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MONOCLONAL ANTIBODIES AGAINST PURIFIED NICOTINIC ACETYLCHOLINE RECEPTOR

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SUMMARY

Eleven stable monoclonal hybridoma cell lines synthesizing antibodies against purified acetylcholine receptor from Torpedo californica were produced. Spleen cells from a rat immunized with acetylcholine receptor and exhibiting experimental autoimmune myasthenia gravis were fused with a mouse myeloma cell line. Studies of the binding of these antibodies to acetylcholine receptor by use of a passive hemagglutination assay in the presence of various cholinergic ligands revealed four general categories of binding specificities: 1) blockade only by alpha bungarotoxin, 2) partial blockade by all ligands, 3) increased titer in the presence of alpha bungarotoxin and benzoquinonium chloride, 4) absence of effect by any ligand.

INTRODUCTION

Anti-sera raised against purified antigens have proven to be useful tools in the identification, quantification, and in situ localization of these antigens (1). Such anti-sera, in general, are made up of multiple populations of antibodies which differ in their immunochemical characteristics and antigenic specificities. Recently developed techniques for the production of monoclonal antibodies (mAbs)[†] (2) have provided a means by which immunoglobulin of uniform structure and specificity may be obtained. Immunochemical reagents prepared in this manner will undoubtedly prove to be powerful probes of macromolecular structures. We have applied this approach to the study of nicotinic acetylcholine receptor (AChR). Although this protein has been intensively studied by

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[†]Abbreviations: mAb, monoclonal antibody; AChR, nicotinic acetylcholine receptor; EAMG, experimental autoimmune myasthenia gravis; αBT, alpha bungarotoxin; Cm, carbamylcholine; dtc, d-tubocurarine; atr, atropine sulfate; Bz, benzoquinonium chloride; Brij-PBS, phosphate buffered saline with 0.05% Brij 35; RBCs, sheep erythrocytes; HA, passive hemagglutination assay.

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biochemists and biophysicists, fundamental questions concerning the relationship between its structure and function remain unanswered. Even the subunit composition of AChR is unresolved,and proposed structures range between 1 and 4 polypeptides (3,4). This protein is thought to have at least 4 distinct functional states (5) the properties of which can be altered through the use of cholinergic ligands, snake neurotoxins, and local anesthetics. In addition, animals immunized with purified Torpedo AChR develop the model disease, experimental autoimmune myasthenia gravis (EAMG), concomitant with the appearance of anti-AChR antibodies (6). Monoclonal antibodies reactive with defined areas on the surface of receptor should provide valuable tools for elucidating the subunit structure of this protein for identifying regions of the molecule important for the binding of pharmacologic agents, and for defining sites on the molecule crucial for the induction of EAMG. In the studies described below, spleen cells from rats immunized with AChR and exhibiting EAMG were fused with a myeloma cell line to produce a number of stable hybridoma cell lines producing anti-receptor antibodies. We report preliminary studies of the binding of these antibodies to AChR in the presence of various substances known to modulate receptor function.

METHODS

Pharmacologic Agents - Alpha bungarotoxin (αBT), $[^{125}I]$αBT, and alpha neurotoxin were kindly supplied by Dr. James Patrick. Carbamylcholine (Cm), d-tubocurarine (dtc), atropine sulfate (atr), choline bromide and physostigmine were obtained from Sigma (St. Louis, MO), neostigmine methylsulfate from Elkins-Simms (Cherry Hill, NJ) and sodium acetate from Fisher (Fair Lawn, NJ). Benzoquinonium chloride (Bz) was a gift of Sterling Drug Company (Rensselaer, NY). All solutions were prepared daily.

AChR Preparation - AChR was prepared from the electroplax of *Torpedo californica* by the method of Berman and Patrick (7,8). Approximately equal proportions of monomeric and dimeric AChR (3.8-4.0 nmoles of αBT binding sites per mg protein) were suspended in phosphate buffered saline containing 0.05% of the non-ionic detergent Brij 35 (Cal Biochem, San Diego, CA) (Brij-PBS).

Immunization - AChR in 0.05% Brij-PBS was emulsified with an equal volume of complete Freund's adjuvant and injected in multiple intradermal sites along the back and in the footpads of 200 gm female Lewis rats. In the primary immunization each animal received 45μg of AChR protein. At the same time each animal was injected with forty-five microliters of killed B.pertussis vaccine (gift of Eli Lilly Co., Indianapolis, IN) at multiple intradermal sites. All animals developed the typical clinical course of EAMG (9) with frank weakness beginning on day 9-12 after inoculation and clearing by day 21. On the 24th day, at which time the acute phase of the disease had cleared but prior to development of the chronic phase, animals received a second injection of AChR, 25 to 100μg without adjuvant, intraperitoneally.

Spleen Cell Hybridization (10) - Three days after the second injection of AChR, spleens were removed from immunized animals, homogenized in a ground glass homogenizer, and centrifuged over a Ficoll-Hypaque gradient (S.G.: 1.094). The mononuclear cell suspension thus obtained was then fused with

the mouse myeloma cell line P3-X63-Ag8 (kindly provided by Dr. C. Milstein). Spleen cells (5×10^7) and myeloma cells (5×10^6) were mixed in 60mm Petri dishes (Falcon) and centrifuged at 250xg for 3 minutes. The cells were then flooded with 50% polyethylene glycol (PEG 1500, Fisher, Fair Lawn, NJ), and after washing, cells were cultured overnight in Dulbecco's modified Eagles medium with 20% heat-inactivated fetal calf serum. The cells were then cultured in hypoxanthine-aminopterin-thymidine (HAT) medium in microtest wells (Costar, Cambridge, MA) (approximately 1×10^4 cells per well). Supernatants from wells with proliferating hybrids were assayed (see below) for anti-AChR activity. Cells from positive wells were then cloned by limiting dilution in microtest wells with irradiated Lewis rat thymocytes serving as a feeder layer. Clones were grown up in bulk in spinner flasks. Some clones were re-cloned by limiting dilution. Antibody Assay - A passive hemagglutination assay (HA) was performed by modification of the method of Aarli et al. (11). AChR (T californica) in 0.05% Brij-PBS was coated onto sheep erythrocytes (RBCs) previously treated with 83.3μg/ml tannic acid. The cells were then washed and resuspended in 0.05% Brij-PBS. The ligand-binding properties of the RBC-bound AChR were tested in a competitive binding assay. AChR-coated RBCs were incubated with various ligands (10^{-5}M) in 0.05% Brij-PBS for 30 minutes prior to a 30-minute incubation with [^{125}I]αBT (10^{-7}M). The cells were centrifuged through a layer of n-butyl phthalate and the pellets counted in a gamma counter.

Serum or culture supernatants were serially diluted with 0.05% Brij-PBS and 1% non-immune heat-inactivated rabbit serum. The AChR-coated sheep erythrocytes were then added to the serum (or supernatant) dilutions in round bottom microwell plates (Falcon, Oxnard, CA), incubated at 37°C for 15 min., and scored for agglutination visually and also after centrifugation at 250xg for 1 min. (12). Assessments of blockade of antibody binding in the HA were carried out by preincubation of AChR-coated cells with pharmacologic agents in 0.05% Brij-PBS or with 0.05% Brij-PBS alone for 30 minutes at 20°C prior to their addition to the serum or supernatant dilutions.

RESULTS

Hybridization: All mAbs studied here resulted from the hybridization of spleen cells from a single rat (Bk) whose serum HA titer to AChR was 2^{17}. Half the wells planted grew out hybrids. Seventy percent of the supernatants from hybrid-positive wells contained anti-AChR activity. Clones were obtained by limiting dilution with 10%-30% of planted feeder-layer wells positive for proliferating hybridomas. All such hybridomas grew within the well as a single patch. Clones were designated by the number of the primary well from which they were derived. (It is possible that some clones derived from the same well are identical.) Five of these antibody-producing clones were re-cloned by limiting dilution. All subclones (n=25) were positive for antibody production suggesting that the original clones were monoclonal. The binding specificities (see below) of the mAbs remained stable as the hybrids were carried through more than 10 passages.

mAb Specificities: The HA titers of culture supernatants from 11 anti-receptor hybridomas are presented in Table 1. Most supernatants were

Table 1

Supernatant Anti-AChR HA Titers (Log_2)

in the Presence of Various Cholinergic Ligands

Clone	Ligand*					
	Control	αBT	dtc	Bz	Cm	atr
60D	13	0	13	12	12	13
56I	29	24	24	21	24	21
56K	22	20	21	22	20	21
57L	16	14	14	14	14	14
62J	23	18	18	20	20	18
73G	17	20	17	21	17	17
38I	13	13	13	13	13	14
58E	9	9	9	9	10	9
58F	6	6	6	6	6	6
77A	19	19	19	19	20	19
77F	20	19	20	20	20	20

*dtc, Bz, Cm, atr - 10^{-5}M; αBT - 10^{-7}M

assayed at least 3 times, with representative results given. Experiments in which 5 replicates were carried out on the same day determined the 95% confidence limits of these titers to be $\pm 2^1$. Differences in HA titers between clones are not significant since the supernatants tested were obtained from cultures containing different cell densities.

Control titers are the result of preincubation of the AChR-coated RBCs in Brij-PBS alone prior to the addition of antibody-containing supernatants. Each supernatant was also tested against uncoated RBCs and tanned RBCs treated with Brij-PBS; no agglutinating activity was detected. The pharmacologic activity of RBC-bound AChR was tested by a competitive radioassay (see Methods). Binding of $[^{125}I]$αBT to AChR-coated RBCs was inhibited by cholinergic ligands in the following order of effectiveness: αBT>dtc>Bz>Cm>atr.

The effects of various cholinergic ligands on antibody binding in the HA are indicated in Table 1. In control experiments each ligand was tested for, and found to lack, agglutinating activity against the AChR-coated RBCs. Preincubation of the receptor-coated RBCs with sodium acetate, choline bromide, neostigmine methylsulfate, and physostigmine

(all 10^{-5}M) had no effect on the HA titers of any of the hybridoma supernatants. The data allow roughly four categories of mAb specificities to be distinguished. In the first category, represented by hybridoma 60D, hemagglutination was completely blocked by αBT with little effect by other ligands. The second type of specificity observed (hybridomas 56I and K, 57L, 62J) was that in which hemagglutination was partially blocked by αBT and by all other cholinergic ligands tested, including the muscarinic antagonist, atr. The third specificity (hybridoma 73G) is that in which none of the ligands tested reduced the HA titer; however,αBT and Bz increased it. The final category includes hybridomas 38I, 58E and F, and 77A and F where HA titers were unaffected by all the ligands tested.

The inhibitory effect of αBT on hemagglutination by antibody from clone 60D was studied in greater detail. Increasing the concentration of αBT in the HA produced a steep inhibition curve (Figure 1) with half maximal effect at αBT concentration between 6×10^{-9}M and 8×10^{-9}M. The possibility that the combination of αBT and 60D supernatant degrades AChR or releases it from the surface of the RBCs was ruled out by the following experiment. AChR-coated RBCs were preincubated with 10^{-7}M αBT and then reacted with 60D supernatant with no resultant agglutination. Resuspension of the cells and addition of a $1{:}2^7$ dilution of 62J supernatant resulted in definite hemagglutination. In another experiment the possibility that antibody from clone 60D could actually be directed against αBT and not AChR was tested. AChR-coated RBCs were preincubated for 30 minutes with 10^{-4}M dtc, a competitive inhibitor of αBT binding. Subsequent incubation with αBT (10^{-7}M for 30 minutes) and then 60D supernatant resulted in HA titer equal to control values for 60D (i.e., 2^{13}). Free αBT thus does not affect hemagglutination by 60D supernatant, indicating that the antibody is directed against AChR and not against the toxin.

DISCUSSION

The experiments presented above represent preliminary investigation of the binding properties (13) of monoclonal antibodies prepared against purified nicotinic acetylcholine receptor. Eleven hybridoma-derived antibodies were studied, and the binding of six of these to erythrocyte-bound AChR could be altered by cholinergic ligands. Four categories of mAb binding specificities could be distinguished. In the first type of specificity (hybridoma 60D) agglutination of receptor-coated RBCs by antibodies is completely inhibited by αBT, but not by other ligands such as dtc which competitively inhibit αBT. This result may be accounted for in several ways. One possibility is that antibody 60D, αBT, and the cholinergic ligands tested all bind to the same site on AChR. αBT is

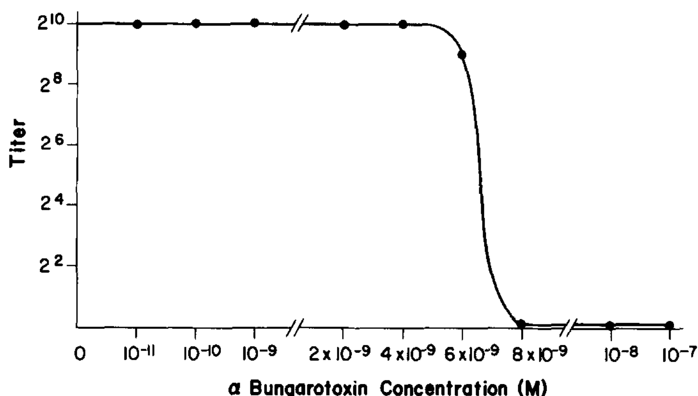

Figure 1 - Anti-AChR HA titers of hybridoma clone 60D supernatant after AChR-coated RBC preincubation with various concentrations of αBT

known to bind to AChR in an essentially irreversible fashion, while the other ligands studied are readily reversible. If antibody 60D binds with much less affinity than αBT but with much greater affinity than dtc, Cm, Bz, and atr, then the type of antibody binding behavior predicted would be similar to that observed. The observation that hemagglutination by 60D antibody can occur in the presence of 10^{-4}M dtc and 10^{-7}M αBT does not, however, support this interpretation and suggests that antibody 60D does not bind to the ligand binding site. An alternative explanation which could account for the data is that antibody 60D binds in close proximity to the ligand binding site. αBT is much larger than any of the other ligands tested (molecular weight approximately 8,000) and would be expected to mask a considerable portion of the AChR surface. Steric hindrance by αBT might be expected to inhibit antibody binding at locations peripheral to the ligand binding site, while smaller ligands would not be effective. A third possibility would be that antibody 60D binds at a site remote from the ligand binding site and that αBT binding, but not other ligand binding, results in a conformational change in AChR which occludes the the 60D binding site.

The partial inhibition by ligands (at the single concentration tested) of hemagglutination by antibodies from hybridomas 56I and K, 57L and 62J and the apparent facilitation of hemagglutination by hybridoma 73G are difficult to interpret. These results suggest that the antibodies are either directed towards antigenic determinants close to ligand binding sites or that they are capable of detecting ligand-induced changes in AChR conformation. Further investigations studying a wide range of ligand con-

centrations, and making use of other antibody assays such as radioimmunoassay (7,8) will be required to distinquish between these possibilities. A somewhat surprising observation is the partial blockade of hemagglutination by atr. We know of no prior data suggesting that this muscarinic antagonist binds to Torpedo AChR, however, at high concentration it is reported to compete with α-neurotoxin for binding to Electrophorus AChR (14).

The hemagglutination titers of the antibodies from hybridomas 38I, 58E and F, and 77A and F are unaffected by the ligands tested. These may bind to sites remote from the ligand binding sites, or, conversely, they may bind with very high affinity to the ligand binding site. Some of the differences in behavior observed in the HA may also derive from structural characteristics of the mAbs. IgM antibodies are particularly active in agglutination assays and might behave differently with respect to ligand blocking than do other immunoglobulins (15). (Preliminary results have identified 60D and 62J as IgG-2a and 77F as IgM.)

The availability of the monoclonal antibodies described above provides new tools by which the nicotinic AChR may be explored. Future investigations using these reagents will include studies of the structure and pharmacology of AChR, of the pathogenesis of EAMG, and of the mechanisms of cholinergic transmission.

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