Antigenic role of single residues within the main immunogenic region of the nicotinic acetylcholine receptor

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The target of most of the autoantibodies against the acetylcholine receptor (AChR) in myasthenic sera is the main immunogenic region (MIR) on the extracellular side of the AChR α -subunit. Binding of anti-MIR monoclonal antibodies (mAbs) has been recently localized between residues $\alpha 67$ and $\alpha 76$ of *Torpedo californica* electric organ (WNPADYGGIK) and human muscle (WNPDDYGGV/K) AChR. In order to evaluate the contribution of each residue to the antigenicity of the MIR, we synthesized peptides corresponding to residues $\alpha 67$ –76 from *Torpedo* and human AChRs, together with 13 peptide analogues. Nine of these analogues had one residue of the *Torpedo* decapeptide replaced by L-alanine, three had a structure which was intermediate between those of the *Torpedo* and human $\alpha 67$ –76 decapeptides, and one had D-alanine in position 73. Binding studies employing six anti-MIR mAbs and all 15 peptides revealed that some residues (Asn⁶⁸ and Asp⁷¹) are indispensable for binding by all mAbs tested, whereas others are important only for binding by some mAbs. Antibody binding was mainly restricted to residues $\alpha 68$ –74, the most critical sequence being $\alpha 68$ –71. Fish electric organ and human MIR form two distinct groups of strongly overlapping epitopes. Some peptide analogues enhanced mAb binding compared with *Torpedo* and human peptides, suggesting that the construction of a very antigenic MIR is feasible.

INTRODUCTION

The human disease myasthenia gravis (MG) is caused by an antibody-mediated autoimmune response to the nicotinic acetylcholine receptor (AChR). Anti-AChR antibodies cause loss and/or blockage of the function of the AChR molecules, resulting in failure of neuromuscular transmission (Drachman, 1987; Willcox & Vincent, 1988; Lindstrom *et al.*, 1988).

AChR is a transmembrane glycoprotein composed of five homologous subunits of the stoichiometry $\alpha_2\beta\gamma\delta$ and with known amino acid sequence (Merlie & Smith, 1986; Numa, 1987; Stroud, 1987). Acetylcholine binds on the two α -subunits regulating the opening of the ion channel. Snake venom α -toxins which bind near the acetylcholine-binding sites, together with agents which non-competitively block channel function, have proved to be excellent probes of the AChR (Changeux & Revah, 1987; Maelicke, 1987). Anti-AChR monoclonal antibodies (mAbs) provide also valuable probes for the study both of the AChR and of MG (Lindstrom *et al.*, 1988; Fuchs *et al.*, 1987; Chinchetru *et al.*, 1989; Tzartos *et al.*, 1990a).

The majority of the mAbs produced in rats immunized with intact AChR compete with each other for binding to an area of the α -subunit called the main immunogenic region (MIR) (Tzartos & Lindstrom, 1980; Tzartos *et al.*, 1982; Kordossi & Tzartos, 1989). Anti-MIR mAbs, when added to muscle cell cultures, cross-link the AChRs and cause their loss (Tzartos *et al.*, 1985). Also, when injected into rats, the mAbs cause experimental MG (Tzartos *et al.*, 1987).

A series of studies with AChR peptides has localized the binding of several anti-MIR mAbs to within residues 1–151 (Barkas *et al.*, 1986), 46–120 (Ratnam *et al.*, 1986), 37–85

(Barkas *et al.*, 1987), 61–76 (Barkas *et al.*, 1988), 65–78 (Wood *et al.*, 1989) and 67–76 (Tzartos *et al.*, 1988, 1989, 1990b) of the α -subunit. Hydrophilicity, flexibility and surface probability data support the antigenicity of the region $\alpha 67$ –76 (Tzartos *et al.*, 1990b). Two-dimensional n.m.r. studies in dimethyl sulphoxide (Me₂SO) solutions showed a rigid folded structure for this segment (Cung *et al.*, 1989a). Electron microscopy studies showed that the MIR on the intact AChR is at or close to the side of the α -subunit, between the synaptic end and the membrane surface (Kubalek *et al.*, 1987).

Single anti-MIR mAbs and their Fab or F(ab)₂ fragments inhibit binding of more than 60% of the anti-AChR antibodies present in sera from immunized rats and MG patients (Tzartos & Lindstrom, 1980; Tzartos *et al.*, 1981, 1982, 1985; Heidenreich *et al.*, 1988; Lennon & Griesmann, 1989). Furthermore, shielding of the MIR of mouse or human cell-bound AChR by Fab fragments of an anti-MIR mAb dramatically protected the AChR against loss induced by the human MG sera (Tzartos *et al.*, 1985; Sophianos & Tzartos, 1989).

Because of the very low and variable binding affinity of the MG antibodies for AChR peptides, reliable epitope localization for the majority of the MG antibodies cannot yet be determined. Therefore it is still uncertain whether competition between anti-MIR mAbs and MG sera is due to MG antibody binding to region $\alpha 67$ –76, or to steric or even to allosteric hindrance. In the intact AChR, $\alpha 67$ –76 may be in contact with various other segments which could participate in the MIR. Recently, an anti-MIR mAb was found to inhibit binding of low-affinity antibodies produced against the peptide $\alpha 125$ –147 (Lennon & Griesmann, 1989). However, antibodies to AChR synthetic peptides often cross-react with several irrelevant AChR peptides, probably

Abbreviations used: MG, myasthenia gravis; AChR, acetylcholine receptor; MIR, main immunogenic region; mAb, monoclonal antibody; Me₂SO, dimethyl sulphoxide; r.i.a., radioimmunoassay; poly(A–K), poly(DL-Ala)–poly(Lys); PBS, phosphate-buffered saline; n.o.e., nuclear Overhauser effect.
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because of conformation and charge pattern similarities (Maelicke *et al.*, 1989). In fact, mAbs binding to peptides $\alpha 65-78$ and $\alpha 125-143$ (Wood *et al.*, 1989) do not compete with each other (Heidenreich *et al.*, 1988), suggesting that the latter segment is not near the MIR.

Various data suggest that the segment $\alpha 67-76$ is important in human MG, as it is in experimental MG. The observations that (1) all testable rat anti-MIR mAbs bind to $\alpha 67-76$, (2) competition among mAbs to several other AChR regions also correlates with peptide mapping (Kordossi & Tzartos, 1987), and (3) the antibody repertoire in MG patients is similar to that in immunized rats (Tzartos *et al.*, 1982) strongly suggest that a large number of the MG antibodies bind on and/or around $\alpha 67-76$. In addition, simply the fact that anti-MIR mAbs to $\alpha 67-76$ block binding and function of the majority of the human MG antibodies (Tzartos *et al.*, 1985; Sophianos & Tzartos, 1989) necessitates extensive structural studies of this segment.

In the present study we attempted to elucidate the role of each residue within region $\alpha 67-76$. We synthesized peptide analogues, corresponding to the *Torpedo californica* AChR $\alpha 67-76$ sequence, in which every amino acid was replaced sequentially by Ala, or in a few cases by an alternative amino acid. We then studied the effect of the amino acid replacement on the binding capacity of the anti-MIR mAbs using three techniques.

MATERIALS AND METHODS

Peptide synthesis and characterization

Fourteen decapeptides, one nonapeptide and one irrelevant octapeptide were synthesized (see Fig. 1). Peptide synthesis was performed using the stepwise solid-phase method (Merrifield, 1963). Peptides were deprotected and cleaved from the resin by HF treatment and purified by chromatographic methods. Their purity was confirmed by t.l.c. and n.m.r. spectroscopy. One- and two-dimensional n.m.r. techniques were used as previously described (Cung *et al.*, 1989a).

mAbs

The production and characteristics of the mAbs used have been described earlier (Tzartos & Lindstrom, 1980; Tzartos *et al.*, 1981, 1983; Ratnam *et al.*, 1986; Barkas *et al.*, 1987, 1988). All had been derived from rats immunized with AChRs from various species. The preparations used were 50% ammonium sulphate precipitates from hybridoma supernatants, dialysed against phosphate-buffered saline (PBS, 8 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 /140 mM- NaCl /3 mM- KCl , pH 7.0) containing 0.05% (w/v) NaN_3 . Some of the characteristics of these mAbs are shown in Table 1.

Peptide mapping

Binding of anti-MIR mAbs to the peptides was tested by a solid-phase r.i.a. Peptides were attached to 96-well plates (Nunc) using either of two techniques. In the first (Barkas *et al.*, 1988, Tzartos *et al.*, 1988), the plates were treated with poly(DL-Ala)-poly(Lys) [poly(A-K)] (Sigma) (20 $\mu\text{g}/\text{ml}$) in 0.1 M- NaHCO_3 overnight at 4 °C, followed by three washings with 10 mM- KH_2PO_4 , pH 7.0 (phosphate buffer). The peptides were diluted in phosphate buffer containing 0.125% (v/v) glutaraldehyde (at a final peptide concentration of 0.2 mg/ml, or as indicated) and added immediately to the plates. After 1 h, the plates were washed three times with phosphate buffer. In the second technique, 0.1 mg of peptides/ml in 0.1 M-carbonate/bicarbonate buffer, pH 9.6, was added directly to untreated plates and incubated overnight at 4 °C.

The following steps were common for both techniques and were all performed at room temperature. The plates were

incubated for 30 min with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween) and then washed twice with the same buffer. They were then incubated for 30 min with PBS/Tween containing 3% (w/v) BSA (200 $\mu\text{l}/\text{well}$) and washed once with PBS/Tween. The test mAb, diluted 1:10 or 1:50 in PBS/Tween containing 2 mg of BSA/ml, was added and incubated for 2.5 h. The plates were then washed three times with PBS/Tween and incubated for 45 min with a 1:150 dilution of rabbit anti-(rat γ -globulin) antibody in PBS/Tween plus 2 mg of BSA/ml, followed by another three washings with PBS/Tween prior to incubation for 30 min with 10^5 c.p.m. of ^{125}I -labelled Protein A per well [radioiodinated by chloramine-T to $(1-2) \times 10^7$ c.p.m./mg]. Finally, the plates were washed six times with PBS/Tween. The bound radioactivity was removed by 1% (w/v) SDS (200 $\mu\text{l}/\text{well}$), transferred to test tubes and measured in a γ -counter. Radioactivity obtained from wells treated either in the absence of peptide or with an irrelevant octapeptide (HDRVYHDDf), but with the corresponding mAb, and from wells treated with the corresponding peptide and with the control mAb 25, was subtracted. Background readings obtained in the absence of peptide were at about the same level as those obtained in the presence of the control peptide (HDRVYHDDf). Incubation volumes were 50 $\mu\text{l}/\text{well}$ except for the cases indicated above. mAb binding to the peptides was estimated as the percentage of its binding to the *Torpedo* peptide under the same conditions and concentrations of mAb and peptide. It is expressed by the following equation:

$$\text{mAb binding (\%)} = \frac{\Delta \text{c.p.m.}(\text{mAb}_x \cdot p_x)}{\Delta \text{c.p.m.}(\text{mAb}_x \cdot p_i)} = \frac{[(\text{mAb}_x \cdot p_x) - (\text{mAb}_x \cdot p)] - [(\text{mAb } 25 \cdot p_x) - (\text{mAb } 25 \cdot p)]}{[(\text{mAb}_x \cdot p_i) - (\text{mAb}_x \cdot p)] - [(\text{mAb } 25 \cdot p_i) - (\text{mAb } 25 \cdot p)]}$$

where mAb_x and p_x are the test mAb and peptide respectively, mAb 25 is the non-binding control mAb, p is the non-specific peptide and p_i is the *Torpedo* peptide. $\text{mAb}_x \cdot p_x$ (etc.) gives the total c.p.m. obtained by binding of mAb_x to peptide p_x (etc.)

Competition experiments

The *Torpedo* peptide was immobilized on the plate using either of the two techniques described above, at concentration of 0.1 mg/ml. An mAb at a dilution of 1:500 was incubated overnight with the test peptide analogue or with the control non-specific peptide (0.2 mg/ml). Subsequently the mixture was added to the plated peptide and the assay was performed as described above.

RESULTS

Synthetic peptides

Fig. 1 shows the synthesized *Torpedo* and human $\alpha 67-76$ peptides, the ten *Torpedo* analogues ($pA^{67}-pA^{76}$, pA^{73}) and the three analogues intermediate between *Torpedo* and human peptides ($pdesA^{70}$, pD^{70} , pV^{75}). Peptide purity was greater than 95%. Their sequence confirmation was achieved by two-dimensional n.m.r. spectroscopy.

Effects of single Ala substitutions on mAb binding

mAb binding to the peptides was tested by two direct r.i.a. techniques. These differed only in the method of plating the peptides to the plastic. Plating was achieved either by direct adsorption or through the use of glutaraldehyde, which covalently bound the peptides to the poly(A-K) previously adsorbed on to the plastic well.

Table 1 shows the raw data obtained for all mAbs tested with the reference *Torpedo* peptide (p_{Tor}) and with a non-specific

peptide. The non-anti-MIR mAbs (142 and 155) gave essentially background radioactivity. The first six mAbs bound 5–35 times more radioactivity to the *Torpedo* peptide than to the control peptide. These six mAbs were used in the subsequent experiments, together with control mAb 25.

Fig. 2 shows the binding pattern of the six anti-MIR mAbs to the ten *Torpedo* peptide analogues. Generally there was a good correlation of the results between the direct peptide plating (■) and the poly(A-K)/glutaraldehyde (■) techniques. However, in some cases very significant differences were observed, due mainly to a dramatically lower binding by all mAbs to certain peptides using the latter technique. Apparently, glutaraldehyde caused

conformational changes which altered the antigenic properties of these analogues.

Substitution of some residues totally inhibited binding of all tested anti-MIR mAbs by either technique. Others were more or less important, but only for some mAbs, whereas some substitutions had little, if any, effect on mAb binding, as follows.

Replacement of residue Trp⁶⁷ with Ala (peptide pA⁶⁷) resulted in complete inactivation of the epitopes using the poly(A-K)/glutaraldehyde technique, but did not dramatically inhibit mAb binding when employing the direct peptide plating technique. Unlike most of the remaining nine residues, substitution of this residue differentiated between the epitopes of the anti-(fish AChR) mAbs [i.e. anti-(*Torpedo* AChR) or anti-(*Electrophorus electricus* AChR) mAbs], its effect varying from inhibition to enhancement of mAb binding. Thus, under the direct plating technique, pA⁶⁷ enhanced binding of the anti-(fish AChR) mAb 22, although it partially decreased binding of another anti-(fish AChR) mAb (no. 50). It also decreased binding of the two anti-(human AChR) mAbs.

Residue Asn⁶⁸ seemed to be the most critical of all of the ten residues tested. Its substitution by Ala in pA⁶⁸ practically eliminated binding of all mAbs, from both groups (anti-fish or anti-human), with both techniques.

Substitution of Pro⁶⁹ by Ala (peptide pA⁶⁹) resulted in a strong, though not complete, inhibitory effect on the binding of all mAbs. Its effect was stronger for the anti-(fish AChR) than for the anti-(human AChR) mAbs. Substitution of Asp⁷¹ had a strong inhibitory effect, second only to that of substituted Asn⁶⁸. Three mAbs did not bind at all to pA⁷¹, whereas the other three bound weakly (i.e. less than 25% of their binding to the *Torpedo* peptide).

Substitution of Tyr⁷² dramatically differentiated between anti-(fish AChR) and anti-(human AChR) mAb epitopes. The aromatic ring of tyrosine seems to be indispensable only for the epitopes of the anti-human mAbs, whereas it did not influence the binding of anti-*Torpedo* mAbs. Interestingly, the effect of Ala substitution on mAb 6 binding (i.e. the only anti-fish mAb cross-reactive with human AChR) was intermediate between the effects of the two groups of mAbs. Use of glutaraldehyde

Peptide name	
pTor	WNPADYGGIK
pA ⁶⁷	A-----
pA ⁶⁸	-A-----
pA ⁶⁹	--A-----
pA ⁷¹	-----A----
pA ⁷²	-----A----
pA ⁷³	-----A----
pdA ⁷³	----- ^d A----
pA ⁷⁴	-----A----
pA ⁷⁵	-----A----
pA ⁷⁶	-----A----
pHum	----D-----V-
pdesA ⁷⁰	----D-----
pD ⁷⁰	----D-----
pV ⁷⁵	-----V----
pCon	HDRVYHDF

Fig. 1. Amino acid sequences of AChR $\alpha 67$ –76 decapeptides from *Torpedo* and human, and various synthetic analogues

Sequences are shown for the *Torpedo* $\alpha 67$ –76 decapeptide (pTor), its synthetic analogues resulting from single L-Ala substitutions (pA⁶⁷–pA⁷⁶) and a D-Ala substitution (pdA⁷³), the human $\alpha 67$ –76 peptide (pHum), three intermediate peptides between the *Torpedo* and human $\alpha 67$ –76 peptides (nonapeptide pdesA⁷⁰, missing residue no. 70, and pD⁷⁰ and pV⁷⁵), and a control peptide of an irrelevant sequence (pCon).

Table 1. Anti-AChR mAbs: some characteristics and their binding to the *Torpedo* peptide $\alpha 67$ –76 (pTor) and to a non-specific control peptide

Data in columns 2 and 3 are from Tzartos *et al.* (1981, 1983, 1988, 1990b), Ratnam *et al.* (1986) and Barkas *et al.* (1988). S.D. $\leq 7\%$. Specificity for intact AChRs from human muscles (H), *Torpedo* (T) or *Electrophorus electricus* (E) electric organs is shown: –, + and ++ indicate the extent of cross-reactivity. Underlining indicates the actual immunogen. Abbreviations: ND, not determined; glut., glutaraldehyde.

mAb no.	Specificity for intact AChR from:				Binding to peptide plated directly to the plate (c.p.m./well)			Binding to peptide plated through poly(A-K)/glut. (c.p.m./well)		
	H	T	E	Epitope	pTor	Control peptide	No peptide	pTor	Control peptide	No peptide
198	++	++	++	MIR, $\alpha 67$ –76*	6423 $\pm$ 254	1438 $\pm$ 96	1329 $\pm$ 47	3512 $\pm$ 42	471 $\pm$ 14	380 $\pm$ 9
203	++	++	++	MIR, $\alpha 67$ –76*	3305 $\pm$ 35	622 $\pm$ 58	619 $\pm$ 16	1698 $\pm$ 146	255 $\pm$ 20	233 $\pm$ 22
6	+	++	++	MIR, $\alpha 67$ –76*	23 508 $\pm$ 162	733 $\pm$ 24	671 $\pm$ 32	10 518 $\pm$ 636	473 $\pm$ 6	411 $\pm$ 19
50	–	++	++	MIR, $\alpha 67$ –76	6580 $\pm$ 90	584 $\pm$ 30	515 $\pm$ 24	3497 $\pm$ 18	310 $\pm$ 35	406 $\pm$ 40
47	–	++	++	MIR, $\alpha 67$ –76	23 217 $\pm$ 203	2279 $\pm$ 124	1814 $\pm$ 19	8952 $\pm$ 294	534 $\pm$ 53	420 $\pm$ 21
22	–	++	++	MIR, $\alpha 67$ –76*	13 532 $\pm$ 190	1915 $\pm$ 60	1821 $\pm$ 92	6516 $\pm$ 74	343 $\pm$ 20	272 $\pm$ 32
28	+	++	++	MIR, $\alpha 67$ –76	1500 $\pm$ 41	921 $\pm$ 39	894 $\pm$ 58	1922 $\pm$ 31	281 $\pm$ 41	238 $\pm$ 22
42	++	++	++	MIR, $\alpha 67$ –76	1710 $\pm$ 134	982 $\pm$ 27	923 $\pm$ 35	2306 $\pm$ 25	1187 $\pm$ 18	940 $\pm$ 40
37	++	++	++	MIR, $\alpha 67$ –76	351 $\pm$ 37	334 $\pm$ 14	308 $\pm$ 10	1150 $\pm$ 22	373 $\pm$ 25	321 $\pm$ 7
177	–	++	+	MIR, $\alpha 67$ –76	512 $\pm$ 44	410 $\pm$ 18	335 $\pm$ 12	ND	ND	ND
142	–	++	–	$\alpha 353$ –359	1433 $\pm$ 13	1429 $\pm$ 25	1482 $\pm$ 35	ND	ND	ND
155	+	++	+	$\alpha 371$ –378	442 $\pm$ 35	455 $\pm$ 18	387 $\pm$ 11	ND	ND	ND
25	–	–	++		345 $\pm$ 33	320 $\pm$ 17	318 $\pm$ 12	272 $\pm$ 39	243 $\pm$ 18	235 $\pm$ 20

* Judging from their relative binding to a $\alpha 55$ –74 peptide, it was concluded that these mAbs bind up to residue $\alpha 74$ (Tzartos *et al.*, 1990b).

Fig. 2. Binding patterns of six anti-MIR mAbs to the AChR $\alpha 67-76$ synthetic peptides and analogues, as obtained by solid phase r.i.a.

■, Binding to peptides which were plated directly; ▨, binding to peptides plated through poly(A-K)/glutaraldehyde. mAb binding was studied at two mAb dilutions (1:50 and 1:10) in three or four experiments per dilution, and results were averaged separately for each dilution. Each bar represents the mean of the average values obtained for the two different mAb dilutions; error bars represent the s.d. between the two means. Binding to the *Torpedo* peptide was considered as 100 % in all cases. pHum, human peptide.

apparently damaged the antigenic conformation of this analogue, as with pA⁶⁷, though not completely.

Substitution of Gly⁷³ by Ala had practically no effect using the direct peptide plating technique. Interestingly, glutaraldehyde apparently fixed the conformation of the peptide so as to resemble a more native-like conformation, resulting in a general enhancement of mAb binding. Substitution of the same residue (Gly⁷³) by D-Ala instead of L-Ala reversed the activation observed with L-Ala into a mild inhibitory effect on binding of most mAbs in both techniques.

Substitution of the second glycine (Gly⁷⁴) by Ala resulted in a distinctly different effect on mAb binding. This substitution was especially critical for the anti-(human AChR) mAbs, thus, like pA⁷², differentiating between the two groups of mAbs. Ala in place of Ile⁷⁵ only weakly affected mAb binding under the direct attachment technique. mAbs 22 and 50 were the only two which were partially affected.

Substitution of Lys⁷⁶ by Ala in most cases had no inhibitory effect. Under the direct technique it differentiated between anti-fish mAbs; this binding pattern resembles that caused by substitution of the first residue of the decapeptide. Hence $\alpha 67$ and $\alpha 76$ seem to be on either edge of the MIR such that some epitopes include them although most do not. pA⁷⁶ had an enhancing effect on binding of some mAbs using both techniques. The very high enhancing effect observed using the poly(A-K)/glutaraldehyde technique may be an exaggeration of the actual effect, since Lys⁷⁶ in the *Torpedo* peptide has probably been dramatically modified. Enhancement of binding observed

when using direct plating of the peptides is analysed further below.

Peptide analogues intermediate in structure between human and *Torpedo* MIRs

Fig. 2 also shows mAb binding to peptides intermediate between *Torpedo* and human AChR $\alpha 67-76$. No mAb bound to the glutaraldehyde-treated human peptide. However, the mAb binding capacity of the same peptide, when attached directly to the plastic, was generally as expected: it marginally, if at all, bound the three specific anti-(fish AChR) mAbs (22, 50 and 47), it exhibited moderate reactivity with the human AChR cross-reactive mAb 6, and its binding was higher with the anti-human mAb 198 than with the *Torpedo* peptide when using the same mAb (170 % compared with 100 % relative binding). However, the anti-human mAb 203 bound rather weakly to this peptide (25 % of its binding to the *Torpedo* peptide). The latter can be explained by the fact that these anti-human mAbs have higher apparent titres (therefore higher affinity) for intact *Torpedo* AChR than for intact human AChR (S. J. Tzartos unpublished work).

Complete elimination of residue $\alpha 70$ (peptide pdesA⁷⁰) resulted in a binding pattern, in the absence of glutaraldehyde, analogous with (though generally with lower binding than) that of the human peptide. This residue is the site of the single conservative substitution between *Torpedo* and human $\alpha 67-76$. Glutaraldehyde enhanced binding of all mAbs as compared with their binding to the directly plated peptides.

Simple substitution of residue Ala⁷⁰ by Asp, i.e. an intermediate structure between the human and *Torpedo* decapeptides, resulted in a peptide (pD⁷⁰) with a binding pattern similar to that of the human peptide, when using either technique. It was shown that Ala⁷⁰ is indispensable for anti-(fish AChR) mAb binding. However, its substitution by Asp enhanced the binding of the anti-human mAbs when compared with their binding to either *Torpedo* or human peptides.

Contrary to the strong effects caused by substitution of $\alpha 70$ observed above, the other intermediate between *Torpedo* and human $\alpha 67-76$, formed by substituting Ile⁷⁵ by Val (peptide pV⁷⁵), had no effect on the binding of anti-fish mAbs, and it had an unexpected mild inhibitory effect on anti-human mAb binding.

In order to investigate the attachment to the plastic of those peptide analogues which did not bind to any mAb under the direct plating technique, the *Torpedo* peptide (at 0.05 mg/ml) was mixed with a low-binding peptide (0.1 mg/ml) (pA⁶⁸, pA⁷¹ or the non-specific peptide) and adsorbed directly on to the plates. Binding of mAb 6 was then tested. This mAb bound to the peptide mixture only by about 25–30 % of the total possible binding had the *Torpedo* peptide alone been used (result not shown). This indicates that the test peptides competed with the *Torpedo* peptide for plating to the plastic, i.e. the test peptides bound satisfactorily to the plastic.

Fig. 3 summarizes the results presented so far, averaging the binding patterns of the anti-human and anti-fish mAbs when peptides are attached directly to the plates. The usually low variations (small s.d.) within each group suggest that each group consists of very similar, though clearly not identical, epitopes.

Peptide competition experiments

For further confirmation of the results obtained by the direct r.i.a., we performed competition experiments between plastic-bound *Torpedo* peptide and soluble peptide analogues for binding to certain mAbs. We tested mAbs 6 (Fig. 4), 47 and 198 (not shown). Although there were significant quantitative differences when compared with the direct r.i.a. techniques, overall the

Fig. 3. Summary of anti-MIR mAb binding to synthetic peptides

Data are derived from Fig. 2. Each bar represents the average binding of the two anti-human mAbs (a) or the four anti-fish mAbs (b) to a peptide analogue as a percentage of binding to the *Torpedo* peptide (pTor) under conditions of direct peptide plating.

Fig. 4. Competition between the immobilized *Torpedo* peptide and its solubilized analogue for binding to mAb 6

The bars represent the extent of inhibition of mAb 6 binding to the plated *Torpedo* peptide (pTor) due to pre-incubation of the mAb with the indicated peptide analogues in solution. ■ and ▨ represent direct and poly(A-K)/glutaraldehyde-mediated pTor plating respectively. pCon, control peptide; pHum, human peptide.

results exhibited the same trend as those of the direct r.i.a. Peptide competition confirmed the critical role of residues Asn⁶⁸, Pro⁶⁹ and Asp⁷¹ for the epitope of mAb 6. In addition, these experiments suggested that Tyr⁷² is in fact important for binding of mAb 6.

Activating peptide analogues

The enhancing effect of analogues pA⁷³ and pA⁷⁶ was studied in more detail by varying mAb and peptide concentrations (Fig. 5). As the actual concentration of the peptides attached to the plastic was unknown, estimation of the absolute affinity constants was difficult. However, the observation that mAb binding to pA⁷³ and pA⁷⁶, as compared to that to the *Torpedo* peptide with equivalent concentrations and conditions, was usually increased (by up to approx. 600 %) by lowering the reactant concentration, suggests that the affinity of these analogues for the mAbs improved substantially. Similar results were obtained when the two peptides were attached to the plates via poly(A-K)/glutaraldehyde (results not shown).

DISCUSSION

In the present study a detailed investigation of the antigenic role of each residue within the segment $\alpha 67-76$ of the AChR, which contains the main loop of the MIR, was performed. In addition to a thorough understanding of the MIR, this kind of study may allow the construction of a synthetic MIR with high affinity for anti-MIR antibodies. Such a peptide could be used in the treatment of MG. Significant progress towards both aims was achieved.

Substitutions of each amino acid with alanine rather than glycine was carried out because the latter often induces major conformational changes in peptides. Alanine, due to the presence of its short side chain, usually does not fundamentally perturb the conformational preferences of the main peptide chain.

Three kinds of r.i.a. were used so as to exclude possible

Fig. 5. Enhancement of binding of mAbs 6 (a, d), 47 (b, e) and 22 (c, f) to peptides pA⁷³ (a-c) and pA⁷⁶ (d-f) expressed as a percentage of binding of the same mAb dilutions to the *Torpedo* peptide under identical conditions

Each point represents the ratio between $\Delta c.p.m.$ for a test peptide of a given concentration and $\Delta c.p.m.$ for the same concentration of the *Torpedo* peptide (pTor); both $\Delta c.p.m.$ values refer to a certain dilution of a certain mAb. ○, ● and △ indicate 0.1, 0.05 and 0.025 mg of peptide/ml respectively used for plating under the direct plating technique. Standard deviations for most points are not shown because of their small size.

artifacts associated with any single technique. In earlier studies, in order to ensure that the peptides would be plated satisfactorily to the plastic wells, they were plated using glutaraldehyde, which covalently bound them to the previously plated poly(A-K) (Tzartos *et al.*, 1988, 1990b). The present study, however, showed that in five of the 15 peptides the use of glutaraldehyde had a dramatic inhibitory effect. In the previous studies the peptides were much longer (18–20 residues long). Large peptides probably acquire a rigid conformation, somewhat related to their conformation in the native protein, and glutaraldehyde may fix this conformation. The small peptides used in the present studies are flexible in aqueous media (Cung *et al.*, 1989a); hence glutaraldehyde probably fixes them at random conformations, a few of which will be capable of mAb binding. This explanation may account for the absence of mAb binding to the human peptide $\alpha 61$ –76 when using glutaraldehyde in the present studies (also J.-M. Gabriel & T. Barkas, personal communication), as compared with the positive binding observed when employing the dimer of the same segment (Tzartos *et al.*, 1988) or the larger peptides $\alpha 63$ –80 and $\alpha 67$ –76 (Tzartos *et al.*, 1988; Barkas *et al.*, 1988). Interestingly, with three other analogues (pA⁷³, pA⁷⁶ and pdesA⁷⁰), glutaraldehyde had a general enhancing effect. However, glutaraldehyde may block side-chain amino groups like those of Trp⁶⁷ and Lys⁷⁶, resulting in altered peptides. This fact probably diminishes the significance of the results obtained under the poly(A-K)/glutaraldehyde coating technique. Parallel use of direct peptide plating was necessary in these studies, and it produced more reliable results.

The relevance of the present results to the actual intact AChR–mAb interaction was confirmed by several internal controls; for example, the expected differential binding of the anti-human and anti-fish mAbs to the human and *Torpedo* peptides and to their intermediates was observed.

mAb binding specificity was very high. Single conservative substitutions (pA⁶⁹, pA⁷⁴) or simple stereochemical changes within a single amino acid (pA⁷³ versus pA⁷³) were sufficient for dramatic alterations in the binding capacity of the mAbs. At least two key positions, i.e. residues essential for binding by any tested anti-MIR mAb, were identified. These were Asn⁶⁸ and Asp⁷¹. It is these two positions in the *Xenopus* AChR sequence $\alpha 67$ –76 (WDPKYGGVK) (Baldwin *et al.*, 1988) that contain the two non-conservative substitutions. Interestingly, *Xenopus* AChR is the only known AChR which does not bind the anti-MIR mAbs (Sargent *et al.*, 1984). This confirms that $\alpha 67$ –76 is the actual site of the MIR on the intact AChR and that residues $\alpha 68$ and $\alpha 71$ play a fundamental role in its antigenicity. A dramatic role of single Ala or Gly substitutions on mAb binding has also been observed with other antigens such as rhodopsin (Hodges *et al.*, 1988) and EDP208 pilus protein (Worobec *et al.*, 1985).

On studying the antigenic role of residues $\alpha 70$ and $\alpha 75$ (which differ between *Torpedo* and human decapeptides), two interesting results appeared. We expected that anti-human mAbs would bind to the two intermediate peptides (pD⁷⁰ and pV⁷⁵) with an affinity intermediate between those with the *Torpedo* and human peptides. However, these mAbs bound more to pD⁷⁰ and less to pV⁷⁵ than to either the human or *Torpedo* peptides. Apparently the sum of the opposing effects of the two substitutions results in the binding pattern of the human MIR.

The binding pattern of mAb 6 was of interest. mAb 6 is the only anti-(*Torpedo* AChR) mAb of those tested which cross-reacts with human AChR. This mAb exhibited a binding pattern (especially with the three analogues intermediate between *Torpedo* and human peptides) which was intermediate between that of the anti-human and anti-*Torpedo* mAbs. This good correlation between antibody binding to intact AChRs and that to the

peptide analogues further supports the specificity of the results in the present study.

The different binding patterns of the two groups of anti-MIR mAbs (anti-fish and anti-human AChRs, Fig. 3) strongly suggest that the two MIRs form two different, though related, structures. Their differences are not restricted to the amino acid substitution at position 70. Other residues, especially Pro⁶⁹, Tyr⁷² and Gly⁷⁴, although common to human and *Torpedo* α -subunits, contribute to a different extent to the two MIRs. It is also shown that the *N*-terminal half of the decapeptide plays the most critical antigenic role. It should be stressed, however, that studying amino acid substitutions by using only Ala may not reflect the actual contribution of each residue to the antigenicity. In addition, substitutions may result in an overall modification of the conformation of the peptide.

Using one- and two-dimensional n.m.r. spectroscopy, the conformation of these peptides in Me₂SO was recently studied (Cung *et al.*, 1989a,b). In aqueous solution these peptides did not exhibit any nuclear Overhauser effect (n.O.e.) connectivities. Water might destabilize intramolecular hydrogen bonds and cause an unfolded structure. In an aprotic solvent such as Me₂SO, the intramolecular hydrogen bonds and the folded conformations are in general less perturbed. Both the presence of strong and multiple short- and long-range n.O.e.s (especially between Asn⁶⁸ and Gly⁷³) in the *Torpedo* peptide and the temperature-dependence measurements argue in favour of a rigid folded conformation. This is stabilized by three interactions involving the Asp⁷¹, Gly⁷⁴ and Lys⁷⁶ amide protons (Cung *et al.*, 1989a).

Comparison of the n.m.r. spectra of the peptide analogues with that of the original *Torpedo* decapeptide showed that the conformation of the decapeptide is significantly affected by any Ala substitution except on $\alpha 75$ (Cung *et al.*, 1989b). It is interesting, however, that substitution at $\alpha 68$ did not dramatically affect conformation, despite the complete loss of the antigenicity of the peptide observed in the present study with this substitution. This further stresses that the effect of substitution at $\alpha 68$ is due to the actual participation of Asn⁶⁸ in the MIR epitope rather than to an overall conformational change to the peptide.

The particular antigenic role of the pA⁷³ analogue suggested the synthesis of a second analogue in which D-Ala replaced L-Ala (peptide pA⁷³). Gly generally has the same conformational behaviour as D-Ala (Boussard *et al.*, 1979). The very significant effect on mAb binding (reversing the activation observed by pA⁷³ by a moderate inhibition by pA⁷³) suggests that the proximity of residues $\alpha 68$ and $\alpha 73$ is critical for mAb binding.

Anti-MIR antibody binding to synthetic peptides is of very low affinity, as it requires high concentrations of reactants. A major improvement to this affinity is required in order for these peptides to be useful in direct MG studies. Enhancement of mAb binding obtained in the case of two peptide analogues (pA⁷³ and pA⁷⁶) suggests that these peptides acquired a conformation which approached, to some degree, that of the intact MIR. This observation opens the way towards constructing a synthetic MIR with binding capabilities very similar to those of the intact MIR. Nevertheless, a lot of work is still needed, as the binding affinity of these peptides is probably still low compared with that of the intact MIR.

We thank Dr. R. Matsas for useful suggestions and A. Kokla and A. Efthimiadis for technical assistance. This work was supported by grants from the Greek General Secretariat of Research and Technology and the Muscular Dystrophy Association of America to S.J.T., the Association Française contre les Myopathies to M.T.C., S.J.T. and C.S., the 'Stimulation' program of the E.E.C. (ST2J-0184) to C.S. and M.M., and the C.N.R.S. to M.T.C. and M.M. E.B. was supported by an EMBO short-term fellowship.

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Received 6 December 1989/6 March 1990; accepted 15 March 1990