MONOCLONAL ANTIBODIES DIRECTED AGAINST THE NEUROTRANSMITTER BINDING SITE OF NICOTINIC ACETYLCHOLINE RECEPTOR

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1. Introduction

The pathogenesis of the disease myasthenia gravis (MG) and its animal model experimental autoimmune myasthenia gravis (EAMG) involves an autoimmune reaction to nicotinic acetylcholine receptor (nAcChR) [1–5], where anti-nAcChR antibodies effectively impair transmission at the neuromuscular junction [1,6]. Hence, the immunogenic properties of nAcChR are of major consideration. The ability to differentiate the antigenic determinants of nAcChR would be of great use by permitting a detailed analysis of the importance of different determinants in MG, as well as providing a new dimension for structural and functional studies of nAcChR.

The lymphocyte hybridization procedure provides an opportunity to develop such antigenic site-specific monoclonal antibodies (mAb) [7]. We have applied the procedure to nAcChR and report the isolation, in particular, of mAbs directed: (i) at the neurotransmitter-binding site; (ii) close to the neurotransmitter-binding site. These mAbs should help delineate the contribution of this region of nAcChR to the pathogenesis of MG.

2. Materials and methods

2.1. *Immunization, fusion and cell culture*

nAcChR was purified from the electric organ of *Torpedo marmorata* [8]. 3 BALB/c mice (3 months old) were injected intraperitoneally with nAcChR (0.5 mg), at day 0 in complete Freund's adjuvant and at day 12 in 150 mM NaCl, 10 mM Na-phosphate (PBS) (pH 7.2).

One mouse was sacrificed 3 days later and the spleen removed. Spleen cells (10^8) were fused with the non-secreting B-lymphocytoma cell line SP2-0Ag14 (10^7 cells) by treating with 50% (v/v) polyethylene glycol 1500 (37°C, 1.5 min) [9]. The cell mixture was distributed in 96 Costar tray wells (2×10^5 cells/well) with a feeder layer of macrophages (4×10^5 cells/well). On the second day in culture, HAT (hypoxanthine/aminopterin/thymidine) medium was added. Supernatants were assayed for anti-nAcChR activity after 21 days in culture, and cells were cloned by limited dilution [10]. Harvested cells were frozen in HAT medium containing 10% (v/v) dimethylsulphoxide, and stored in liquid nitrogen.

2.2. *Radioimmunoassays*

The antigen used was ^{125}I-labelled nAcChR (^{125}I-nAcChR) [11]. The specific activity of different preparations varied from $2.4–3.0 \times 10^5$ cpm/pmol toxin binding site, as calibrated by the filtration assay [12]. Initially, the radioimmunoassay (RIA) was performed by a double antibody procedure, incubating (37°C, 60 min) conditioned medium with ^{125}I-nAcChR (0.3 pmol) and precipitating the immune complex with rabbit anti-mouse immunoglobulin G (RAMIG) serum (37°C, 60 min then 4°C, 16 h). Later, a solid-phase RIA was used, based on an antibody adsorbent of bacterial protein A (PA) that was prepared and stored as a 10% (v/v) suspension [13]. RIA was performed in PBS containing 0.05% (v/v) Emulphogen BC720 (PBSEO5) (total vol. 0.25 ml) by incubating (room temp., 30 min) conditioned medium with ^{125}I-nAcChR (0.3 pmol). Immune complexes were precipitated by adding excess PA (5 min incubation) and centrifuging ($2500 \times g$, 10 min, 4°C). The pellet was washed with 2×4 ml PBSEO5,

then counted. In some clones, it was necessary to include RAMIG (incubation at room temperature, 30 min) to act as a link between mouse IgG subclass I–nAcChR complexes and PA [14].

2.3. *Effects of ligands on binding of mAbs to nAcChR*

Effects of various cholinergic ligands on binding of antibodies to ^{125}I-nAcChR were tested as follows. The ligand was preincubated for 30–60 min at room temperature with receptor (0.3 pmol), then with conditioned medium (30 min). The immune complex was harvested as above, using the PA procedure.

3. Results

3.1. *Cell fusion and cloning*

All 96 Costar wells contained growing hybridomas, of which 61 (63.5%) were secreting anti-nAcChR antibodies. The cells from 37 of the original wells were frozen.

The extent to which conditioned medium inhibited binding of ^{125}I-labelled mono-iodo α-bungarotoxin (α-Bgt) to nAcChR-rich membrane fragments was used as a preliminary screening process for selecting cells for cloning (not shown) as we wished to identify mAbs binding to nAcChR exposed at the cell membrane. The cells from 3 wells showing a high degree of inhibition of toxin binding were cloned. Five, stable, monoclonal lines were established. These are labelled 1–5

Table 1
Effect of RAMIG on the protein A assay

Clone	Maximal-fold increase in titre in presence of RAMIG
1	64.0
2	65.1
3	2.5
4	2.0
5	No effect

RIA was performed as in section 2. Aliquots of conditioned medium were incubated first with ^{125}I-nAcChR (0.3 pmol) then with increasing amounts (0.1–12.5 μg) of purified RAMIG. Sufficient PA was added to precipitate all IgG. Results are the averages of 2 expt. and are expressed as: Titer (presence of RAMIG)]/[Titer (absence of RAMIG)]

3.2. *Effect of RAMIG on assay of mAb*

Table 1 illustrates the effects of adding RAMIG in RIA using PA. The large increases in titers for clones 1 and 2 indicate that they belong to mouse IgG subclass I [14]. RAMIG had a less marked effect on the titers of clones 3 and 4, and had no effect on clone 5.

3.3. *Effect of cholinergic ligands on mAb binding to nAcChR*

The results are depicted in table 2. mAbs of clones 1 and 2 were inhibited by all ligands tested, whilst those of clones 4 and 5 were inhibited only by α-Bgt. mAbs of clone 3 were unaffected by the two nicotinic antagonists tested, and atropine, but showed

Table 2
Effects of ligands on RIA using protein A

Clone	Control binding (cpm)	Binding in the presence of ligand (% of control binding)			
		Carbamylcholine	d-Tubocurarine	Atropine	α-Bgt
1	10 271 ± 387	56.9 ± 3.7	4.3 ± 1.1	78.7 ± 2.3	0
2	22 827 ± 600	69.9 ± 1.5	2.8 ± 1.9	85.7 ± 1.1	0
3	10 833 ± 643	85.3 ± 2.0[a]	98.3 ± 3.6	101.4 ± 1.6	103.7 ± 2.8
4	4513 ± 32	100.1 ± 3.2	105.4 ± 7.5	104.9 ± 7.6	17.7 ± 1.8
5	1297 ± 87	95.4 ± 3.5	115.5 ± 4.1[a]	115.1 ± 8.3	0

[a] Significant at $p = 0.05$ using Student's t-test

The antibody activities of the monoclonal lines were assayed using PA, after preincubation of ^{125}I-nAcChR (0.3 pmol) with cholinergic ligands. With clones 1–4, RAMIG (10 μg, 5 μl) was included in the incubation mixture. Control binding was established in the absence of ligands. Ligand was 1×10^{-4} M (carbamylcholine, d-tubocurarine and atropine) and 1×10^{-6} M (α-Bgt). Results are expressed as % of control binding ±SD ($n = 4$). The analyses were performed on at least 2 separate occasions

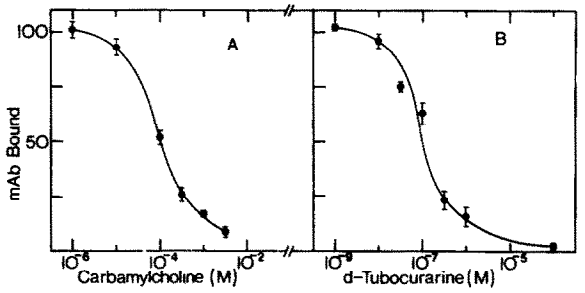

Fig.1. Dose–response curves for the effects of carbamylcholine (A) and d-tubocurarine (B) on binding of mAbs of clone 1 to ^{125}I-nAcChR (section 2). RAMIG (10 μg, 5 μl) was included in the incubation mixture. Results ±SD (n = 4) are expressed as % of binding in the absence of ligands ((A) 7655 ± 142 cpm; (B) 14460 ± 216 cpm). Analyses were performed on at least 2 separate occasions.

a significant, 15% decrease in the presence of the agonist carbamylcholine. Clone 1 was studied in greater detail. Dose–response curves indicated that half-maximal inhibition (I_{50}) of antibody binding to nAcChR was achieved at 1×10^{-4} M carbamylcholine and 1×10^{-7} M d-tubocurarine (fig.1). A similar analysis using α-Bgt gave an I_{50} of 2.3×10^{-9} M (fig.2A). An identical value was obtained with α-Bgt using clone 5 (fig.2B).

4. Discussion

One approach to studying MG is the induction of EAMG by passive transfer of anti-nAcChR antibodies [2,3]. Combined with mAbs, it may allow the identification of determinants of particular importance

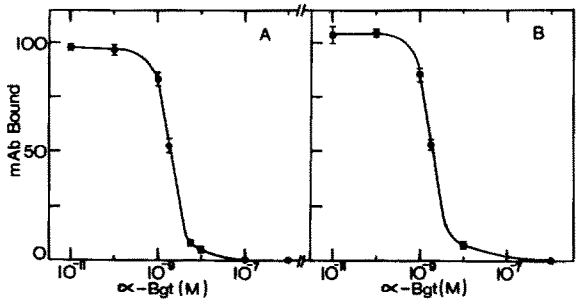

Fig.2. Dose–response curves of the effects of α-Bgt on binding of antibodies of clones 1 (A) and 5 (B) to ^{125}I-nAcChR (section 2). For clone 1, RAMIG (10 μg, 5 μl) was included in the incubation mixture. Results ±SD (n = 4) are expressed as % of binding in the absence of α-Bgt ((A) 8062 ± 621 cpm; (B) 5551 ± 240 cpm). Analyses were performed on at least 2 separate occasions.

in the disease. Obviously, it necessitates that the mAbs bind to nAcChR in situ and a preliminary requirement is to identify such antibodies. Four of the five clones established meet this requirement.

Clones 1 and 2 are probably secreting the same mAb as results with RAMIG (table 1) and cholinergic ligands (table 2) are similar. Moreover, dose–response curves, most notably those with the small mol wt ligands (fig.2A,B) indicate that the antibody is binding at the neurotransmitter binding site of the receptor. The I_{50} values reflect the affinities of the ligands for nAcChR [15,16]. Atropine also inhibits binding of the mAb to nAcChR, although to a much lesser extent and only at a high concentration (10^{-4} M). The binding of mAbs of clones 4 and 5 is inhibited by α-Bgt; none of the smaller ligands had an effect (table 2, fig.3B). These mAbs bind close to, but not at, the neurotransmitter-binding site of nAcChR. Moreover, the studies with RAMIG (table 1) suggest that these antibodies are not identical and they may therefore be directed against different determinants.

The mAb secreted by clone 3 was partially affected only by the presence of the nicotinic agonist carbamylcholine. One possible explanation is that the antibody binds to a determinant affected by conformational changes induced in nAcChR by binding of the agonist. A stereochemic inhibition of binding of the mAb to nAcChR is ruled out because toxin had no effect.

Four reports have appeared describing mAbs against nAcChR [17–20]. Gomez et al. [17] describe one mAb whose binding to nAcChR is completely inhibited by α-Bgt, but no other ligand. Thus, it is similar to mAbs 4 and 5 above. Tzartos and Lindstrom (one monoclonal line, [18]) and Lennon and Lambert (2 monoclonal lines, [19]) both report the passive transfer of EAMG with mAbs whose determinants are remote from the neurotransmitter binding site of nAcChR.

Thus the importance of different antigenic determinants of nAcChR for inducing EAMG is slowly being unravelled. The mAbs described in this report will be particularly useful for analysing the contribution of determinants in the region of the neurotransmitter binding site.

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References

[1] Drachmann, D. B. (1978) New Engl. J. Med. 298, 136–142.
[2] Drachmann, D. B. (1978) New Engl. J. Med. 298, 186–193.
[3] Lindstrom, J. (1978) in: The biochemistry of myasthenia gravis and muscular dystrophy (Lunt, G. and Marchbanks, R. M. eds) pp. 135–156, Academic Press, London, New York.
[4] Fuchs, S. (1979) Curr. Top. Microbiol. Immunol. 85, 1–29.
[5] Heilbronn, E. and Stalberg, E. (1978) J. Neurochem. 31, 5–11.
[6] Zurn, A. D. and Fulpius, B. W. (1979) Progr. Brain Res. 49, 435–440.
[7] Kohler, G. and Milstein, C. (1975) Nature 256, 495–497.
[8] Zurn, A. D. and Fulpius, B. W. (1978) Eur. J. Immunol. 7, 529–532.
[9] Galfrè, G., Howe, S. C., Milstein, C., Butcher, G. W. and Howard, J. C. (1977) Nature 266, 550–552.
[10] Hengartner, H., Luzzati, A. L. and Schreier, M. (1978) Curr. Top. Microbiol. Immunol. 81, 92–99.
[11] Hunter, W. M. and Greenwood, F. C. (1962) Nature 194, 495.
[12] Klett, R. P., Fulpius, B. W., Cooper, D., Smith, M., Reich, E. and Possani, L. D. (1973) J. Biol. Chem. 248, 6841–6853.
[13] Kessler, S. W. (1975) J. Immunol. 115, 1617–1624.
[14] Goding, J. W. (1978) J. Immunol. Methods 20, 241–253.
[15] Heidmann, T. and Changeux, J.-P. (1978) Annu. Rev. Biochem. 47, 317–357.
[16] Raftery, M. A., Vandlen, R. L., Reed, K. L. and Lee, T. (1975) Cold Spring Harbor Symp. Quant. Biol. 40, 193–202.
[17] Gomez, C. M., Richman, D. P., Berman, P. W., Burres, S. A., Arnason, B. G. W. and Fitch, F. W. (1979) Biochem. Biophys. Res. Commun. 88, 575–582.
[18] Tzartos, S. J. and Lindstrom, J. M. (1980) Proc. Natl. Acad. Sci. USA 77, 755–759.
[19] Lennon, V. A. and Lambert, E. H. (1980) Nature 285, 238–240.
[20] Moshly-Rosen, D., Fuchs, S. and Eshhar, Z. (1979) FEBS Lett. 106, 389–392.