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Expression of human–*Torpedo* hybrid acetylcholine receptor (AChR) for analysing the subunit specificity of antibodies in sera from patients with myasthenia gravis (MG)

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SUMMARY

The nicotinic AChR, a pentamer composed of $\alpha_2\beta\gamma$(or ϵ)δ subunits, is the autoantigen in the human autoimmune disease MG. Anti-AChR antibodies in MG sera bind mainly to conformational epitopes, therefore determination of their specificities requires the use of intact AChR. Indirect antibody competition studies have suggested that most MG antibodies are inhibited from binding to AChR by MoAb to the main immunogenic region (MIR) on the α-subunits. More recently, based on the knowledge that MG antibodies show little detectable cross-reaction with *Torpedo* AChR, we have shown, using mouse–*Torpedo* hybrid AChR, that most MG antibodies that detectably cross-react with the mouse AChR bind to the α-subunit. To analyse the whole anti-AChR antibody repertoire in MG sera, we expressed on stably transfected fibroblasts a novel human α+ *Torpedo* $\beta\gamma\delta$ AChR and compared the antibody titres against human, *Torpedo*, and the hybrid AChR. Direct information was provided for the subunit specificity of several MoAbs and sera from 50 MG patients. On average, at least 48% of the anti-AChR antibodies in the sera were directed against the α-subunit. Interestingly, the anti-α-subunit antibodies predominated in low titre (0·6–7·4 nM) but not in high titre (10–386 nM) sera, where they comprised on average 68% *versus* 23% of the antibodies, respectively. Finally, the directly determined anti-α-subunit antibodies and the anti-MIR antibodies defined by antibody competition were significantly correlated, thus suggesting that at least a significant fraction of the anti-MIR antibodies in MG sera bind to the α-subunit.

Keywords myasthenia gravis acetylcholine receptor epitope mapping

INTRODUCTION

The nicotinic AChR of the fish electric organ and vertebrate neuromuscular junction is a cation channel composed of four subunits in a stoichiometry of $\alpha_2\beta\gamma\delta$ (or $\alpha_2\beta\epsilon\delta$), with an overall molecular mass of $\approx$ 290 000. Acetylcholine and other agonists and competitive antagonists, such as α-bungarotoxin (α-BT), bind to the two α-subunits, regulating the function of the ion channel [1–4]. The AChR subunit genes and cDNAs from a number of species have been cloned and sequenced, and the homology of the deduced amino acid sequences indicates structural similarities among the four subunits and between AChR species [5].

The AChR is the autoantigen in the human autoimmune disease MG. Anti-AChR antibodies decrease the number of effective AChR at the endplate, leading to failure of synaptic transmission, manifested by muscle weakness and proneness to fatigue. Anti-AChR antibodies are detectable in $\approx$ 90% of myasthenic patients' sera. However, population studies have shown that the anti-AChR antibody titre is not well correlated with the severity of clinical symptoms [6,7], raising the possibility of heterogeneity in the pathogenic potential of the different anti-AChR antibody specificities in the sera. Therefore, the determination of the epitopes on the AChR which may be responsible for inducing or maintaining MG could lead to more direct and specific treatments of the disease. Several laboratories have provided results from indirect competition studies indicating that the binding to intact AChR of the majority of anti-AChR antibodies from MG patients can be inhibited by MoAb directed against the main immunogenic region (MIR), an extracellular area of the AChR α-subunit, which is not involved in neurotransmitter binding [8–12]. However, the observed inhibition could be due to steric or allosteric effects, rather than binding to a common epitope [13,14]. Direct approaches to defining the subunit or epitope specificities of MG antibodies using individual subunits or subunit fragments are restricted by the fact that most MG antibodies recognize only conformational epitopes on native AChR [6]. To overcome this problem, we have

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expressed fully functional surface hybrid mouse–*Torpedo* AChR pentamers on stably transfected fibroblasts. Since MG antisera cross-react considerably with mouse AChR, but little with *Torpedo* AChR, by using these mouse–*Torpedo* AChR hybrids as antigens we were able to provide the first direct evidence for a major anti-α-subunit specificity in MG antisera [15]. However, this approach could only be applied to those human antibodies detectably cross-reacting with mouse AChR.

In order to characterize the whole antibody repertoire in MG sera, in the present study we expressed a human α+ *Torpedo* $\beta\gamma\delta$ (HαT) hybrid AChR. On the basis of direct antibody binding assays to the AChR from the human muscle cell line TE671, the *Torpedo* electric organ and the hybrid fibroblast cell line HαT, we have defined the anti-α-subunit specificity of a large number of anti-AChR antibodies, both previously characterized MoAbs and MG sera of unknown specificity. Our results show that in 25/50 MG sera, anti-α-subunit antibodies represent 50–100% of the immune response against AChR. An interesting differentiation between low- and high-titre sera, showing that antibodies to the α-subunit are not predominant in high-titre sera but are highly predominant in low-titre sera, may have clinical implications.

MATERIALS AND METHODS

Cell lines and transfections

The human cell line TE671 expresses functional muscle-type AChR [16]; it was maintained in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal calf serum (FCS; DMEM–FCS) at 37°C, under 5% CO_2, and the cells were used 4–6 days after reaching confluency.

All-11 is a *Torpedo* AChR-expressing fibroblast cell line established as described previously [17]; it was maintained in DMEM containing 10% newborn calf serum (NCS) and HAT (15 μg/ml hypoxanthine, 1 μg/ml aminopterin, 5 μg/ml thymidine) in 37°C, 5% CO_2. To induce surface expression of AChR, the culture medium of confluent cells was supplemented with 20 mM sodium butyrate and cells were incubated at 26°C for 10–12 days.

The HαT cell line, which stably expresses the human α+ *Torpedo* $\beta\gamma\delta$ hybrid AChR, is a clonal isolate derived by co-transfection of the appropriate AChR subunit cDNA engineered into pSV_2 vectors and the neomycin resistance gene (pSV_2-neo) into murine NIH3T3 fibroblasts. Full-length cDNA clones of the human (TE671) AChR α-subunit [16] and the *Torpedo* AChR β, γ, and δ subunits [17] were kindly provided by Drs J. Lindstrom (Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, PA) and T. Claudio (Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT), respectively. For transfection, a calcium phosphate precipitation procedure was followed, essentially as described [18]. After $\approx$ 10 days, well separated colonies were isolated, grown into stable cell lines and screened for surface binding of ^{125}I-labelled α-BT (^{125}I-α-BT), as described below. Hybrid AChR expression was induced by growing confluent HαT cell cultures in selection medium containing 10 mM sodium butyrate for either 24 h at 37°C, then 42 h at 26°C, or for 7–9 days at 26°C.

MoAb and MG antisera

The MoAbs used (Table 1) were derived from rats immunized with intact or SDS-denatured AChR from several species; some of their characteristics have been previously described (for references see Table 1). Stock solutions of MoAb were produced from concentrated supernatants of hybridoma cultures in DMEM–10% FCS, after dialysis against PBS (137 mM NaCl, 8 mM Na_2HPO_4, 2·7 mM KCl, 1·5 mM KH_2PO_4, pH 7·4) containing 0·05% (w/v) NaN_3. Selected MoAbs were coupled to Sepharose-CNBr-activated beads (Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's instructions (MoAb-Sepharose).

Serum samples were collected from Greek myasthenic patients and their titres determined by radioimmunoassay (RIA) using human AChR from the TE671 cell line [26]. Positive sera were defined as those with titres $\geqslant$ 0·6 nM, compared with normal human sera (NHS) with titre values of $\leqslant$ 0·2 nM.

Cell lysis, immunoprecipitations and immunoblots

Confluent cell cultures (10-cm dishes) were washed with cold PBS containing 0·01% $CaCl_2$ (PBS–Ca^{2+}), scraped and centrifuged at 500 ***g***, 4°C, for 5 min. All subsequent steps were carried out at 4°C. The cell pellets were resuspended in 0·5 ml of lysis buffer (150 mM NaCl, 50 mM Tris pH 7·4, 5 mM EDTA, 2 mM EGTA, 0·03% NaN_3, 1% Triton X-100) containing fresh 2 mM PMSF and 1 μg/ml each of aprotinin and pepstatin. The lysates were vortexed several times over a 30-min period and centrifuged at 12 000 ***g*** for 10 min to remove insoluble material.

Cell extracts were pretreated by incubation with an excess of MoAb 25-Sepharose (a negative control MoAb, specific for *Electrophorus* AChR) for 1 h. After pelleting of the beads at 12 000 ***g*** for 5 min, a predetermined saturating amount of pooled anti-α AChR subunit MoAb-Sepharose (MoAbs 198 and 192 or 6) was added to the supernatant and the mixture incubated by rocking overnight. Samples were then centrifuged and the pellets washed twice with lysis buffer containing 0·5 M NaCl and three times with lysis buffer alone. After the final centrifugation, the immunoprecipitates were subjected to SDS–PAGE [15] and immunoblotting [27]. The nitrocellulose filters were blocked in Tris-buffered saline (TBS; 20 mM Tris–HCl pH 7·5, 150 mM NaCl) containing 5% non-fat milk for 2 h at room temperature and then incubated with an excess of a pool of MoAbs 155 (anti-α), 124 (anti-β), 168 (anti-γ) and 7 (or 166, anti-δ), diluted in TBS–0·5% bovine serum albumin (BSA) (TBS–BSA) overnight at 4°C. After three washes with TBS–BSA, filters were exposed to rabbit anti-rat immunoglobulin coupled to peroxidase (Dako, Glostrup, Denmark) at 1:500 dilution in TBS–BSA for 2 h, at room temperature and washed again, as above. The immune complexes were detected by incubation in TBS containing fresh 0·06% diaminobenzidine, 0·03% $NiCl_2$ and 0·03% H_2O_2. The reaction was stopped by washing the filters with H_2O.

^{125}I-α-BT binding and sucrose gradient sedimentation

To measure the surface AChR, the cells were washed once with PBS containing 0·1% BSA and 0·01% $CaCl_2$ (PBS–BSA–Ca^{2+}), incubated with 5–10 nM ^{125}I-α-BT (specific activity 600–700 ct/min per fmol) in PBS–BSA–Ca^{2+} for 2 h at room temperature, washed three times with PBS–BSA–Ca^{2+}, collected with 0·5 N NaOH, and radioactivity was counted in a γ-counter. Background values were obtained from parallel plates incubated with ^{125}I-α-BT plus 5–10 mM unlabelled α-BT. To label the total cellular AChR, cells were lysed as described above and the lysates were incubated with 10–20 nM ^{125}I-α-BT for 3 h at 4°C. Surface AChR turnover rates were determined under conditions of blocked protein synthesis, in the presence of cycloheximide, as described

earlier [28]. The cells were labelled with ^{125}I-α-BT, then incubated in culture medium at 37°C and the cell-associated radioactivity monitored with time.

For gradient fractionations, cell extracts, labelled with ^{125}I-α-BT, were layered on 5 ml 5–20% linear sucrose density gradients prepared in lysis buffer and the gradients centrifuged in a SW 50·1 rotor (Beckman Instruments, Palo Alto, CA) at 155 000 ***g*** for a total $\omega^2 t = 8{\cdot}9 \times 10^{11}$ at 4°C. Fractions of $\approx 0{\cdot}3$ ml were collected from the top of the gradient and subjected to RIA using anti-AChR subunit-specific MoAb-Sepharose. Parallel gradients of TE671 cell lysates and of *Torpedo* AChR reduced extracts were run in order to determine the sedimentation coefficient of native AChR pentamers.

Radioimmunoassays

Cells grown in 10-cm dishes were either directly labelled with 10 nM ^{125}I-α-BT and lysed as described above or were first solubilized and the lysates incubated with 10–20 nM ^{125}I-α-BT. *Torpedo* electric organ membranes, containing a predetermined concentration of AChR, were labelled with an excess of ^{125}I-α-BT for 1 h at 4°C, washed twice with PBS, solubilized for 2 h at 4°C in PBS containing 0·5% Triton X-100 (PBS–T) and the supernatants collected after centrifugation at 12 000 ***g***, for 30 min at 4°C. Direct RIAs were carried out as described [26], with some modifications. Briefly, 50 μl, containing $0{\cdot}5 \times 10^{-9}$ M of ^{125}I-α-BT-labelled AChR (^{125}I-α-BT-AChR), were incubated with 10 μl of MoAb or MG serum dilution in PBS–0·1% BSA containing, respectively, as carrier when required, normal rat serum or NHS at a minimum total volume of 0·1 μl of undiluted serum for 16 h at 4°C. Immune complexes were quantitatively precipitated by a predetermined amount of rabbit anti-rat immunoglobulin (in the case of MoAb) or goat anti-human immunoglobulin (in the case of MG antiserum) in PBS–T. After 4 h of incubation at 4°C, the precipitates were washed twice with PBS–T and radioactivity was counted in a γ-counter. Background radioactivity was determined in the presence of MoAb 25 or NHS and did not exceed 5% of the radioactivity precipitated by an excess of MoAb 192 or 6, considered as the positive controls when TE671 and HαT or *Torpedo* AChR were used in the assay, respectively. Antibody titres were defined according to [26] at antibody dilutions that precipitated about 20% of the ^{125}I-α-BT binding sites in the assay mixture and were expressed as moles of ^{125}I-α-BT binding sites precipitated per litre of antibody.

Competition assays were performed as described [8], with some modifications. Aliquots of $0{\cdot}3 \times 10^{-9}$ M ^{125}I-α-BT-AChR were incubated overnight at 4°C with an excess of protecting MoAb 192 (anti-MIR) or negative control MoAb 25 in a total volume of 50 μl; 10 μl of a suitable MG serum dilution in PBS–0·1% BSA, previously determined as sufficient to precipitate $\approx$ 80% of the ^{125}I-α-BT-AChR (supplemented when necessary with NHS to a total of 0·1 μl of serum), were added and the mixtures incubated for 4 h at 4°C. Non-specific precipitation was determined by: (i) use of high excess unlabelled α-BT together with the ^{125}I-α-BT; and (ii) use of NHS instead of MG serum. Immune complexes were quantitatively precipitated by incubation for 3 h at 4°C with goat anti-human immunoglobulin absorbed with normal rat serum in order to avoid cross-precipitation of the rat MoAb 192. The precipitates were washed twice with PBS–0·5% Triton X-100 and the radioactivity counted in a γ-counter. After subtraction of background radioactivity precipitated by NHS, the inhibition of binding of MG antibodies by MoAb 192 was calculated from the ratio of the radioactivity precipitated in the presence of MoAb 192 (*A*) to that precipitated in the presence of MoAb 25 (*B*), according to the equation:

$$\%\text{inhibition} = 100 \times \left(1 - \frac{A}{B}\right)$$

RESULTS

Characterization of hybrid AChR

It has been previously shown for a number of AChR-producing cell lines established using cDNA engineered under the control of the SV40 promoter that AChR expression is significantly amplified by sodium butyrate. In addition, temperature dependence has been observed in all cell lines expressing *Torpedo* subunit-containing AChR, with surface appearance of ^{125}I-α-BT

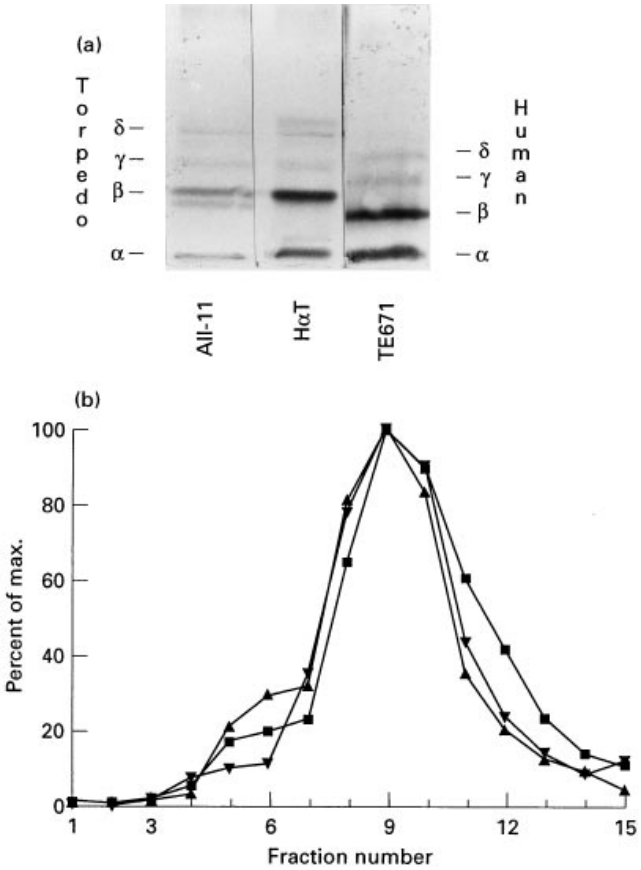

Fig. 1. Expression of AChR in the HαT cell line. (a) Subunit composition of AChR from All-11, HαT and TE671 cell lines. Cell extracts were immunoprecipitated by a mixture of anti-main immunogenic region (MIR) MoAb-Sepharose (MoAbs 198 and 192 or 6) and resolved by electrophoresis on 7·5% SDS–PAGE and immunoblotting using, as probes, a pool of AChR subunit-specific MoAbs: 155 (anti-α), 124 (anti-β), 168 (anti-γ) and 7 (or 166, anti-δ). The positions of *Torpedo* and human AChR subunits are indicated by α, β, γ, δ to the left and right of the blot, respectively. (b) Sucrose gradient sedimentation profiles of ^{125}I-α-bungarotoxin (α-BT)-labelled HαT (▲), *Torpedo* (■) and TE671 (▼) AChR on 5–20% linear sucrose density gradients. Fractions of $\approx 0{\cdot}3$ ml were collected and immunoprecipitated with MoAbs 192 or 6. The top of the gradient corresponds to fraction 1. Normalized profiles (% of maximum radioactivity) are shown.

Table 1. Characterization of anti-AChR MoAbs

	Earlier available data			Present data			Proposed subunit specificity	
	Binding to intact AChR*			Precipitated AchR (%)†				
MoAb	H	T	Subunit, region and sequence specificity*	H	T	HαT	From present data	From all available data‡
a. MoAbs of earlier determined subunit specificity								
6	+	++	α67–74, MIR	60	100 (100)	55		Tα/Hα
35	+	++	α, MIR	40	100 (80)	51		Tα/Hα
198	++	++	α67–74, MIR	59	92 (92)	57		Hα/Tα
124	++	++	β354–359, cytoplasmic	45	60	35		Tβ/Hβ
148	++	++	β354–359, cytoplasmic	28	62	21		Tβ/Hβ
16	–	++	α, MIR	0	60	2	Tα	Tα
195	++	–	α, MIR	93	0	92	Hα	Hα
202	++	–	α, MIR	98	0	84	Hα	Hα
192	++	–	α, conformational, MIR	100	0	100	Hα	Hα
64	++	–	α, not MIR	84	1	56	Hα	Hα
5	–	++	α357–360, cytoplasmic	0	100	0	Tα	Tα
73	+	–	β, near MIR	72	2	2	H(non-α)	Hβ
66	+	–	γ, near MIR, extracellular	12	0	0	H(non-α)	Hγ
67	+	–	γ, near MIR, extracellular	6	0	0	H(non-α)	Hγ
168	±	++	γ365–372, cytoplasmic	0	36	55	T(non-α)	Tγ
7	±	++	δ389–396, cytoplasmic (and γ382–387)	0	99 (27)	29	T(non-α)	Tδ (Tγ)
112	–	++	β354–350, cytoplasmic	1	65 (5)	4		Tβ
137	±	++	δ376–392, cytoplasmic	2	77 (3)	2		Tδ
139	±	++	δ374–385, cytoplasmic	1	41 (4)	2		Tδ
141	±	++	δ385–392, cytoplasmic	1	53 (2)	2		Tδ
166	±	++	δ374–385, cytoplasmic	3	91 (8)	4		Tδ
b. MoAbs of unknown subunit specificity								
190	++	–	Conformational, MIR	91	0	91	Hα	Hα
196	+	–	Conformational, MIR	39	4	55	Hα	Hα
1	–	++	Conformational, MIR	0	100	1	Tα	Tα
4	–	++	Conformational, MIR	0	66	1	Tα	Tα
12	–	++	Conformational, near MIR	2	100	18	Tα + (non-α)	Tα + (nonα)
14	–	++	Conformational, near MIR	0	88	72	T(non-α)	Tγ (near α)
69	+	–	Not MIR, near α-BT site	5	0	0	H(non-α)	H(near α)
25	–	–	Negative control					

* From[10,11,15,19 (plus references 14, 26, 28 and 30 therein), 20–25]. H, human; T, *Torpedo;* -, ±, +, ++ indicate the extent of MoAb binding (from no binding to very good binding). α67–76, etc., indicate subunit and epitope specificity (the numbers denote the position of the epitope on the subunit amino acid sequence).

† ^{125}I-α-bungarotoxin (α-BT)-labelled AChR were incubated with a molar excess of MoAb and then precipitated by rabbit anti-rat Immunoglobulin. Non-specific binding determined in the presence of MoAb 25 was subtracted. Data are expressed as percentage of total AChR immunoprecipitated by MoAb 192 (for TE671 and HαT AChR) or 6 (for *Torpedo* AChR). Numbers in parentheses under column T represent results from immunoassays using *Torpedo* AChR from All-11 cells rather than AChR from *Torpedo* electric organs. Displayed values are the mean of three determinations (s.e.m. < 10%).

‡ T/H indicates that the corresponding MoAb binds to both *Torpedo* and human subunit; α, β, γ or δ indicates that the epitope is formed by the indicated subunits. In deciding the subunit specificity of a MoAb, both present and earlier data were taken into account.

binding sites being seen only at temperatures < 30°C [15,17]. Thus, in this study, in order to determine the optimal conditions for AChR expression, HαT cells were incubated with increasing concentrations of sodium butyrate (5, 10, 15 and 20 mM), for various times (1–12 days) at several temperatures (26–37°C). Optimum expression was induced by growing confluent HαT cell cultures in medium containing 10 mM sodium butyrate for 7–9 days at 26°C. Under these conditions, the expression of cell surface HαT AChR per 35-mm dish reached 400–500 fmol of ^{125}I-α-BT binding sites. Compared with All-11, a *Torpedo* AChR expressing fibroblast cell line [15,17], the HαT cell line, expressing an AChR differing only in the α-subunit, is a much higher producer (> 10 times). Similar results have been reported for *Torpedo* and human α+ *Torpedo* βγδ AChR expressed in *Xenopus* oocytes [29].

Monitoring of the HαT cell-associated ^{125}I-α-BT with time showed that the surface HαT AChR were degraded at 37°C as typical extrajunctional AChR with a $t_{1/2}$ of 11–12 h [28]. Association of ^{125}I-α-BT with, and dissociation from, the HαT AChR was found to follow kinetics similar to that of the TE671 AChR (not shown).

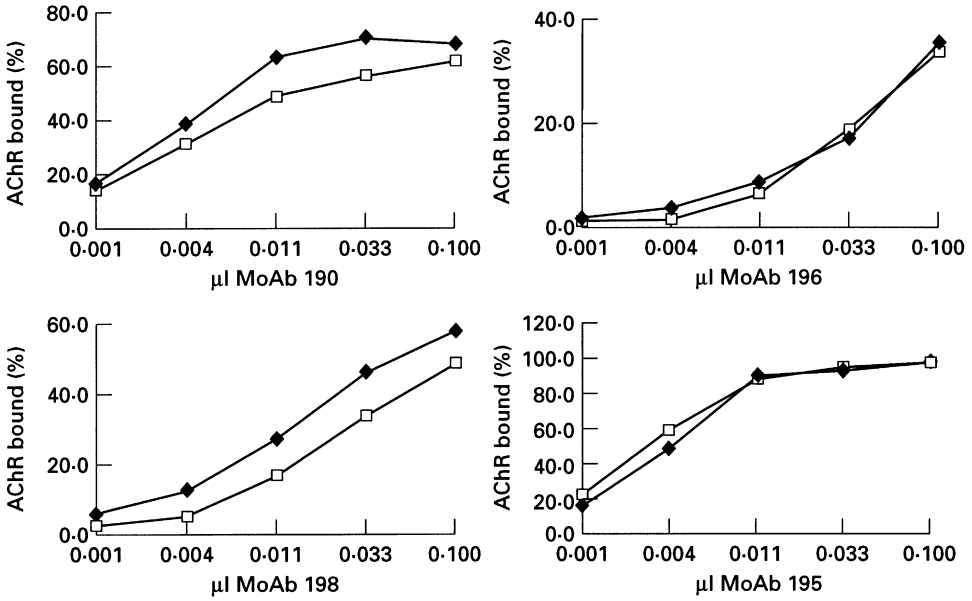

Fig. 2. Binding of four anti-main immunogenic region (MIR) MoAbs on ^{125}I-α-bungarotoxin (α-BT)-labelled human (◆) and HαT (□) AChR. Binding was determined by radioimmunoassay at various MoAb concentrations, as described in Materials and Methods. Total AChR was determined by the use of excess MoAb 195.

To examine the subunit composition of the novel hybrid AChR type, cell lysates were immunoprecipitated with anti-α-subunit MoAb-Sepharose and subsequently analysed by SDS–PAGE and immunoblotting, using a mixture of MoAbs specific for each of the four AChR subunits. Similarly treated lysates from All-11 and TE671 cells were analysed in parallel as controls for *Torpedo* and human AChR subunits, respectively. As shown in Fig. 1*a*, HαT cells expressed four polypeptides with apparent molecular weights identical to those of the respective AChR subunits from All-11 and TE671 cells, thus suggesting that the human α-subunit is associated with the *Torpedo* β, γ, and/or δ subunits to form AChR complexes. Further evidence supporting the association of the α-subunit with the other subunits was provided by co-immunoprecipitation of ^{125}I-α-BT binding sites in HαT cell lysates by several non-α-subunit-specific MoAbs directed against the β (MoAbs 124 and 148), γ (MoAb 168) or δ (MoAb 7) subunits (see below and Table 1).

Analysis of the ^{125}I-α-BT-labelled subunit complexes by velocity sedimentation on sucrose gradients following immunoprecipitation with anti-α (Fig. 1b) or non-α-subunit MoAb (not shown), showed that HαT cells expressed complexes migrating with a sedimentation coefficient of 9S, identical to those of the monomeric *Torpedo* and the TE671 AChR [29], strongly suggesting that the HαT AChR has a pentameric stoichiometry of $\alpha_2\beta\gamma\delta$.

Characterization of MoAb specificities

The reliability of the human–*Torpedo* hybrid AChR system for mapping the subunit specificity of uncharacterized anti-AChR antibodies was first validated by testing the binding of a panel of anti-AChR MoAbs, of known species and subunit specificity, to the HαT hybrid (Table 1). Most of these MoAbs are directed against non-conformationally dependent epitopes, i.e. they bind detectably to denatured AChR subunits or even smaller peptides, although in many cases with an affinity several orders of magnitude lower than that for the intact AChR. Since this weak binding to the isolated subunits and/or peptides may not reflect their actual subunit/epitope specificity on the intact antigen, confirmation of such mapping information was necessary. The comparison of direct binding assays of MoAb to human, *Torpedo* and HαT hybrid AChR, as undertaken here, may provide an alternative approach for the direct determination of their subunit specificity on the intact AChR. Under the assay conditions used (MoAb present in molar excess over AChR), near-maximal precipitation was achieved, thus any differences in the extent of immunoprecipitation reflect the binding capacity of the test MoAb for each AChR type.

As shown in Table 1a, the binding of most MoAbs to the HαT AChR was consistent with earlier data. The first six MoAbs, which react with both *Torpedo* and human AChR, recognized also the HαT hybrid, thus confirming the structural integrity of the corresponding epitopes. The following 11 species-specific MoAbs showed a binding pattern to the HαT AChR as predicted from their known species and subunit specificities. In fact, among the anti-α-subunit MoAbs tested, the *Torpedo*-specific MoAbs 16 and 5 did not bind to HαT AChR, whereas MoAbs 192, 194, 195, 202 and 64, which are mammalian-specific, recognized the HαT hybrid. Similarly, the mammalian-specific anti-β subunit MoAb 73 and anti-γ subunit MoAbs 66 and 67 did not bind to the hybrid, whereas the *Torpedo* AChR-specific MoAb 168 (anti-γ) and 7 (anti-δ), recognized HαT AChR.

An unexpected reactivity was seen with five *Torpedo* AChR-specific MoAbs directed against epitopes located on the cytoplasmic side of the molecule. The anti-β subunit MoAb 112 and anti-δ subunit MoAbs 137, 139, 141 and 166, which react well with *Torpedo* electric organ AChR, did not efficiently immunoprecipitate either the HαT AChR hybrid or the *Torpedo* AChR from All-11 cells. Since all the cDNA clones used for establishing the above fibroblast cell lines are reported to encode intact AChR subunits [17], it was hypothesized that these cytoplasmic epitopes are much more readily proteolysed in the fibroblast than in *Torpedo* membrane extracts, due to possibly higher protease/protein ratios in the former preparations. However, preincubation of *Torpedo* membrane extracts with AChR-depleted lysates from All-11 cells (All-11 AChR was eliminated by immunoabsorption with anti-AChR

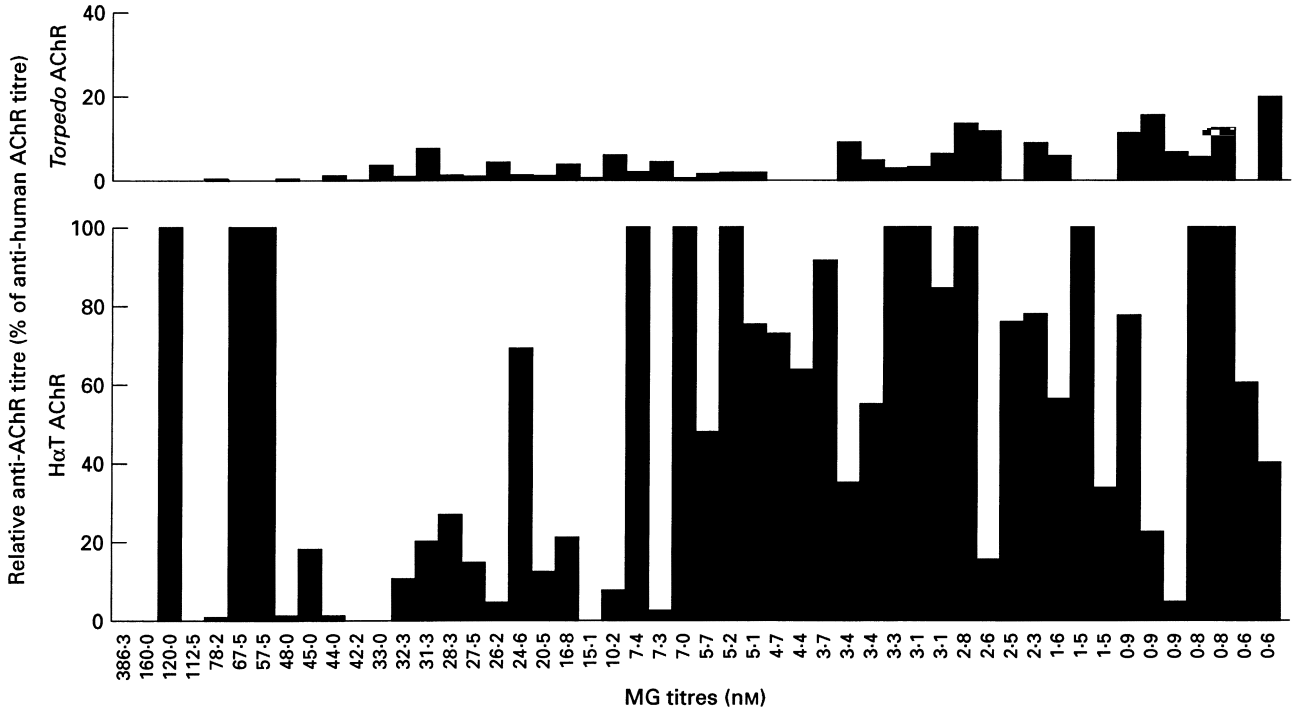

Fig. 3. Relative anti-AChR titre of myasthenic antisera against HαT and *Torpedo* AChR. The apparent titres of 50 independent myasthenia gravis (MG) serum samples against TE671, *Torpedo* and HαT AChR were determined by direct radioimmunoassays as described in Materials and Methods. Data are expressed and plotted as the percentage of each serum titre against HαT or *Torpedo* AChR relative to the corresponding titre against TE671 AChR. Displayed values represent the mean of three determinations (s.e.m. < 10%).

MoAb-Sepharose) did not diminish the subsequent MoAb binding to *Torpedo* AChR (not shown), thus excluding this hypothesis. Another possibility is that the relevant epitopes undergo distinct post-translational modifications in *Torpedo* electrocytes compared with mouse fibroblast cells. It is also likely that these epitopes are hidden in the fibroblast-expressed AChR, or their conformation has been distorted.

Finally, the subunit specificity of several conformationally dependent MoAbs was defined for the first time (Table 1b). The binding pattern of MoAbs 1, 4, 190 and 196 (identified previously by antibody competition experiments as anti-MIR antibodies) [24] to the HαT hybrid, clearly indicated their anti-α-subunit specificity. In addition to the results of Table 1, where MoAb excess was used, several MoAbs were tested with the three AChR types at various limited MoAb concentrations which would identify differences in their affinity for binding to the different AChR. Figure 2 shows that no significant differences could be detected in the binding of the four anti-MIR MoAbs 190, 196, 198 and 195. This observation confirmed that, on the intact AChR, the whole epitope(s) of the anti-MIR MoAb is/are entirely within the α-subunit.

New information was also obtained for MoAbs 12 and 14, two *Torpedo*-specific conformationally dependent MoAbs, which, on the basis of partial competition with certain anti-MIR MoAbs, were proposed to bind to the α-subunit, near the MIR [10,24]. The positive, but low, binding of MoAb 12 to HαT AChR suggests that the α-subunit indeed contributes to its epitope, but only partially; one or more of the β, γ or δ subunits must participate also in the formation of this MoAb epitope. In contrast, the fact that the binding of MoAb 14 to the HαT and *Torpedo* AChR was very similar suggests that this MoAb is a non-α-subunit antibody. Combining the present results with those reported in [30] it is concluded that MoAb 14 is anti-γ-subunit (see Discussion). In addition, the fact that MoAb 69 (anti-calf AChR), which binds weakly to human AChR, did not react at all with the HαT hybrid, suggests that it is not an anti-α-subunit MoAb, despite its ability to compete partially with α-BT for binding to the human AChR [25].

Analysis of MG serum antigenic specificities

Since MG antisera rarely cross-react detectably with *Torpedo* AChR [6,15], by performing in parallel direct immunoassays using solubilized *Torpedo*, human–*Torpedo* and human AChR, we were able to investigate the proportion of subunit-specific antibodies in MG antisera. Figure 3 demonstrates the relative titres of 50 myasthenic sera against *Torpedo* and HαT AChR compared with the anti-human AChR titres. Thirty percent of the sera did not react appreciably with either *Torpedo* or HαT AChR, and therefore bound to non-α human subunits; 20% reacted partially with HαT and therefore bound to α-subunit and to other human subunits; the remaining 50% bound predominantly to the HαT AChR and therefore reacted predominantly with the α-subunit. Overall, the average anti-HαT AChR titre from the 50 MG serum samples was 48 ± 39% of the anti-human AChR titre.

Interestingly, the anti-human AChR titres of 21 of the 25 sera containing a large percentage of anti-α-subunit antibodies did not exceed 7·4 nM, whereas the anti-human AChR titres of most sera showing negligible or low binding to the HαT hybrid were higher than 10 nM (Fig. 3). The occurrence of anti-α-subunit antibodies in sera with low (< 7·4 nM) and high (> 10 nM) titres against human AChR was 68 ± 31% and 23 ± 34% of the antibody repertoire, respectively. It seems that anti-α-subunit antibodies constitute by

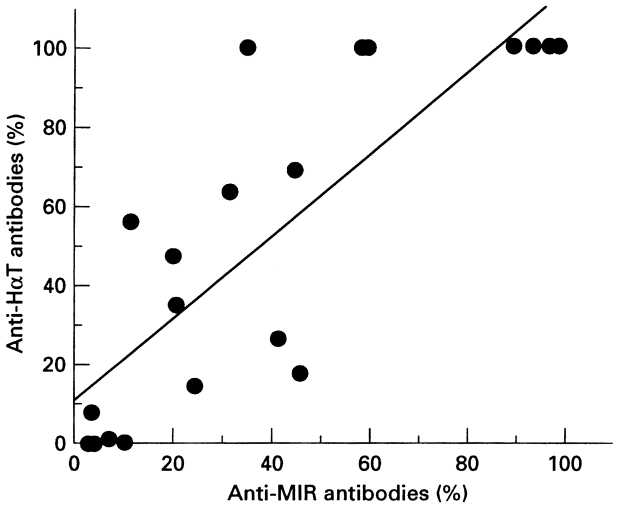

Fig. 4. Correlation of direct binding and competitive inhibition of binding of myasthenia gravis (MG) sera to AChR. Twenty MG sera were selected from the 50 sera shown in the Fig. 3 in order to have high (> 50%), moderate (10–50%) and negligible (< 10%) relative anti-HαT AChR titres. Sera from each subgroup were chosen randomly. Direct binding to HαT AChR, indicating the fraction of anti-α-subunit antibodies, was determined and expressed as described in the legend to Fig. 3. For competition assays, ^{125}I-α-bungarotoxin (α-BT)-labelled TE671 AChR were incubated with an excess of anti-main immunogenic region (MIR) MoAb 192, then exposed to MG serum and the complexes precipitated by goat anti-human immunoglobulin. The percentage inhibition of MG serum binding by MoAb 192, indicating the fraction of anti-MIR antibodies, was determined according to the equation described in Materials and Methods. A linear regression line was fitted to the data with the following parameters: correlation coefficient $r = 0{\cdot}82$, slope $= 1{\cdot}03$ (s.e.m. $= 0{\cdot}17$).

far the predominant specificity in MG sera with low anti-human AChR titres, whereas they are not predominant in most high-titre sera. Similar results were obtained with 20 further MG serum samples with anti-human AChR titres ranging from 2 nM to 140 nM (not shown). To investigate the potential pathogenic significance of this phenomenon, we compared the clinical condition of MG patients with high and low anti-HαT AChR relative titres; mild to severe myasthenic symptoms were seen, with a similar frequency, in both groups (not shown).

Finally, we asked whether the antibodies of myasthenic patients, indirectly determined as anti-MIR, are directed against the α-subunit or not. To answer this question we looked for a relationship between the direct antibody binding to HαT hybrid AChR and the indirect antibody competition approach by testing the inhibition of the binding of 20 representative MG sera to TE671 AChR, induced by an anti-MIR MoAb. Three groups of test sera were selected according to their relative anti-HαT AChR titre (high, moderate, negligible). Sera from each subgroup were chosen randomly. The results from the direct immunoassays using HαT AChR (indicating anti-α-subunit antibodies) and the competition experiments between sera and the anti-MIR MoAb 192 (indicating anti-MIR antibodies) were combined and are shown in Fig. 4. In most cases, the anti-MIR antibody fraction correlated significantly with the relative anti-HαT AChR titre of each serum. This suggests that a large fraction of the anti-MIR antibodies are probably directed against the α-subunit and confirms earlier data on MoAbs [14] indicating that the observed competitive inhibition is not due to gross allosteric phenomena, but results from shielding of the MIR by the MoAb used.

DISCUSSION

The determination of AChR epitopes which may be involved in inducing or maintaining MG is crucial to the understanding of the disease. Competition experiments using MG sera and anti-AChR MoAb indicate that the main MG antibody fraction binds to the MIR [6,8,14], and direct immunoassays using mouse–*Torpedo* hybrid AChR suggest that most MG antibodies, detectably cross-reactive with mouse AChR, bind to the α-subunit [15]. In the present study, by establishing a novel cell line which stably expresses human α+ *Torpedo* $\beta\gamma\delta$ hybrid AChR (HαT), we aimed: (i) to directly analyse the whole anti-AChR antibody repertoire in individual MG sera, rather than the subpopulation detectably cross-reacting with mouse AChR; and (ii) to examine a random large population of MG sera with various antibody titres, rather than a restricted number of sera selected to cross-react sufficiently well with the mouse AChR.

Initially, by testing the binding to the HαT AChR of several well characterized MoAbs (directed against sequential or conformational epitopes), we confirmed that this hybrid is a suitable antigen for analysing the subunit specificity of uncharacterized anti-AChR antibodies. In fact, the observed binding profiles confirmed, in most cases, earlier information concerning the species- and subunit-specificity of the MoAb, which had been determined by alternative methods (Table 1). New information was obtained for the subunit specificity of seven conformationally dependent MoAbs, four of which (MoAbs 1, 4, 190 and 196), that competed mutually and efficiently for binding to the MIR [24], were found to be α-subunit-specific. The epitopes of two other MoAbs, nos 12 and 14, which partially competed with certain anti-MIR MoAbs but not between themselves [10,24], were currently mapped between the α and another adjacent subunit (MoAb 12) and on a non-α-subunit next to the α-subunit (MoAb 14). Green & Claudio [30], using cell lines stably expressing *Torpedo* AChR subunit oligomers, showed that MoAb 14 could bind to the $\alpha\gamma$ dimer but not to the $\alpha\beta$, $\alpha\delta$, or α subunits. They concluded that MoAb 14 requires a certain conformation on the α-subunit which can be provided only by its joining with the γ-subunit. Since the present results showed that this MoAb does not bind to the α-subunit, it becomes clear that it is specific for the γ-subunit. Finally, it was shown that MoAb 69, although it partially competes with α-BT for binding to the AChR [25], is not directed to the α-subunit.

The binding of sera from 50 MG patients to human, *Torpedo* and HαT AChR was subsequently determined (Fig. 3). As expected from earlier studies [6,15], the cross-reactivity of the sera with *Torpedo* AChR ranged from undetectable to very low. Of the 50 sera, 15 did not distinguish HαT hybrid AChR from *Torpedo* AChR, thus they should primarily contain antibodies against β, γ, δ and/or intersubunit determinants. It is possible, however, that certain anti-α-subunit antibodies are present, but they cannot bind to the HαT AChR because their epitopes have been modified in the fibroblast expression system, as is probably the case for the epitopes of MoAbs 112, 137, 139 and 166 (Table 1). Antibodies against the α-subunit were detected in all the remaining 35 sera, as indicated by their binding to HαT AChR. In fact, in 25 sera (50%), average anti-HαT AChR titres reached 84·3% of the corresponding anti-human AChR titres.

An interesting observation was that in sera with low anti-human AChR titres (< 8 nM), anti-α-subunit antibodies constituted a large fraction (on average 68 ± 31%), while in sera with high anti-human AChR titres (> 10 nM), anti-α-subunit antibodies were not predominant (on average 23 ± 34%). The higher occurrence of anti-HαT AChR antibodies in the low-titre sera is also apparent when considering the absolute values of the serum titres against HαT AChR. Thus, taking into account all sera with anti-human AChR titres > 1 nM, the anti-HαT AChR titre was < 1 nM in 50% (11/22) of the high-titre sera, but only in 19% (4/21) of the low-titre sera.

The above difference has not been clearly noted before, probably because no considerable quantitative studies on the α-subunit specificity of MG sera have been performed: (i) Conroy *et al*. [29] used the partially mature unassembled α-subunit population that is present in TE671 cells to study 45 MG sera. They found that the MG sera bound to the α-subunit with an average 14-fold lower titre, apparently due to the altered conformation of the unassembled subunit. Contrary to our results, there was a surprisingly very good correlation between anti-human and anti-α-subunit titres; (ii) Ashizawa *et al*. [31] tested the binding of nine MG sera on α-subunit synthetic peptides; four sera had titres for human AChR 0·9–8·18 nM, whereas the remaining five had titres 40–264 nM. Despite the great differences in titre the sera responded comparably to the various peptides; in fact, the low-titre sera often exhibited rather better peptide binding (see Fig. 3 in the paper), which presents some similarity with the present results. No effort was made to calculate the fractions of MG antibodies that bound to these peptides; (iii) in our study on the cross-reactivity of nine MG sera with mouse–*Torpedo* AChR hybrids [15] no correlation of antibody titre with the fraction of anti-α-subunit antibodies could be detected; this may be due to the limited fraction of antibodies tested (i.e. only those detectably cross-reactive with mouse AChR) and the bias in the selection of the test sera (i.e. those well cross-reactive with mouse AChR). Other studies have only proved qualitatively the presence of antibodies to recombinant human α-subunit polypeptides in MG sera [32].

It is also interesting that in earlier indirect competition studies on the occurrence of anti-MIR antibodies in myasthenic sera, no lack of such antibodies in high-titre sera was observed [8,9,28,33]. It should be noted, however, that there is a significant difference in the definition between the anti-MIR MoAb and the anti-MIR fraction of MG sera. A MoAb is considered as anti-MIR when it nearly completely competes with other anti-MIR MoAbs from binding to the AChR. All such anti-MIR MoAbs that could be tested for subunit specificity, both earlier [10,11,15,19,24] and in the present study (see Table 1 and Fig. 2), were found specific for the α-subunit. In contrast, partial competition of MoAb with an anti-MIR MoAb has been interpreted as due to mere propinquity of the corresponding epitopes [8,10,11,24]. Such MoAbs have been found to bind to the α-, β-, or γ-subunits [8,24,34]; see, for example, MoAbs 73, 66, 67, 12 and 14 in Table 1. Therefore, the fraction of MG antibodies that is inhibited by an anti-MIR MoAb from binding to the AChR probably contains antibodies both against the MIR (those which are completely inhibited) and against sites near the MIR (those that are partially inhibited); these two subpopulations cannot be easily distinguished from each other. Furthermore, the sum of the MG antibodies inhibited by an anti-α (MIR), an anti-β and an anti-γ MoAb (which partially compete with each other) is usually much higher than 100% [8,33]; see also similar studies of other groups [9,35]. These facts may partially account for the differences in the occurrence of anti-MIR and anti-α-subunit antibodies in high-titre sera.

Despite the above, a significant correlation was observed between the anti-HαT AChR titre of representative MG sera and their anti-MIR specificities (determined by competition against an anti-MIR MoAb, Fig. 4). This suggests that a significant fraction of the MG antibodies which compete with anti-MIR MoAbs for binding to the MIR bind indeed to the AChR α-subunit.

As mentioned before, the relative pathogenicity of the anti-AChR antibody specificities could be one of the factors explaining why, in population studies, antibody titres are not well correlated with the clinical condition of MG patients [6,7]. The fact that the symptoms of MG patients with low and high anti-human AChR titres do not differ significantly might suggest that the presence of a moderate amount of anti-α-subunit antibodies (usually of one to a few nM) is sufficient to cause MG symptoms, whereas, in the absence of anti-α-subunit antibodies, a much higher amount of non-α-subunit antibodies is required for inducing symptoms of a similar severity. This hypothesis needs further investigation.

Beeson *et al*. have transfected TE671 cells with the cDNA of the human muscle ϵ-subunit, so that these cells produce both embryonic (with γ-subunit) and adult (with ϵ-subunit) AChR [36]. Some sera of very low (equivocal) titre for the embryonic TE671 AChR were found positive for the transfected TE671 AChR [37]. It will be very informative to compare the binding of MG sera to HαT with that to the ϵ-subunit containing AChR.

In conclusion, in this study the subunit specificity of a large number of MG sera and anti-AChR MoAbs was directly evaluated by using the system of stably transfected fibroblasts expressing HαT AChR. Antibodies directed against the human AChR α-subunit were found to dominate the antibody repertoire in the large majority of MG patients with low-titre sera, but not in those with high-titre sera.

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