inhibited on average 69% of the anti-AChR antibodies in a group of 24 MG sera. $F(ab)_2$ fragments of this MAb and of MAb 35 were equally effective.

Arguments Against a Major Role of the MIR in Human MG

As mentioned above, the available data cannot yet ensure that the MIR, conceived as a small region on the α subunit, is the target of the majority of human anti-AChR antibodies.

Lennon and Griesmann (1989) have argued that MG patients' sera do not recognize the MIR as a main target of their anti-AChR antibodies. The argument was based on the observations that: (1) The anti-MIR MAb, which indeed inhibited binding of the MG antibodies, crossreacted with both *Torpedo* and human AChR, whereas the MG antibodies did not bind to *Torpedo* AChR; and (2) The same MAb also blocked binding of an antiserum raised against the peptide α125–147 of the human AChR.

In our view, the above observations confirm that the putative anti-MIR antibodies of human sera are not of a single clone, but they are not in disagreement with the notion of a major role of the MIR in human MG. Concerning the first observation (i.e., the heterogeneity in crossreactivity), several data have shown that differences in species-specificity or crossreactivity between antibodies should not be taken as an indication of a major difference in the corresponding epitopes. In fact, anti-MIR MAbs that clearly bind to α67–76 (mainly to α67–71), as shown previously, exhibited dramatic species-specificity differences (Tzartos et al., 1981,1983) attributable to specific preferences for certain amino acids in positions that differ between *Torpedo* and human AChR (Papadouli et al., 1990,1991).

Concerning the second observation, competition between MAbs binding to α67–76 (MIR) and antisera against the synthetic peptide α125–147 could simply mean that the two segments are

located very near each other on the intact AChR. It could even mean that parts of α125–147 constitute the probably existing, but yet unidentified, complementary parts of the intact MIR. An additional explanation for the above competition may be that some of the serum antipeptide antibodies simply crossreact with α67–76. Unexpected crossreactivities of antibodies raised against synthetic peptides have been observed, probably because of conformation and charge pattern similarities (Maelicke et al., 1989).

In conclusion, there is not much solid evidence either in favor or against a major role of the strictly defined MIR in human MG. The fine correlation that has been observed in the anti-AChR MAb studies between antibody-competition experiments and peptide-mapping experiments proved that those antibody competitions depicted with an unexpected accuracy the actual epitope location on the AChR. This correlation strongly favors the possibility that competition between MAbs and MG sera may have also depicted the actual antibody specificities in MG sera.

Direct proof for the actual antigenic regions on human AChR in MG should come from more direct experiments. Unfortunately we cannot see how peptide-mapping experiments can be used reliably with the heterogeneous antibody mixtures of the human MG sera, which probably vary greatly in their affinities for the denatured AChR and its synthetic peptides. On the other hand, it is currently possible to express in eukaryotic cells various mutants of human AChR with substitutions on various sites. Such mutants may uncover the actual antigenic structure of the AChR in MG.

Function of the Anti-MIR Antibodies in Human MG Sera

It was shown previously that the anti-MIR MAbs are very potent in inducing MG in experimental animals and in causing antigenic modulation of the AChR in animals and in muscle-cell cultures. Unfortunately there is not yet an efficient method of directly testing the effect of the anti-MIR antibodies from human sera, since for the moment we cannot isolate this antibody fraction in a quantitative manner. Therefore, indirect approaches have been used, in which the MIR is shielded by an Fab fragment of an anti-MIR MAb and then the MG serum is added in order to measure its capacity to downregulate the protected AChR (Fig. 1B). Fab fragments bind well to the AChR, but, being univalent, do not crosslink the AChR molecules; therefore they do not accelerate the AChR internalization rate, i.e., they do not cause antigenic modulation.

By the Fab-protection experiments, the capacity of human anti-MIR antibodies to cause antigenic modulation of the AChR was studied, initially on the mouse muscle cell line BC3H1 (Tzartos et al., 1985). Fabs of MAb 35 were added to shield the MIR of the AChR on the cell surface. The MG sera added subsequently could bind to all regions other than the protected MIR. Thus, the resulting antigenic modulation was measured and was compared with that of nonprotected AChR. For 27 tested MG sera the anti-MIR Fab protected the AChR, on average, by 68 ± 16%. This was interpreted as the contribution to antigenic modulation of the anti-MIR antibodies in the same sera. In contrast, an anti-β-subunit MAb (No. 73), which binds to the extracellular side of the AChR, was a very weak protector.

Similar experiments were subsequently performed on human cell cultures. Human cells present the major advantage that the whole anti-AChR repertoire of the MG antibodies, rather than the small percentage that are crossreactive with animal AChR human antibodies, are tested. Because normal human-muscle cell cultures produce low amounts of surface AChR, a richer source of human AChR, the TE671 cell line, was used. These cells were initially considered to be of medulloblastoma origin (McAllister et al., 1977), but it was later suggested that they are of rhabdomyosarcoma origin (Stratton et al.,1989). They express a nicotinic AChR apparently identical with the human-muscle AChR (Luther et al., 1989). Using the above protection experiments,

it was found that Fab of anti-MIR MAbs inhibited the modulatory effect of the human anti-MIR antibodies by approx 80% (Sophianos and Tzartos, 1989), i.e., an even higher percentage than that obtained when using the mouse-cell cultures. The anti-β-subunit MAb was again only a weak protector. These data suggest that the human anti-MIR antibodies (as defined by the antibody-competition experiments) are mainly responsible for the loss of human AChR via antigenic modulation. In vivo experiments are in progress in order to study the pathogenic effect of the anti-MIR antibodies, possibly through all the pathogenic mechanisms in MG.

The MIR and Anti-MIR Antibodies as Tools for AChR Studies and for Therapeutic Approaches to MG

Probes for AChR Studies

At least three groups of uses of the anti-MIR MAbs as tools for the study of the AChR can be identified:

1. As markers of the α subunit and the AChR. As such they have been used in numerous studies that are not discussed in the present review.
2. As markers of the proper conformation of the α subunit and the AChR. Because anti-MIR MAb binding is heavily conformation-dependent, anti-MIR MAbs have proved valuable as probes of the proper AChR conformation, at least at the corresponding specific site. Thus anti-MIR MAbs were used in conjuction with α-bungarotoxin and MAbs to conformation-independent epitopes for monitoring α-subunit synthesis, maturation and assembly with the other AChR subunits in AChR expression systems, either endogenously expressed or after introducing specific RNA or DNA to various cells (Merlie and Smith, 1986; Fujita et al., 1986; Blount and Merlie, 1988; Paulson and Claudio, 1990; Conroy et al., 1990; Saedi et al., 1991), or for the renaturation of the denatured AChR α subunit (Tzartos and Changeux, 1984).
3. For the identification, isolation, and characterization of neuronal AChRs. Because of the considerable phylogenetic stability of the MIR, anti-MIR MAbs have been used successfully for the identification and characterization of AChRs from various species and tissues. They have also proved particularly valuable for the study of neuronal AChRs. MAbs to the MIR of electric-organ AChRs bound to non-α-bungarotoxin-binding chick brain and ganglionic molecules, though not to mouse or rat brain molecules (Swanson et al., 1983; Jacob and Berg, 1988). The chick molecules were subsequently isolated by affinity chromatography (using the same MAbs) and were characterized as functional AChRs. Unlike muscle AChR, these AChRs consist of only two kinds of subunits, α and β (Whiting and Lindstrom, 1986; Lindstrom et al., 1987). In addition to the chick, the anti-MIR MAbs also crossreacted with goldfish brain AChR (Henley et al., 1988), bovine chromaffin cell AChR (Higgins and Berg, 1987), and frog retina and optic tectum molecules (Sargent et al., 1989), and have been used in extensive studies of these molecules.

MAbs raised subsequently against the chick brain AChR were found to bind also to rat-brain AChR and were used for its purification (Whiting et al., 1987). In turn, MAbs raised against rat brain AChR crossreacted with human and bovine brain AChR and were used for their purification and study as well (Lindstrom et al., 1987; Whiting and Lindstrom, 1988).

Binding of anti-MIR MAbs to chick neuronal AChR but not to rat brain AChR, which delayed progress in this field, can now be explained on the basis of the amino acid sequences of these molecules. Antigenically critical residues within the MIR segment α67–76 (especially α68 and α71) have been conserved in most known sequences of chick brain AChR α subunits, but not in most rat neuronal AChR α subunits (Cauley et al., 1989; Nef et al., 1988; Wada et al., 1988) (Fig. 2B).

The use of anti-AChR MAbs, including the anti-MIR ones, as probes for AChR studies has been recently reviewed (Tzartos, 1990; Lindstrom et al., 1991).

Potential Tools for Therapeutic Approaches to MG

If it is proven that the majority of the human anti-AChR antibodies bind on the α67–76 segment, then the design of an MIR analog with the conformation, and therefore the binding properties, of the intact MIR, and its availability in large quantities, will lead to interesting therapeutic applications.

The peptide-analog studies for the identification of the antigenically critical residues within α67–76 showed that it is possible to construct a modified MIR peptide with high binding activity. Substitution of Gly73 or Lys76 by Ala in the *Torpedo* α67–76 peptide resulted in significant enhancement of the binding for most tested MAbs (Papadouli et al., 1990). Subsequently, we produced analogs of both *Torpedo* and human α67–76 with various amino acids on α73 or on both α73 and α76. Certain analogs, especially with His or Arg substitutions, exhibited distinct enhancement of MAb binding. Such enhancement was especially dramatic with anti-MIR MAbs, which bound very weakly, if at all, to the original decapeptides. Most characteristically, MAb 35, which does not bind to either *Torpedo* or human decapeptides, did bind to a *Torpedo* analog and weakly to three human α67–76 analogs (Papadouli et al., 1991). It is likely, therefore, that a mixture of selected α67–76 analogs, rather than a single analog, each partially mimicking the conformation of the actual antigen on the AChR surface, could be used for specifically immunoabsorbing the anti-MIR antibodies from the patients' blood. Also, peptide–toxin hybrid molecules could be formed with such analogs for the selective targeting and killing of AChR-specific lymphocytes. Finally, MIR analogs administered to patients could possibly bind to and inactivate their endogenous anti-MIR antibodies.

The use of anti-idiotypic antibodies against the anti-MIR antibodies deserves further elaboration. As mentioned above, the anti-idiotypes against the anti-MIR MAbs studied so far do not cross-react well among the different anti-MIR MAbs (Killen et al., 1985; Verschuuren et al., 1991). This can now be explained by the fact that the anti-MIR MAbs bind to almost, but not quite the same epitope, and the CDRs on their antigen-binding sites, though quite similar, differ significantly. However, since all these antibodies do bind to a single structure (i.e., to the MIR), one should be able to find a "universal" antiidiotypic MAb with the actual MIR structure, thus capable of binding all anti-MIR antibodies. The binding site of such an antiidiotype will simply have the structure of the MIR. If α67–76 indeed plays a major role in MG, such a search would probably be laborious but rewarding.

Independently of whether MG MAbs bind mainly on and around a67–76 or their inhibition by anti-MIR MAbs from binding to the AChR is attributable to other mechanisms, the fact that the AChR can be protected against the activity of MG antibodies by a single MAb has considerable therapeutic potential, even though we cannot envisage complete cure from this approach. Several requirements should be met for the success of such an approach:

1. The used MAbs should not induce MG. In fact, Fab fragments of the anti-MIR MAbs are expected (and to some extend have already been seen) to be pathogenically inactive, since Fab cannot crosslink their antigen nor can they bind complement.
2. Fab–MAb binding should not interfere with any aspect of the neuromuscular junction; since anti-MIR MAbs do not interfere with the gating of the channel, this requirement is also very likely to be satisfied.
3. The Fabs should not be immunogenic; thus either novel human anti-MIR MAbs or "humanized" MAbs (Riechmann et. al., 1988) must be designed by engineering the available animal MAbs.
4. Their affinity for the AChR must be very high in order to bind practically irreversibly on the junctional AChR, so that small and infrequent doses are sufficient. Such very high affinity Fabs of anti-MIR MAbs will probably also have to be engineered from the available ones or, alternatively, new MAbs will have to be produced.

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