arations (Fig. 2; $P < 0.01$), confirming our earlier findings [2, 5]. However, release stimulated by DNP, RR or caffeine was similar in both preparations, suggesting that ethanol tolerance was not likely to be associated with an increase in the capacity of either mitochondrial or endoplasmic reticulum to store Ca^{2+}. It should be pointed out that we did not measure the concentration of intracellular Ca^{2+} following *in vitro* treatments but evidence from previous experiments has indicated that caffeine, DNP and RR all induce an increase in Ca^{2+} concentration [8–10], resulting in increased transmitter release from synaptosomes [9] or slices [22].

Our results suggest that in ethanol tolerance, enhanced [^{3}H]DA release from striatal slices results from an increase in the sensitivity of the release process to Ca^{2+} and not from an increase in capacity of intracellular stores to sequester and probably later release Ca^{2+}. This does not rule out a role for stored Ca^{2+} in the enhanced release of neurotransmitter, however, since it has been reported that inositol phosphate production on depolarization is enhanced in preparations from ethanol-tolerant animals [23, 24]. Inositol triphosphate has been shown to release intracellular Ca^{2+} from a variety of cell types [25, 26] so that its increased production in the ethanol-treated preparations may contribute to enhanced neurotransmitter release.

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Recognition of muscarinic acetylcholine receptor ligands by monoclonal antibodies against propylbenzilylcholine mustard

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Muscarinic acetylcholine receptors are involved in neurotransmission in the autonomic and central nervous systems. Ligand binding studies indicate that these receptors are composed of two subclasses [1]. Some muscarinic ligands can discriminate between the receptor subclasses, and binding of these ligands to a portion of the total receptor population results in selective biochemical and physiological responses [1–4]. It is likely that detailed classification of muscarinic ligands and accurate description of the ligand binding properties of the receptor will lead to the identification of selective drugs with enhanced therapeutic usefulness.

In this report we describe the production of two monoclonal antibodies directed against propylbenzilylcholine mustard (PrBCM*), the affinity alkylating muscarinic antagonist [5]. These antibodies selectively recognized muscarinic, but not nicotinic, cholinergic ligands and may be used for obtaining information concerning the specificity of muscarinic ligands as well as the binding and biochemical properties of the receptor itself.

Materials and methods

Materials. Propylbenzilylcholine mustard and [^{3}H]quinuclidinyl benzilate (35.5 Ci/mmole) were from the Amersham Corp. BSA, *p*-nitrophenyl phosphate and cholinergic drugs were from Sigma, except for quinuclidinyl benzilate (Hoffmann-LaRoche Laboratories), which was a gift from W. A. Catterall, and McNA343 and pirenzipine, which

* Abbreviations: BSA, bovine serum albumin; ELISA enzyme-linked immunosorptive assay; PrBCM, propylbenzililylcholine mustard; and QNB, quinuclidinyl benzilate.

were gifts of McNeil and Boehringer Ingelheim respectively. Cell culture medium components were from Gibco. Goat anti-mouse immunoglobulins conjugated to alkaline phosphatase were from Cappel. All other chemicals were of reagent grade.

Synthesis of PrBCM-protein conjugates. PrBCM was diluted to 6×10^{-4} M with 10 mM sodium phosphate, pH 7.4, and incubated at room temperature for 30 min. BSA or ovalbumin from a 100 mg/ml stock was added to a final concentration of 2.5 mg/ml. The mixture was incubated at room temperature for 4–6 hr and dialyzed four times against 500 vol. of 10 mM sodium phosphate, pH 7.4.

Immunization and fusion. Balb/C mice were immunized by intraperitoneal injection of 50 μl of PrBCM-BSA conjugate emulsified with an equal volume of Freund's complete adjuvant, boosted twice at 3-week intervals with conjugate and incomplete adjuvant, and killed 3 days following the last boost. The spleen cells were fused with SP-2 myeloma cells by the procedure of Galfrey *et al.* [6] as modified by de St. Groth and Scheidegger [7]. Clones were screened for reactivity by an ELISA [8, 9] starting on day 10. Positive clones were subcloned by limiting dilution until stable, and the hybridoma cells were grown as ascites tumors as described by Galfrey *et al.* [6]. Ascites fluid was first centrifuged at 200 *g* to remove cells and then again at 15,000 *g*. The supernatant fraction was stored at −70°.

ELISA. Samples (50 μl) of target antigen (50 μg/ml) were aliquoted into microtiter wells and incubated for 2 hr at room temperature. After rinsing the wells four times with 5% horse serum in 0.8% NaCl, 0.02% KCl, and 0.12% $NaHPO_4$ (pH 7.5), 50 μl of culture medium was added to the wells and incubated for 2 hr. Wells were washed again and incubated with 50 μl (1:250 dilution) goat anti-mouse IgG conjugated to alkaline phosphatase for 2 hr, washed, and then incubated with 100 μl of 0.2 mg/ml *p*-nitrophenyl phosphate in 1 M Tris, pH 8.0, until a yellow reaction product developed. Absorbance was read using a Titertek ELISA reader.

Radial immunodiffusion. The subclasses of the antibodies were determined using an Ouchterlony immunodiffusion technique (Miles typing kit) as described in the instructions of the manufacturer.

Binding assays. The binding of ligands to the antibodies was assayed by a modification of the ammonium sulfate precipitation assay of Hurko [10]. A final 1-ml reaction volume containing 1:50,000 ascites fluid, 1×10^{-9} M [^{3}H]-QNB, and the competing ligand at the concentration indicated in 50 mM sodium phosphate, pH 7.4, was incubated at room temperature for 60 min. A 10 mM concentration of sodium phosphate was used when PrBCM was the competing ligand. The reaction was stopped and filtered over GF/C discs as described by Hurko. The discs were placed in 4 ml of scintillation fluid [11] for counting. Nonspecific binding, the amount of binding that remained in the presence of 1 mM atropine sulfate, was less than 10% of the total.

Results

The fusion of spleen cells from a mouse immunized with a PrBCM-BSA conjugate and SP-2 myeloma cells resulted in viable clones in 40% of all wells. Initial screening indicated that four clones out of two hundred were reactive in an ELISA when PrBCM-BSA, but not BSA, was used as the target antigen. After subcloning, two of the four clones retained activity specific for PrBCM and were amplified in ascites. Subtype analysis indicated that clone 12-2G7 was IgG type 3 and clone 12-5B11 was IgG type 1.

The antibodies in spent culture medium from the two clones were tested for their abilities to recognize PrBCM in an ELISA (Fig. 1). The antibodies exhibited significantly greater reactivity towards PrBCM linked to BSA or ovalbumin than to either BSA or ovalbumin alone, indicating

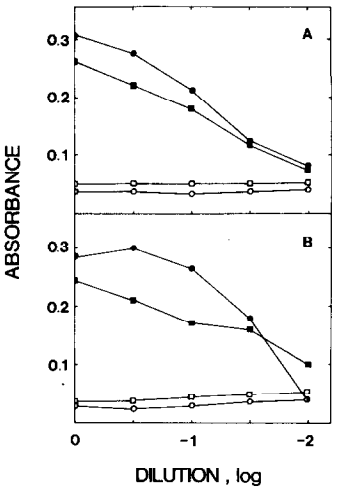

Fig. 1. Immunoreactivity of antibodies 12-2G7 (A) and 12-5B11 (B) toward PrBCM. PrBCM-BSA (●), PrBCM-ovalbumin (■), BSA (○), or ovalbumin (□) was plated at 50 μg/ml protein concentration and incubated with the indicated dilutions of culture medium from clone 12-2G7 or 12-5B11. Antibody binding was detected by goat anti-mouse antibody conjugated to alkaline phosphatase and measured by the reaction with *p*-nitrophenyl phosphate. Each point represents the average of duplicate samples. Standard deviations are less than 10% of the mean values shown.

that the antibodies recognize the muscarinic antagonist PrBCM.

A filter binding assay employing the muscarinic antagonist [^{3}H]QNB was used to quantitate ligand binding to the antibodies. Both antibodies bound [^{3}H]QNB with high affinity and specificity (Fig. 2). Scatchard analysis of the data revealed the ligand bound to a single class of sites with K_D values of 2.0×10^{-9} M and 2.0×10^{-8} M for 12-2G7 and 12-5B11 respectively.

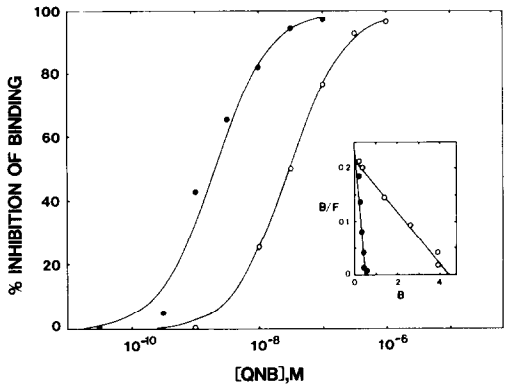

Fig. 2. Binding of QNB to antibodies 12-2G7 and 12-5B11. Ascites fluid containing antibody 12-2G7 (●) or 12-5B11 (○) was assayed for [^{3}H]QNB binding in the presence of competing concentrations of unlabeled QNB. The points represent observed inhibition of binding, and the curves are drawn through the EC_{50} calculated by Hill analysis. Scatchard analysis of the data is shown in the inset. The values represent averaged results from two independent competition experiments. Standard deviations are within 10% of the mean values shown.

The affinities of the antibodies for several muscarinic and non-muscarinic ligands, calculated from their abilities to compete with the binding of [^{3}H]QNB, are summarized in Table 1. ([^{3}H]QNB was used in competition binding studies to avoid complications in interpreting data obtained from experiments using the irreversible ligand, [^{3}H]PrBCM.) Antibody 12-2G7 displayed the highest affinity for PrBCM and high affinities also for QNB, atropine, scopolamine, and the M_1-subtype selective antagonist pirenzipine. Antibody 12-5B11, in contrast, exhibited its highest affinity for QNB with high affinities also for PrBCM, atropine and scopolamine but displayed a much lower affinity for the selective antagonist pirenzipine.

Table 1. Affinities for cholinergic ligands

Drug	K_D (M)	
	12-2G7	12-5B11
PrBCM	4.1×10^{-10}	1.3×10^{-7}
QNB	2.0×10^{-9}	2.0×10^{-8}
Atropine	9.3×10^{-6}	2.0×10^{-5}
McNA343	1.2×10^{-5}	1.6×10^{-3}
Pirenzipine	6.4×10^{-5}	9.5×10^{-3}
Scopolamine	1.2×10^{-4}	1.0×10^{-3}
Pilocarpine	5.3×10^{-3}	6.6×10^{-3}
Oxotremorine	$> 10^{-2}$	3.8×10^{-3}
Carbachol	1×10^{-2}	$> 10^{-2}$
Decamethonium	$> 10^{-2}$	$> 10^{-2}$
Hexamethonium	$> 10^{-2}$	$> 10^{-2}$

The affinities of antibodies 12-2G7 and 12-5B11 for various cholinergic ligands were calculated by the abilities of the compounds to compete for the binding of [^{3}H]QNB. The K_D of each ligand was calculated from the concentration which inhibited 50% of [^{3}H]QNB binding, using the method of Cheng and Prusoff [12]. The values represent the mean from at least three separate competition curves. Standard deviations are, in most cases, less than 10% of the mean values shown, and in all cases, less than 15%.

A variety of muscarinic agonists were tested for their binding to the antibodies. Antibody 12-2G7 displayed its highest affinity for the selective agonist McNA343 and had a slightly lower affinity for pilocarpine. 12-2G7 also bound oxotremorine and carbachol, but only at high concentrations ($K_D > 10^{-2}$). The antibody 12-5B11 bound the agonist pilocarpine with an affinity similar to that of 12-2G7 but bound the selective agonist McNA343 with an affinity 100 times less than that of 12-2G7. Neither antibody significantly bound nicotinic cholinergic drugs such as *d*-tubocurarine, decamethonium, or hexamethonium.

Discussion

The synthesis of the immunogen PrBCM-BSA took advantage of the highly reactive arizidinium ion of PrBCM to couple the compound to BSA. This technique is easy and convenient and could be applied to other drugs that possess a nonspecific reactive alkylating group.

The monoclonal antibodies generated in this study against a muscarinic antagonist were capable of distinguishing muscarinic ligands from nicotinic ligands, indicating that these compounds possess some common structural features which distinguish them from other classes of drugs. Both antibodies bound classical muscarinic antagonists with high affinity. Antibody 12-2G7 bound PrBCM with much higher affinity than 12-5B11, whereas QNB, atropine and scopolamine bound similarly to both antibodies. Both antibodies also recognized several muscarinic agonists but did so with lower affinity. The muscarinic receptor itself also has lower affinities for agonists than antagonists [13, 14].

Interestingly, antibody 12-2G7 additionally recognized the novel ligands pirenzipine and McNA343 with affinities approximately 100 times those of 12-5B11. These ligands bind preferentially to the M_1 subtype of muscarinic receptors (M1) [1, 2]. Therefore, 12-2G7 binds M1 selective drugs with higher affinity than 12-5B11. While we do not know whether the differences in the affinities of the two antibodies for pirenzipine and McNA343 reflect the differences in the binding sites of the M1 and M2 receptors, these results suggest that these antibodies may be useful in characterizing subtype-selective muscarinic ligands. The availability of large quantities of monoclonal antibodies with differential affinities for muscarinic subtype selective drugs may also be useful in the elucidation of structural features of the binding sites that recognize M1 and M2 ligands.

The abilities of muscarinic ligands to compete with the binding of PrBCM indicate that both antibodies were directed against a portion of the PrBCM involved in binding to the receptor. Preliminary experiments indicate that, although the antibodies did not recognize PrBCM once it was bound to the native receptor, proteolytic digestion of the prelabeled receptor yielded a labeled fragment that was specifically recognized by the 12-2G7 antibody (M. W. Gainer and N. M. Nathanson, unpublished results). Therefore, this antibody may be useful for purification of the portion of the receptor near the ligand binding site.

The high affinities of the two anti-PrBCM antibodies for muscarinic ligands indicate that these antibodies should be useful in the production of anti-idiotype antibodies that bind to the muscarinic receptor. Antibodies made against other anti-ligand antibodies have been isolated, and these anti-idiotypic antibodies specifically recognize the ligand binding sites of the β-adrenergic, nicotinic acetylcholine, and insulin receptors [15–17]. Since the antibodies described here have different affinities for subtype-selective muscarinic ligands, anti-idiotypic antibodies generated against these two anti-PrBCM antibodies may recognize different subclasses of the receptor.

We have described the production of two monoclonal antibodies which recognize muscarinic but not nicotinic ligands. One of the antibodies displayed high affinity for M1 subtype selective ligands. Both antibodies exhibited a single homogeneous population of binding sites and were readily available in large quantities. These antibodies provide an excellent system for studying antibody–hapten interactions and may yield not only valuable information concerning the binding properties of the muscarinic receptor and its ligands but also perhaps about the regulation of immunological responses to small molecules recognized by membrane neurotransmitter receptors as well.

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A re-evaluation of the competitive protein binding assay for methotrexate binding to dihydrofolate reductase

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Competitive ligand binding assays are widely used for the measurement of hormone [1], vitamin [2], and drug [3] levels. For small molecular weight ligands, activated charcoal is employed frequently to adsorb ligand that is not protein bound, leaving the protein-bound portion in the supernatant fraction after centrifugation [2]. Myers *et al.* [3] utilized charcoal adsorption and centrifugation steps in their assay for methotrexate (MTX), an important antineoplastic agent. In recent experiments designed to measure the "on" and "off" rates of methotrexate with its target enzyme, dihydrofolate reductase (DHFR), the binding protein used in drug assays, we noted that the stability of this complex was highly dependent on the concentration of free MTX and its cofactor NADPH, both of which are required for the formation of a stable ternary complex with the enzyme. At low NADPH concentration, the complex rapidly dissociated. The addition of charcoal to solutions containing ternary complex led to rapid dissociation of the complex through adsorption of free NADPH, a problem aggravated by the time required for centrifugation in order to separate charcoal from the aqueous solution. In this report we present a filtration-based method for separating protein-bound and free methotrexate; this method permits a more rapid and, therefore, more accurate quantitation of the drug–enzyme–NADPH complex for purposes of drug assay and biochemical study.

Materials and methods

Charcoal (acid-washed), albumin (fraction V), and dextran (mol. wt. 200,000–400,000) were purchased from the Sigma Chemical Co. (St. Louis, MO). Unlabeled MTX was obtained from the Drug Research and Development Branch of the National Cancer Institute (Bethesda, MD). [3′,5′,9(n)-^{3}H]Methotrexate (18 Ci/mmole) was purchased from Moravek Biochemicals (Brea, CA). NADP[^{3}H] (5 Ci/mmole) was a gift of Dr. James Phang of the National Cancer Institute. Acro-LC13 disposable filters (0.45 μm) were obtained from Gelman Scientific (Ann Arbor, MI). Human DHFR [4] was a gift of Dr. Bernard Kaufman of the National Cancer Institute (sp. act. 27.3 μmoles/min/mg at 37°). *Lactobacillus casei* DHFR was purchased from the New England Enzyme Center (Boston, MA) (sp. act. 0.64 μmole of tetrahydrofolate formed/min/mg at 37°).

MTX binding assay. Ternary complex was formed with tritiated MTX, enzyme, and 10^{-4} M or 10^{-8} M NADPH. After ternary complex formation reached an equilibrium (10 min), the unbound drug was removed by adsorption to a mixture of albumin–dextran-coated charcoal. All assays were performed at 23° and were carried out by the sequential addition of the following: (1) 0.1 to 1.0 nCi [^{3}H]MTX in 150 μl of water; (2) 200 μl of aqueous solutions containing various concentrations of unlabeled MTX in water; and (3) 50 μl of 0.5 M potassium phosphate buffer, pH 6.2 or 7.2, containing 0.0027 units of DHFR and 5×10^{-8} or 5×10^{-13} moles of NADPH. Following addition of all assay components, tubes were vortexed and allowed to equilibrate for 10 min. To each tube was then added 50 μl of a charcoal suspension prepared as follows: Norit A-activated untreated charcoal, 10 g; (2) bovine serum albumin, fraction V, 2.5 g; and (3) high molecular weight dextran, 0.1 g in a total volume of 20 ml. The pH of the charcoal suspension was adjusted to either 6.2 with 1 N HCl or pH 7.2 with 1 N NaOH immediately prior to use. After addition of the charcoal, the tubes were processed in one of two ways: (1) *Conventional method:* Tubes were vortexed, allowed to stand for up to 10 min, and then centrifuged at 1200 *g* for 30 min. A 200-μl aliquot of supernatant fraction was added to 10 ml of scintillant (Ready-Solv, Beckman) and counted in a Searle liquid scintillation counter. (2) *Filtration method.* Tubes were vortexed and then transferred to a 3-ml syringe fitted with a Gelman Acro-LC13 filter; at exactly 60 sec after addition of charcoal, the sample was filtered into a counting vial, thus separating the charcoal containing non-enzyme-bound [^{3}H]MTX from that associated with the ternary complex.

Charcoal adsorption of NADPH and MTX. To demonstrate that NADPH is adsorbed by charcoal, [^{3}H]-NADPH was added to unlabeled NADPH to obtain final concentrations of 10^{-9} M to 10^{-4} M. A 0.45-ml aliquot of various concentrations of labeled NADPH solutions was adjusted to pH 6.2 or pH 7.2. Charcoal slurry (50 μl) adjusted to the appropriate pH was added to the NADPH solutions and vortexed, and the NADPH was allowed to remain in contact with the charcoal for various amounts of time prior to separation of charcoal from the aqueous solution with an Acro-LC13 filter. In a similar fashion, solutions of [^{3}H]MTX with concentrations up to 10^{-4} M were incubated with charcoal for various amounts of time prior to separation with an Acro-LC13 filter.