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MONOCLONAL ANTIBODIES AGAINST THE HUMAN ACETYLCHOLINE RECEPTOR

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Monoclonal cell lines synthesizing antibodies against partially purified acetylcholine receptor from human muscle (H.AChR) were produced. Eleven clones secreted antibodies against H.AChR. Four were obtained in ascitic form. Two of them have been exhaustively studied. Specificity and affinity for H.AChR were demonstrated. Cross-reactivity with mouse AChR was shown but not with torpedo or porcine AChR at the same concentration. Purified IgG injected intravenously provoked an obvious muscular weakness. Inhibition experiments on myasthenia gravis sera binding have demonstrated that monoclonal antibody specificity is directed against an antigenic determinant shared by human and mouse AChR.

Monoclonal antibodies (mAbs) against the acetylcholine receptor (AChR) have been prepared using purified AChR from torpedo and eel eletric organs, and have been used to study structural and functional properties of AChR. Antibody specificities defined by the capacity to bind to neurotoxin and cholinergic sites (1,2,3), AChR structure (4,5), species specificities(6,7) and pathogenicity (3,6,8) have been demonstrated. The preparation of mAbs directed against human AChR determinants would greatly aid in the investigation of the multispecificity of antibody responses to AChR during myasthenia gravis (MG). These studies are difficult because of the low yield of human AChR preparations and hence the further purification of antigenic subunits. This paper reports the preparation of mAbs against a partially purified

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human muscle AChR and demonstrates that it is possible to obtain mAbs with different properties.

MATERIALS AND METHODS

AChR preparation - AChR from various species were prepared as previously described (9), from human amputated limbs, mouse legs, porcine intercostal muscles and torpedo electric organ tissue (gift to J. Giraudat, Institut Pasteur, Paris). Human AChR (H.AChR) was dialyzed against phosphate buffer 0.02M, pH 7.4, and was chromatographied on DEAE Sepharose CL6B using a discontinous phosphate gradient. The purified human AChR was eluted in the 0.15M fraction (H.AChR.F15).

Immunization and fusion - Four C57Bl/6 mice (2 months old) were injected in their footpads on day 0 with H.AChR.F15 (1.4 pmoles AChR in CFA) and were boosted s.c. on day 13 with the same antigen concentration. Two additional inoculations were made with 7.4 pmoles in CFA. Muscular weakness was observed and anti-AChR titers were determined throughout the immunization process. The mouse with the highest anti-AChR titer was sacrificed 3 days after the last booster and the spleen was removed. Spleen cells were fused with the non secreting myeloma cell line P3-X63-Ag 8653 as previously described (10). Normal mouse spleen was used as control. Supernatants were assayed for anti-AChR activity. Cells from the positive wells were cloned and subcloned by limiting dilution and grown as tumor ascites in (C57BL/6 x BALB/c)F_1 mice primed with Pristane.

Radioimmunoassays (RIA) - Anti-AChR titers were determined with an ^{125}I- α bungarotoxin-AChR complex (^{125}I- αBgt-AChR). 50-200 µl of supernatant per well were incubated with 200-300 fmoles of ^{125}I- α Bgt-H.AChR (room temperature 90 min) in a final volume of 500 µl. The immune complexes were precipitated with goat anti-mouse immunoglobulin G serum (MILES YEDA LTD and PEL-FREEZ Biologicals) after the addition of 1 µl of normal mouse serum (NMS). After 60 min, the complex was centrifuged (3000 rpm 10 min) and the supernatant was discarded. The pellet was washed one with 2 ml of borate buffer 0.1M, pH 8.4, containing 8% polyethylene glycol 6000. The final pellet was counted in an Intertechnique gamma counter. 1-10 µl of ascitic fluid were assayed under the same conditions without the addition of NMS. Ascitic fluidsfrom hybrids producing mAbs against other antigens were used as controls.

Inhibition of MG sera binding to AChR by mAbs - 7.5 - 15 fmoles of ^{125}I- αBgt-AChR in 100 µl of PBS were incubated for 90 min at room temperature with mAbs (12-15 fmoles) or PBS in a final volume of 200 µl. Then 15-20 fmoles of anti-AChR contained in MG sera (titers 15 to 650 x 10^{-9}M) were added and incubated overnight at 4°C. Goat anti-human immunoglobulin G serum (ICL SCIENTIFIC) previously depleted of antibodies cross-reactive with mouse IgG (incubation with NMS overnight at 4°C) was added. After 90 min, the pellet was washed with PBS containing 1% BSA, centrifuged (3000 rpm) and the radioactivity measured. Controls mAbs were used at the same antibody and protein concentrations. The percentage of inhibition was determined after subtraction of the radioactivity counted in the assay incubated without mAbs from that measured in presence of mAbs.

Passive experimental autoimmune myasthenia gravis (EAMG) - Monoclonal IgG from ascitic fluid was purified on a DEAE Tris Acryl (IBF) chromatography column after gel filtration on AcA 202 (IBF). 200-500 µg of lyophilized purified mAbs IgG in 200 µl of a physiological solution were injected intravenously into C57BL/6 mice. Normal mouse IgG was used as controls. Mice were observed 5,18 and 24 hours after the injection and compared to controls. Signs of weakness were scored according to the capacity of the mouse to grip the cage repetitively in a tensile test.

General methods - Protein determinations were made according to Lowry et al. (12). The AChR concentration was determined after binding with ^{125}I- αBgt (15.5 µCi/µg, New England Nuclear). Specific binding was tested in the presence of αBgt (from Bungarus multicinctus SIGMA) by the ammonium sulphate method of Meunier et al. (13) modified by Aharonov et al., (14) Mouse immunoglobulin subclasses were determined by the radial immunodiffusion method (MELOY).

RESULTS

After the last booster, sera from mice immunized with H.AChR.F15 had positive anti-AChR titers ($0.45 \pm 0.15 \times 10^{-9}$M for controls). Table I shows the evolution of antibody titers for the mouse whose spleen was used for the fusion. The last titer was significantly higher than the control titer (more than 6 times). Muscle weakness was exhibited by all the mice injected. Among the 19 hybridomas obtained, 12 were positive in the RIA. After cloning by limiting dilutions, 7 of the 12 tested supernatants had titers > 12×10^{-12}M , which was the

TABLE I

EVOLUTION OF ANTIBODY TITER IN MOUSE SERUM AFTER IMMUNIZATION WITH PURIFIED HUMAN AChR

Injection		Day of determination	Anti-AChR ($\times 10^{-9}$M)
N°	Day		
I	0	0	0.58
II	13	18	1.14
	"	44	1.20
III	112	125	2.60
IV	126	130	3.60

I and II 1.4×10^{-12}moles of H.AChR•F15 in CFA in footpads and (s.c)
III and IV: 7.4×10^{-12}moles of H.AChR•F15 in CFA (s.c.)

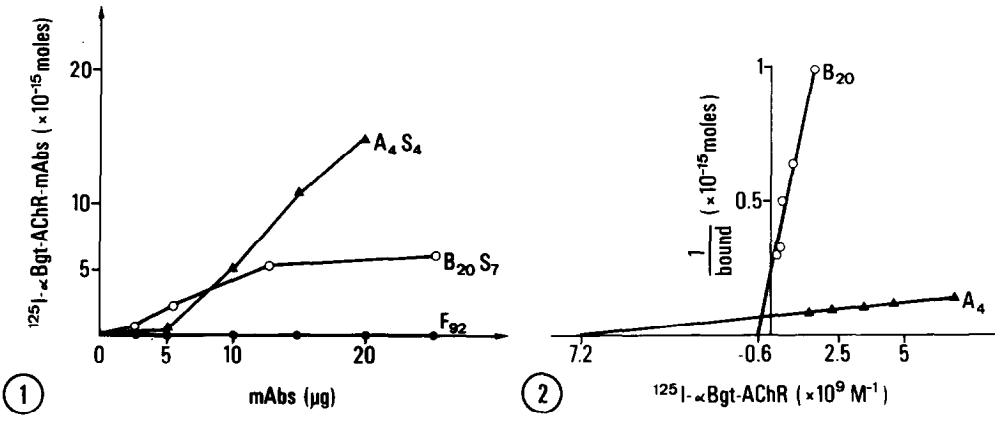

Figure 1 Binding of mAbs to H.AChR related to antibody concentrations.
Anti-AChR titers were determined as described in materials and methods in the presence of 286 fmoles of H.AChR and 2 - 20 µl ascites.

Figure 2 Reciprocal plot of mAbs binding to H.AChR.
To determine the apparent Km of mAbs towards H.AChR, incubations were made at the initial rate. The R.ACh-mAbs complexes were precipitated after 30 min at 37°C.

highest titer given by the control. Four of the clones with antibody titers of 13.5, 19.5, 29.3 and 55.8 x 10^{-12}M were subcloned three times and passed in ascites. All the mAbs discussed here are IgG1. For the same clone, the antibody concentration in the ascites varies from one mouse to another (from 0.74 mg/ml to 2.55 mg/ml).

Two clones (A4 and B20) have been exhaustively studied. Figure 1 shows the specificity of these two mAbs against H.AChR. The apparent Km of mAbs was 1.60 x 10^{-9}M for B20 and 0.14 x 10^{-9}M for A4 (Figure 2). These mAbs, tested against AChR preparations from other species, show cross-reactivities with mouse AChR (Table II).

The effect of these mAbs on human serum antibodies was assayed by inhibition experiments. The two mAbs inhibit the immune reaction for only one of the tested sera (14.1% inhibition for A4 and 9.0% for B20). This serum (n°9) shows a total cross-reactivity with both human and mouse antigen used to

TABLE II

CROSS-REACTIVITY BETWEEN mAbs AGAINST HUMAN ANTI-AChR AND AChR FROM VARIOUS SOURCES*

mAbs	Human	Mouse	Porcine	Torpedo
A4	13.3	3.2	0	0
B20	5.4	3.5	0	0

* Assays with 250 fmoles of ^{125}I-α Bgt AChR at a constant protein concentration (i.e. 8 mg/ml) and 15 µg of mAbs. Controls for each determination were performed with α-Bgt (10^{-6}M). Results are expressed as fmoles of ^{125}I-α Bgt-AChR precipitated. Values obtained with control mAbs (mAbs anti-DNA) at the same IgG1 concentration were subtracted.

determine the anti-AChR titers (Table III). For the other sera the cross-reactions are weak (from 0 to 20%) regardless of the titer. The A4 and B20 mAbs inhibit the binding of serum 9 antibodies to mouse AChR by 16.0% and 11.8% respectively (Fig. 3).

TABLE III

ANTI-AChR ANTIBODY TITERS (nM) IN MG PATIENTS' SERA

Serum	AChR from human	AChR from mice	% cross-reactivity
Controls	0.3	0	-
1	13.3	2.7	20.3
2	13.5	1.1	8.2
3	14.6	1.5	10.3
4	17.8	1.5	8.4
5	21.6	0	0
6	26.5	0	0
7	30.2	1.1	3.6
8	33.1	1.9	5.7
9	159.7	155.9	97.6
10	399.0	12.0	3.0
11	674.0	70.8	10.5

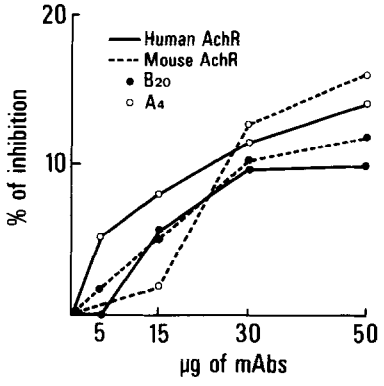

Figure 3 Inhibition curves of serum 9 binding to human and mouse antigens. Experiments were performed with 15 fmoles of H.AChR and 7.6 fmoles of mouse AChR previously complexed with an excess of ^{125}I- αBgt.

Subsequent to injection with the IgG purified from clones A4 and B20, mice exhibited muscular weakness and were more rapidly fatigable than control mice (2 to 3 more times) when assayed in our clinical test. After 4-5 days, the mAbs injected mice regained normal muscle control.

DISCUSSION

The immunization of mice by a partially purified fraction of H.AChR (H.AChR.F15) has permitted us to prepare mAbs specifically directed against the human antigen. The immunized mice had significant anti-H.AChR antibodies and they exhibited obvious muscular weakness throughout the immunization process. Other assays performed in the laboratory with a crude H.AChR preparation gave clones whose supernatants contained antibodies that titered at the limit of a positive detection. Using H.AChR.F15 as antigen, we obtained four clones adapted to ascitic form and expressing positive anti-H.AChR activity. All these clones are IgG1. This subclass represents 38% of the mAbs prepared in rat against eel, torpedo and calf AChR (15). Two of the mAbs have been exhaustively studied and have been shown to be distinct in regard to their biochemical and

immunological properties. Affinities towards the human antigen have been determined and have been shown to be weak as compared to human sera antibody affinities (16). Cross-reactivity experiments were positive with mouse AChR and were negative when torpedo AChR was used. This result shows a better cross-reactivity between mammalian receptors, in agreement with observations made using polyclonal sera (17). We failed to demonstrate a cross-reaction with porcine AChR, but this latter preparation had a very low AChR concentration as compared to other preparations. This negative result could be due either to the experimental conditions or to the proteolysis of the crude extract, this latter phenomenon has been observed in our laboratory and reported by others (17).

The inhibition of human MG serum binding to H.AChR by mAbs was obtained for only one of the tested sera (serum 9) at approximate ratios of 9 and 16% of the reaction. This result would be expected due to MG serum polyclonality. The two mAbs inhibited in the same way the binding of serum 9 to mouse AChR. For this serum the cross-reactivity between human and mouse antigens was total. The poor cross-reactivity of the other sera is in agreement with results of other authors (18).

The data obtained in both cross-reactivity and inhibition experiments could be explained by the presence in serum 9 of antibodies having the same specificity as the mAbs. This specificity is probably directed against a determinant shared to human and mouse antigens which is also suggested by the direct binding of the mAbs to mouse AChR. The identification of those determinants responsible for the disease state remains to be made.

We have been able to produce a transient pathogenic effect in mice with the purified IgG from mAbs. The IgG1 from clone

B20 was more efficient than that from A4. Immunofluorescent studies (not reported here) performed on newborn mouse muscle cells confirm the latter observation, by showing a greater positivity with the B20 mAb. This could be explained by a better accessibility of this clone to membrane AChR.

Our results support the findings that the mAbs produced are directed against one or more of the shared human and mouse AChR determinants. They would be distinct from the "main immunogenic region" described by Lindstrom et al (3) as shown by the failure of inhibition experiments with most of the sera.

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