MOLECULAR PHARMACOLOGY, 24:513–520

Production and Characterization of Separate Monoclonal Antibodies to Human Acetylcholinesterase and Butyrylcholinesterase

STEPHEN BRIMIJOIN,[1] KEITH P. MINTZ,[1] AND MICHAEL C. ALLEY[2]

Departments of Pharmacology and Rheumatology, Mayo Clinic, Rochester, Minnesota 55905

Received March 14, 1983; Accepted June 14, 1983

SUMMARY

Butyrylcholinesterase purified from human plasma and acetylcholinesterase purified from human red blood cells were used to immunize separate groups of BALB/c mice. A solid-phase immunoadsorbance assay was developed to screen and characterize antibodies specific for the cholinesterases. Immunized spleen cells were fused with a non-immunoglobulin-secreting myeloma cell line (FO). After two subcultures at limiting dilution, several clones secreting antibodies to acetylcholinesterase or butyrylcholinesterase were obtained. Selected clones were expanded as ascites tumors in immunosuppressed BALB/c mice. All tested immunoglobulins consisted of *kappa* light chains and either G_1 or G_{2b} heavy chains. Two-dimensional gel electrophoresis confirmed the monoclonal nature of each isolated antibody. None of the antibodies to acetylcholinesterase cross-reacted with butyrylcholinesterase, and vice versa. All tested antibodies exhibited high avidity for human enzyme, independent of the tissue source (apparent dissociation constants: 1–3 nM for acetylcholinesterase antibodies; 2–13 nM for butyrylcholinesterase antibodies). Treatment of enzymes with monoclonal antibodies increased the sedimentation coefficients (from 6.5 S to 12 S for acetylcholinesterase, from 11 S to 18 S or 20 S for butyrylcholinesterase). All of the monoclonal antibodies displayed marked species specificity. Several antibodies reacted only with human enzyme; others reacted with enzyme from nonhuman primates as well. A few of the butyrylcholinesterase antibodies cross-reacted weakly with enzyme from dog, cat, and horse, but none reacted with the enzyme from rat, guinea pig, and chicken. One acetylcholinesterase antibody cross-reacted with acetylcholinesterase of rabbit and guinea pig. The avidity, species selectivity, and other properties of these antibody reagents will be useful in future studies on the regulation and disposition of cholinesterases.

INTRODUCTION

There is much interest in the regulation and interrelations of AChE[3] (EC 3.1.1.7) and BuChE (EC 3.1.1.8). Accordingly, there is a need for specific molecular probes that could be used to study the origin, localization, and fate of the cholinesterases. Ideal probes would be high-affinity ligands that permit (a) direct measurements of the amounts of enzyme protein per sample, (b) "tagging" for histochemical experiments, and (c) selective extraction of enzyme from crude mixtures. Monoclonal antibodies are a close approximation to this ideal.

Recently Fambrough and co-workers (1) obtained monoclonal antibodies to the AChE of human red blood cells. These antibodies cross-reacted with enzyme from human muscle and, to a limited extent, with enzyme from other species, but were said to be unsatisfactory for immunohistochemical staining of brain or spinal cord (1). Quantitative data on comparative avidities were not published.

Antibodies to BuChE have received less attention. A polyclonal antibody to mouse brain AChE (2) was found *not* to cross-react with the BuChE of rat (3) or cat (4). Polyclonal antibodies to BuChE have been used to show that the normal and atypical variants of the human serum enzyme are immunologically similar (5). No monoclonal antibodies to BuChE have yet been reported.

It is clearly desirable to produce and characterize additional antibodies to the cholinesterases. In order to facilitate the comparison of AChE and BuChE, we undertook to raise monoclonal antibodies to both antigens, as isolated from human plasma and red blood cells. The properties exhibited by the antibodies obtained are described below.

METHODS

BuChE purification. BuChE was purified from outdated normal human plasma (dibucaine number, 80) by means of affinity chromatography on *N*-methylacridinium-Sepharose 4B as described by Brimijoin

This work was supported in part by Grant NS 11855 from the National Institutes of Health.

[1] Department of Pharmacology.

[2] Department of Rheumatology.

[3] The abbreviations used are: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; IgG, immunoglobulin G: IMDM, Iscove's modified Dulbecco's medium; HAT, hypoxanthine/aminopterin/thymidine.

0026-895X/83/060513-08$02.00/0

et al. (6). Each liter of plasma yielded approximately 0.34 mg of protein with about 15% of the total starting BuChE activity. The specific activity of the product, 643 units/mg of protein, was 46,000 times greater than that of the starting material and was 95% of that previously reported for electrophoretically homogenous human plasma BuChE (7).

AChE purification. Red cell ghosts were prepared from packed human red blood cells by the procedure of Pazmino and Weinshilboum (8). AChE was extracted from the ghosts by homogenization in 4 volumes of 10 mM Tris-HCl (pH 8) containing 1% Triton X-100 and 1 mM EDTA. The purification scheme (Table 1) was essentially similar to the one for BuChE except that the material eluted from the *N*-methylacridinium column was further purified by affinity chromatography on phenyltrimethylammonium-Sepharose 4B, prepared according to the method of Traylor and Singer (9). The final specific activity of 820 units/mg, in relation to previously reported specific activities (10), indicated that the AChE was approximately 25% pure.

Enzyme assays. For routine assay, the activities of AChE and BuChE were measured at room temperature by the method of Ellman *et al.* (11) with 1 mM acetylthiocholine or butyrylthiocholine (Sigma Chemical Company, St. Louis, Mo.) as the substrate. Dilute samples were assayed by the radiometric method of Johnson and Russell (12), with [^{3}H]acetate-labeled acetylcholine (New England Nuclear Corporation, Boston, Mass.) as a substrate. With crude enzyme preparations, the selective inhibitor BW284C51 (final concentration 1 μM, Burroughs Wellcome, Research Triangle Park, N. C.) was used to inactivate AChE, or ethopropazine (10^{-4} M, Sigma Chemical Company) was used to inactivate BuChE. These inhibitors were omitted in work with highly purified enzymes. Enzyme activities were calculated in units of micromoles of substrate hydrolyzed per minute. Dibucaine numbers were determined by the method of Kalow and Genest (13).

Protein determinations. Protein was measured by the dye-binding method (14) with bovine serum albumin as a reference standard.

Screening procedures. A solid-phase immunoadsorbance assay was used to screen samples for the presence of antibodies directed against AChE or BuChE. Rabbit antiserum to mouse immunoglobulin (Miles Laboratories, Elkhart, Ind.) was immobilized on protein A-bearing *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring, San Diego, Calif.) in order to ensure the precipitation of IgG. To coat the cells, a suspension of Pansorbin was pelleted by low-speed centrifugation, washed with buffer [50 mM Tris-HCl (pH 7.4)/0.1% bovine serum albumin], and resuspended in buffered rabbit antimouse Ig (10%, v/v). After incubation for 1 hr at 37°, the coated cells were washed again and resuspended in buffer.

For the screening assay, rabbit antimouse Ig-Pansorbin (100 μl) was pelleted and resuspended in 230 μl of 0.1% bovine serum albumin with 50 mM pH 7.4 buffer (Tris in AChE experiments, sodium phosphate in BuChE experiments). To this suspension were added 10 μl of the antibody sample and 10 μl of antigen (7 milliunits of the crude extract of red cell AChE, or 14 milliunits of an ammonium sulfate fraction of plasma BuChE). In control tubes, the antibody sample was replaced with 10 μl of appropriately diluted nonimmune mouse serum.

The suspensions of antibody, antigen, and rabbit antimouse Ig-Pansorbin were incubated for 1 hr at 37° in a shaking water bath and then centrifuged at low speed. The pellets were washed twice with buffer and resuspended in 1.8 ml of 100 mM sodium phosphate (pH 8). Cholinesterase activity in the resuspended pellets was determined by the method of Ellman *et al.* (11). Reactions were stopped after 10 min by the addition of a selective anticholinesterase. Antigen binding was evaluated qualitatively (yellow color) or quantitatively (optical density at 412 nm).

For the purposes of clonal selection, a positive hybridoma culture was defined as one whose conditioned medium contained enough antibody in a 10-μl volume to bind all of the added antigen. Typically, the nonspecific binding was about 5%, so the signal-to-noise ratio in the screening assay was 20:1.

Media and cells. IMDM, antibiotics, and glutamine were purchased from Grand Island Biological Company (GIBCO, Grand Island, N. Y.); newborn calf serum was purchased from Flow Laboratories (Dublin, Va.); all other reagents were obtained from Sigma Chemical Company. For the initial isolation of hydridomas, HAT-fortified medium was made by supplementing IMDM with newborn calf serum (10%), penicillin (100 IU/ml), streptomycin (100 μg/ml), *l*-glutamine (2mM), mercaptoethanol (20μM), hypoxanthine (0.1 mM), aminopterin (40 μM), and thymidine (16 μM). HT medium was the same, but lacking aminopterin. Nonsecretory, FO mouse myeloma cells (15) were provided by Dr. C. G. Fathman (Mayo Clinic, Department of Immunology).

Immunization. Young adult female BALB/c mice (Jackson Laboratories, Bar Harbor, Me.) were initially immunized sc with 100 μg of purified BuChE or partially purified AChE, emulsified in complete Freund's adjuvant (DIFCO Laboratories, Detroit, Mich.). Four weeks later, 50 μg of antigen in 0.9% NaCl was administered iv. Mice with relatively high serum antibody titers were selected for fusion. Each animal received a final immunization (50 μg), ip, 3 days before *in vitro* splenocyte-myeloma cell hybridization.

Cell hybridization and cloning. Cells were hybridized by a modification (15) of the Köhler-Milstein procedure (16). Spleen cell suspensions were prepared by gentle mechanical disaggregation and filtration through nylon mesh (100 μm). For each fusion, 10^8 nucleated spleen cells were combined with 5×10^7 FO cells. Fusion was induced by treatment with 1 ml of IMDM and polyethylene glycol 1540 [50% (v/v); J. T. Baker Company, Phillipsburg, N. J.]. Afterward, the cells were centrifuged, gently resuspended in HAT-fortified medium, and distributed among three 24-well trays (1 ml/well). A control tray was prepared with unfused FO cells. Each tray well contained irradiated macrophages obtained from AJ mice and plated as a feeder layer 1 day earlier. The

TABLE 1

Purification of AChE from human red blood cells

One or more units of packed red cells were used as the starting material for each batch of enzyme. The specific activity in the ghosts represents a large increase in purity over the crude hemolysate. All buffers contained Triton X-100 (0.1% or more). Enzyme eluted from the acridinium column by a choline chloride gradient (0–50 mM) was applied without dialysis to the phenyltrimethylammonium column, which was eluted in turn with a decamethonium gradient (0–100 mM).

Purification step	AChE					
	Volume	Units (total)	Protein	Specific Activity	Yield	Purification
	ml		*mg*	*units/mg*	%	
Red blood cell ghosts	375	1127	800	1.4	100	—
Acridinium affinity chromatography	162	655	ND[a]	ND	58	—
Phenyltrimethylammonium affinity chromatography	2.3	439	1.33	330	39	236
Bio-Gel A-1.5 m gel filtration	0.94	648	0.79	820	57	585

[a] ND, Not determined.

trays were incubated at 37° in the presence of 5% CO_2/95% air and 100% relative humidity. Culture media were replaced daily for 3 days and then at 3-day intervals (HT medium was substituted for HAT medium when the control tray exhibited evidence of complete growth arrest).

As cell densities approached confluency (10–20 days), the culture supernatants were screened for immunoreactivity (see Screening Procedures). Primary cultures whose media contained the highest titers were cloned by subculture at limiting dilution (average of ⅓ cell per well) in HT medium, on feeder layers of irradiated BALB/c spleen cells. From a stochastic model, the chance that given cultures would be monoclonal was calculated as

$$\frac{1}{3(e^{1/3} - 1)} = 0.84$$

Each antibody-producing culture was cloned by a second subculture at limiting dilution to ensure stability of antibody production as well as the clonal nature of the cells synthesizing antibody. After the second cloning, the probability of monoclonality was estimated as $1 - (1 - 0.84)^2 = 0.975$.

Ascites tumors. Female BALB/c mice, 6–8 weeks old, were primed by ip injection of 0.3 ml of the immunosuppressive agent, Pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Company, Milwaukee, Wisc.). One week later, 10^6 hybridoma cells were injected ip into each mouse. After 1–2 weeks, ascites fluid from anesthetized mice was collected through a sterile 19-gauge needle. Cells were removed by centrifugation, and supernatant fluids were enriched in antibody by fractionation with ammonium sulfate (50% saturation). Fractionated immunoglobulins were dialyzed against 50 mM Tris (pH 7.4) and stored at 4° in the presence of 0.02% NaN_3.

Isotyping. Immunoglobulins were characterized with the aid of an enzyme-linked immunoassay kit (Zymed Laboratories, San Francisco, Calif.). Immunoplates (GIBCO) were coated with 0.1–0.2 μg of purified AChE or BuChE. To these plates were applied (a) conditioned media or ascites fluid, (b) isotype-specific rabbit antimouse immunoglobulin, and (c) β-galactosidase-conjugated goat antirabbit Ig. *O*-Nitrophenyl-β-D-galactopyranoside was then added, and color development was scored by a "blinded" observer.

Gel electrophoresis. Immunoglobulins were subjected to 2-dimensional gel electrophoresis (horizontal electrofocusing followed by vertical electrophoresis in the presence of sodium dodecyl sulfate), and were silver-stained according to the method of Tracy *et al.* (17).

Statistics. The standard error of the mean was used as the measure of variation. Least-squares nonlinear regression analysis of binding data was carried out on a Hewlett-Packard Model 85 microcomputer (Hewlett-Packard, Corvallis, Ore.) with the aid of a program written by Peck and Barrett (18).

RESULTS

Evidence for monoclonality. Two mice immunized against AChE and two immunized against BuChE were used for spleen cell-myeloma fusions. When conditioned media from primary hybridoma cultures were screened, graded binding of AChE or BuChE was observed. "Positive cultures" (those secreting sufficient antibody to bind all added antigen) were subcultured at limiting dilution. Most of the resulting subcultures failed to secrete any detectable antibody to AChE or BuChE, but a few were again positive (the incidence of positive subcultures ranged from 1% to 20% in different experiments). All daughter cultures derived from these positive subcultures via a second limiting dilution were found to secrete specific antibody. These patterns of immunoreactivity are consistent with the selection of individual clones from a mixed culture.

Although many antibody-secreting clones were finally identified, the number of different cell lines in each primary hybridoma culture was probably small. Several of our clones could therefore have stemmed from the same cell. Accordingly, we selected only a few antibodies for analysis in detail. Immunoglobulins from eight clones were subjected to isotype analysis. Each protein proved to be either IgG_1 or IgG_{2b} and was found to possess a *kappa* light chain. Two-dimensional gel electrophoresis indicated that each of these immunoglobulins consisted of a restricted number of charge-isomers of single-sized heavy and light chains (Fig. 1). Guided by these immu-

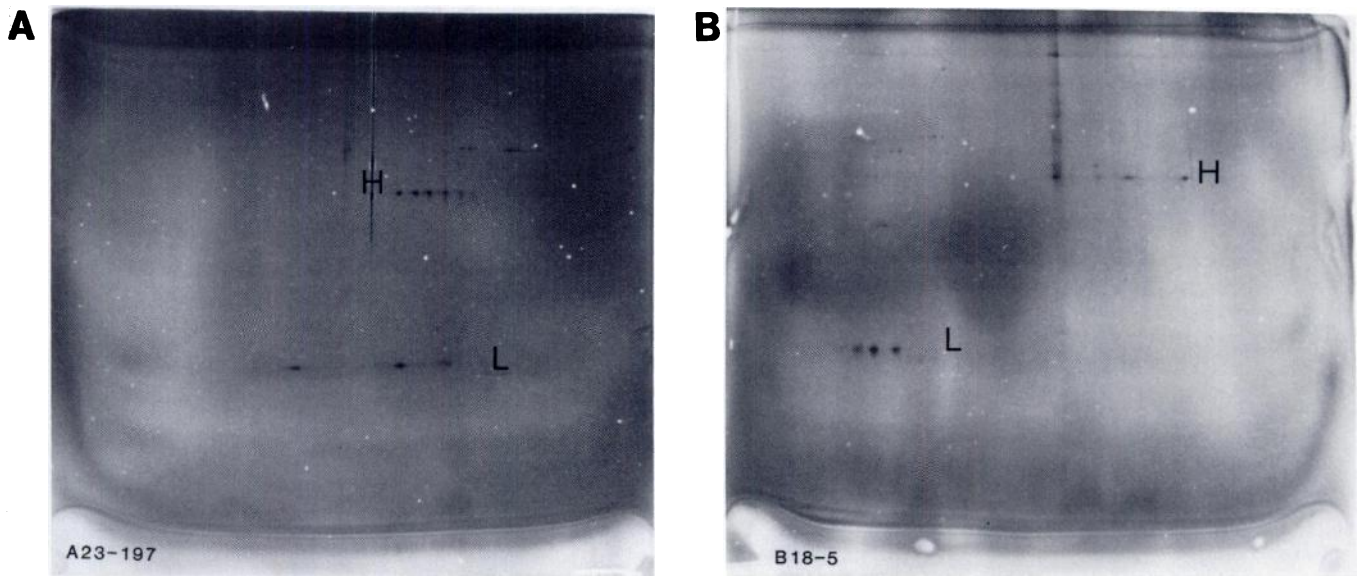

FIG. 1. *Two-dimensional gel electrophoresis of immunoglobulins*
A, Antibody A_1 23–197 to human erythrocyte AChE; B, antibody B_2 18–5 to human plasma BuChE. Gels were loaded with 5-μg portions of the immunoglobulin fractions of ascites fluid. The central streaks are staining artifacts. A few charge isomers of individual heavy chains (*H*) are visible in the *upper right* quadrants; charge isomers of individual light chains (*L*) are in the *lower portion* of the gels. These patterns, which were typical of the antibodies from the expanded cell lines, are consistent with a monoclonal nature.

nochemical characteristics, we chose to expand as ascites tumors three clones secreting antibodies to AChE and two secreting antibodies to BuChE.

Avidity. In order to assess their avidity for antigen, monoclonal antibodies from the expanded clones were tested in varying dilutions. As shown in Figs. 2 and 3, nanogram quantities of the IgG fractions were able to bind measurable amounts of antigen. If one assumes that binding in the solid-phase assay follows mass-action laws, with one epitope per enzyme monomer and two recognition sites per IgG molecule, one can calculate an apparent dissociation constant for the antigen-IgG complex. Since the concentration of antigen was low (~0.08 nM), binding obeyed the equation

$$b = \frac{2[Ab]}{2[Ab] + K_d}$$

where b is the *fraction of epitope* bound, $[Ab]$ is the molar concentration of antibody, and K_d is the dissociation constant. However, the attachment of even one antibody probably sufficed to bind a multimeric cholinesterase to the rabbit antimouse Ig in the solid phase (see Methods). Therefore, the fraction of enzyme bound was

$$B = 1 - (1 - b)^n$$

where n is the number of epitopes per molecule.

Least squares, nonlinear regression analyses of the dilution curves in Fig. 2 gave excellent fits to this equation with $n = 2$, as would be expected of dimeric AChE with one epitope per catalytic subunit. The apparent K_d values calculated by computer were 1.0 ± 0.1 nM for the A_1 23–197 antibody, 2.9 ± 0.3 nM for the A_1 63–63 antibody, and 1.8 ± 0.4 nM for the A_2 45–16 antibody (not shown in Fig. 2). The true K_d values could have been somewhat lower since the immunoglobulin fractions may have contained small quantities of transferrin and albumin in addition to IgG.

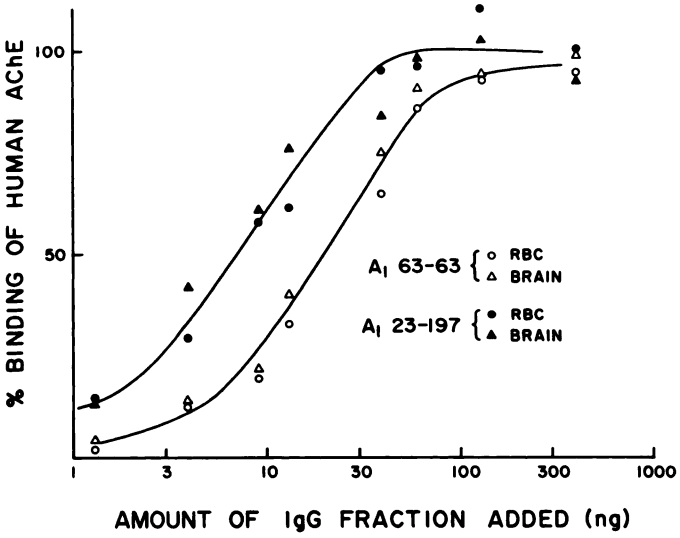

FIG. 2. *Dilution curves of anti-AChE monoclonal antibodies*
All experiments were performed with 7 milliunits of AChE extracted from red blood cells (*RBC*) or from human caudate nucleus (obtained at autopsy approximately 6 hr post mortem). The standard screening assay procedure was followed (see Methods) with a reaction volume of 0.25 ml and an incubation for 1 hr at 37°. AChE activity was measured in the bound phase and expressed as a percentage of the activity added. The *solid lines* were fitted by eye. Apparent dissociation constants were calculated by means of an iterative least-squares nonlinear regression fit to the binding equation given in the text.

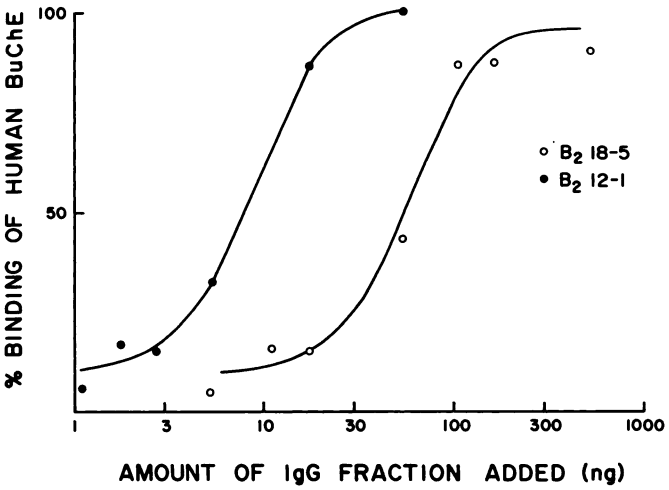

FIG. 3. *Dilution curves for anti-BuChE monoclonal antibodies*
All experiments were performed with 7 milliunits of BuChE from normal human plasma. The *solid lines* were fitted by eye, and apparent dissociation constants were calculated as described in the legend to Fig. 2.

A similar experiment carried out with AChE extracted from human caudate nucleus in place of red cell enzyme gave essentially the same results. As evident from Fig. 2, the tested antibodies readily bound the AChE of brain. Allowing for the fact that brain-AChE is a tetramer with, presumably, four epitopes per molecule, the K_d values calculated for the antibody complexes were not significantly different from those for the complexes with AChE from the red blood cell. It is therefore likely that the AChEs of brain and blood are similar in regard to the epitopes recognized by our monoclonal antibodies.

A dilution curve was constructed to test the binding of human serum BuChE by the IgG fractions of ascites fluid from clones B_2 18–5 and B_2 12–1. The results (Fig. 3) resembled those obtained with the monoclonal antibodies against AChE. Apparent dissociation constants were 2.2 ± 0.3 nM for the B_2 12–1 antibody and 13 ± 2nM for the B_2 18–5 antibody.

We also compared the reactivity of these antibodies against BuChE from brain and plasma. Unfortunately, our sample of caudate nucleus contained much more AChE (about 20 units/g) than BuChE (about 0.2 unit/g). Therefore we could not be sure of eliminating cross-contamination in the assays, even with the aid of selective inhibitors. This uncertainty made it difficult to determine the exact percentage of brain BuChE bound. Nevertheless, the results were consistent with the view that brain and serum BuChE are immunologically equivalent. Thus, within experimental error, half-maximal binding of each antigen required the same amount of antibody (30–60 ng of B_2 18–5 or 8–12 ng of B_2 12–1).

Another point of interest was whether the inherited variants of human serum BuChE would be immunologically distinguishable. We compared the reactivity of antibodies from our expanded clones with normal BuChE (dibucaine number, 80) and homozygous atypical enzyme

(dibucaine number, 25). As expected, the antibody dilution curves were similar. Half-binding of both normal and atypical BuChE required the same amount of IgG (70 ng of B_2 18–5 or 12 ng of B_2 12–1).

Cross-reactivity between types of cholinesterase. Culture media from all of the cell lines secreting antibodies against AChE were tested for cross-reactivity with BuChE, and vice versa. Even though these samples were tested without dilution, no specific binding of the "inappropriate" antigen was observed. We concluded that the two types of cholinesterase are highly distinct immunologically.

Recognition of cholinesterases from different species. Other experiments were carried out to compare the reactivity of the monoclonal immunoglobulins with the corresponding antigens from several animal species. Preliminary results showed that none of the antibodies had measurable affinity for AChE or BuChE from rat. For further comparisons, cholinesterase activity in the binding assays was standardized (7 milliunits of AChE, 14 milliunits of BuChE) to ensure roughly equivalent amounts of antigen. Even allowing for variations in homospecific activity, antigen concentrations were expected to be well below the K_d values for human enzyme. Clones secreting antibodies to BuChE were tested with plasma from squirrel monkey, dog, cat, horse, rabbit, guinea pig, and chicken (AChE was inactivated with BW284C51). Clones secreting antibodies to AChE were tested with red blood cell extracts from monkey, dog, burro, sheep, goat, pig, and guinea pig (BuChE was inactivated with ethopropazine). Chicken and rabbit red cells were not used, owing to their low content of AChE, but extracts of rabbit brain were tested.

In general, the antibodies were highly species-selective (Figs. 4 and 5). At least three different types of binding

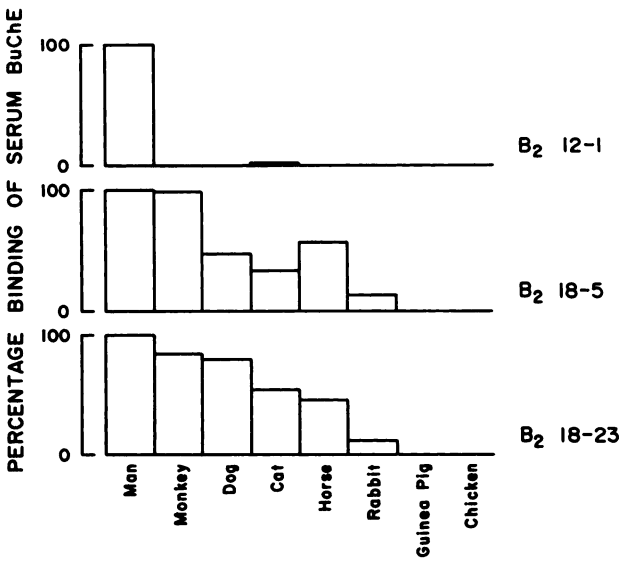

FIG. 4. *Reactivity of anti-BuChE monoclonal antibodies with plasma BuChE from various animal species*

Conditioned medium (10 μl) from the microtiter wells of cultures passed through two limiting dilutions was used as the source of antibody. A variable amount of plasma was added (5–50 μl), so that exactly 14 milliunits of BuChE were present from each species. The reaction volume was 0.25 ml in all cases.

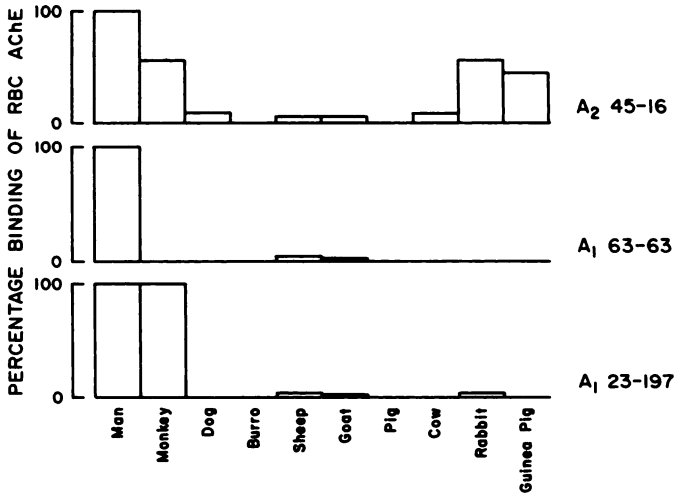

FIG. 5. *Reactivity of anti-AChE monoclonal antibodies with erythrocyte AChE from various animal species*

The experimental design was as described in the legend to Fig. 4. Erythrocyte extracts were prepared by homogenizing red cell ghosts in 10 volumes of Tris-HCl (10 mM, pH 7.4) and 1% Triton. A variable amount of extract was added (10–35 μl), so that 7 milliunits of AChE were present from each species. Rabbit AChE was from a Tris-Triton extract of brain (see text).

patterns were seen: (a) binding of human enzyme only (A_1 63–63 and B_2 12–1); (b) binding of primate enzyme only (A_1 23–197); and (c) more widespread binding, including antigen from species as remote as horse (B_2 18–5) or guinea pig (A_2 45–16). Subtle differences in binding profiles were occasionally observed. For example, the properties of clone B_2 12–6 (not shown) resembled those of B_2 18–23 (Fig. 5) except that medium from the former was weak in binding the BuChE of horse serum. One sample from a nonclonal, primary culture bound BuChE from man and dog almost exclusively. This result suggests that clones with other interesting patterns of species selectivity could be obtained.

Sedimentation effects. We also examined how the antibodies from selected clones affected the sedimentation of AChE and BuChE on linear sucrose density gradients. In the absence of antibody, BuChE centrifuged as a single symmetrical peak with a sedimentation coefficient of approximately 11 S (Fig. 6). Such behavior is characteristic of the globular tetrameric form (19). The 11 S form was totally converted to an 18 S form in samples that had been preincubated for 1 hr with the B_2 18–5 antibody in a concentration about 100 times the apparent K_d (Fig. 6). Incubation with B_2 12–1 antibody gave an even larger shift, to a sedimentation coefficient of 20 S. The shifted peaks were still symmetrical in appearance, and there was no evidence of intermediate peaks or of residual BuChE with properties of the native enzyme.

Similar experiments were performed with AChE and its antibodies. In a control gradient (Fig. 7), AChE activity from crude extracts of human red blood cells centrifuged as a symmetrical peak with a sedimentation coefficient of 6.5 S. This behavior is characteristic of the globular dimeric form of the enzyme (20). The antibody-treated AChE sedimented much faster than normal: most of it appeared as a 12.5 S peak, although about 10%

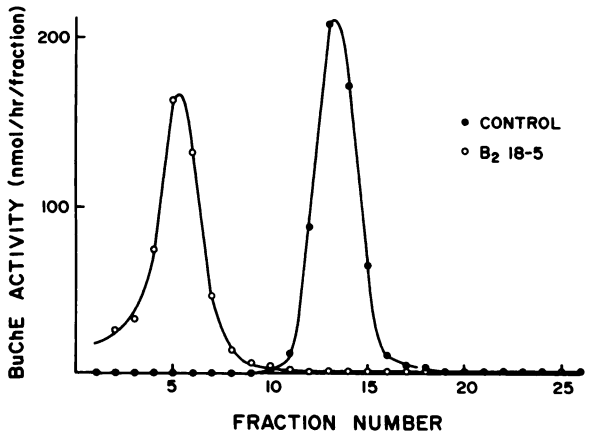

FIG. 6. *Effect of anti-BuChE monoclonal antibody on sedimentation of BuChE*

Approximately 5 μg of IgG were incubated for 1 hr at 37° with 14 milliunits of partially purified human plasma BuChE, in 50 mM sodium phosphate (pH 7.4) (total volume, 100 μl). The entire reaction mixture was layered on a 5-ml linear sucrose density gradient (5–20%) containing 150 mM NaCl and 50 mM phosphate buffer (pH 7.4). The gradients (calibrated with catalase, 11.2 S) were centrifuged for 16 hr at 35,500 rpm (L2-65B centrifuge, SW 50.1 rotor). The control enzyme behaved as a BuChE tetramer. The effect induced by the B_2 18–5 antibody is shown as a typical result.

sometimes sedimented as an 8 S peak (Fig. 7). This behavior contrasted with that induced by polyclonal mixtures (e.g., conditioned medium from primary cultures), which gave rise to broad, asymmetrical peaks of enzyme activity sedimenting near the bottom of the gradient tubes.

DISCUSSION

Monoclonality. The antibodies from all of the expanded cell lines have properties that point unequivocally to a monoclonal nature. These properties include (a) possession of one-sized heavy chain and one-sized light chain: (b) induction of smooth symmetrical shifts in sedimentation of the target antigen; (c) stability of key characteristics on subcloning (i.e., selectivity and avidity for antigen). Arguing from the latter criterion and from statistical considerations, it is likely that the majority of the other antibody-secreting cell lines were also monoclonal.

The accelerated sedimentation of the cholinesterases in the presence of specific antibodies is consistent with the observations of Fambrough *et al.* (1). The sharp, symmetrical peaks showed that single types of complexes predominated, as would be expected of monoclonal antibodies binding to single epitopes. The nature of the complexes cannot be determined without additional information about their shapes or diffusion coefficients. However, the most likely complex with plasma BuChE would be an entity of approximately 940,000 M_r, consisting of four IgG molecules (150,000 M_r) and one enzyme tetramer 340,000 M_r). In the case of erythrocyte AChE, one might anticipate a complex of 460,000 M_r, consisting of two IgG molecules and one enzyme dimer (160,000 M_r). The sedimentation pattern of Fig. 7 suggests that smaller complexes also formed.

Nature and number of epitopes recognized. Even though our clones were obtained from only a few parental cultures and still fewer immunized mice, there was a good deal of variation in the characteristics of the antibodies. Differences in species selectivity were especially remarkable. These differences showed clearly that each of the five expanded clones produced antibodies against a different epitope. However, it is quite possible that many of our other antibody-secreting cell lines were producing antibodies against the same epitopes. Additional experiments on the stoichiometry of binding and on the competition between these antibodies are required to determine the true number of epitopes recognized. Meanwhile, it is clear that none of our antibodies bind to the active site of AChE or BuChE, since none of them was able to inactivate either enzyme.

Applications. Relatively few of the antibodies recognized nonprimate enzyme. When they did, the binding was never complete, even though the antibodies were added in large excess. Evidently the affinity for foreign enzyme was low. Nevertheless, immunoaffinity columns based upon the A_2 45–16 antibody have proved useful for purifying the AChE of rabbit brain.[4] A new generation of monoclonal antibodies with high affinity for rabbit AChE is therefore within reach.

The more species-selective antibodies may have other applications. In particular, they could provide information about the antigenic relationships among cholinesterases of the higher primates. The species and population distributions of the epitopes recognized by antibodies such as B_2 12–1, which fails to bind even the BuChE of squirrel monkey, would be of special interest in a phylogenetic context (see ref. 21).

It is noteworthy that none of our antibodies distinguished the enzymes of blood from those of brain. This result establishes the utility of the antibodies for investigation of neural cholinesterases, even though the immunizing antigens came from plasma and red blood cells.

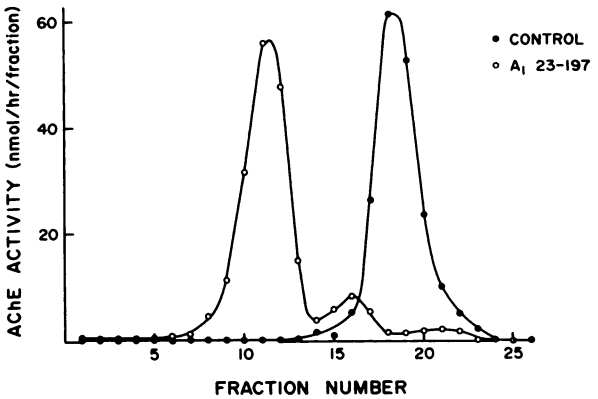

FIG. 7. *Effect of anti-AChE monoclonal antibody on sedimentation of AChE*

The experimental design was as described in the legend to Fig. 6, except that AChE was used as the antigen (7 milliunits of enzyme from the crude extract of human red blood cell ghosts). The control enzyme behaved as an AChE dimer. The shift induced by incubation with the A_1 23–197 antibody is shown as a typical result.

[4] K. P. Mintz and S. Brimijoin, unpublished results.

In themselves, however, the similar immunological profiles of the antigens from different tissues do not prove that the enzyme structures are identical. At most, these findings indicate that the epitopes recognized by our panel of antibodies are shared by the cholinesterases produced in different cells.

The hazards of stressing apparent immunological equivalence are illustrated by the failure of B_2 18–5 and B_2 12–1 antibodies to differentiate between normal plasma BuChE and homozygous atypical BuChE. There is ample evidence that the amino acid composition of the variant differs from that of the normal enzyme (22), but the alteration is almost certainly confined to the active site (23). This fact probably accounts for the undiminished reactivity of antibodies that are noninhibitory, whether they be polyclonal, as in the experiments of Eckerson *et al.* (5), or monoclonal, as in the present case.

There would be many uses for antibodies that could bind to the active sites of cholinesterases. Such antibodies might provide a convenient alternative test for BuChE variants, for example, to predict succinylcholine-induced apnea. We selected against active site-directed antibodies by measuring enzyme activity in the bound phase only, in order to avoid interference from soluble cholinesterases in the samples tested. Another way to avoid such interference would be to bind antibodies to the solid phase and rinse them before adding antigen. The active site-directed antibody could then be recognized by loss of enzyme activity from the supernatant without compensatory activity in the pellet. However, many clones would have to be screened, with a low probability of success. Alternatively, one might be able to obtain active-site directed antibodies by immunization with appropriate enzyme fragments (24).

Relationship between AChE and BuChE. Because absolute specificity will facilitate experiments on regulation and disposition, it is fortunate that the monoclonal antibodies show no cross-reactivity between AChE and BuChE. Yet in a broader perspective this lack of cross-reactivity is disturbing. Current thinking holds that AChE and BuChE are distinct proteins encoded by distinct genes (25), but a closer relationship has been suggested (26), and similarities in substrate preference and mechanism of action argue for a common evolutionary origin (27). It will be surprising if the amino acid sequences do not ultimately show some degree of homology. Meanwhile, attention might be directed to the possibility that the sugar residues of the BuChE glycoprotein, which is 25% carbohydrate by weight (28), mask antigenic determinants that this enzyme shares with AChE.

Conclusion. Additional characterization of our monoclonal antibodies is warranted. It needs to be determined whether any of them can discriminate among the different molecular forms or the electrophoretically defined isozymes of AChE and BuChE. It is also important to learn whether any of the antibodies will be useful as histochemical markers, especially in neural tissue. Studies along these lines are in progress, as are attempts to raise antibodies with further useful properties. At present one can look forward to performing many interesting experiments on the biology and pharmacology of the cholinesterases with the aid of these new reagents.

ACKNOWLEDGMENTS

We thank Dr. C. G. Fathman for his gift of FO myeloma cells, Dr. Vanda Lennon for valuable advice and material aid in the early stages of this work, Dr. William Burlingham for assistance with the animal immunization, and Dr. Russell Tracy for performing the 2-dimensional gel electrophoresis on our immunoglobulin samples. We are indebted to Dr. Haruo Okazaki for an autopsy specimen of human caudate nucleus and to Drs. James Harper and Mary Burrit for an authenticated blood sample from a patient with homozygous atypical BuChE. Luanne Wussow's expert assistance in preparing the manuscript and charts is gratefully acknowledged.

REFERENCES

1. Fambrough, D. M., A. G. Engel, and T. L. Rosenberry. Acetylcholinesterase of human erythrocytes and neuromuscular junctions: homologies revealed by monoclonal antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **79**:1078–1082 (1982).
2. Adamson, E. D. Acetylcholinesterases in mouse brain, erythrocytes and muscle. *J. Neurochem.* **28**:605–615 (1977).
3. Vigny, M., V. Gisiger, and J. Massoulie. "Nonspecific" cholinesterase and acetylcholinesterase in rat tissues: molecular forms, structural and catalytic properties, and significance of the two enzyme systems. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2588–2592 (1978).
4. Koelle, G. B., K. K. Richard, and G. A. Ruch. Interrelationships between ganglionic acetylcholinesterase and nonspecific cholinesterase of the cat and rat. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6012–6016 (1979).
5. Eckerson, H. W., A. Oseroff, O. Lockridge, and B. LaDu. Immunological comparison of the usual and atypical human serum cholinesterase phenotypes. *Biochem. Genet.* **21**:93–108 (1983).
6. Brimijoin, S., K. P. Mintz, and F. G. Prendergast. Dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide: a potent and selective fluorescent inhibitor of butyrylcholinesterase. *Biochem. Pharmacol.* **32**:699–706 (1983).
7. Lockridge, O., and B. N. LaDu. Loss of the interchain disulfide peptide and dissociation of the tetramer following limited proteolysis of native human serum cholinesterase. *J. Biol. Chem.* **257**:12012–12018 (1982).
8. Pazmino, P. A., and R. M. Weinshilboum. Human erythrocyte phenol-*O*-methyltransferase: radiochemical microassay and biochemical properties. *Clin. Chim. Acta* **89**:317–329 (1978).
9. Traylor, P., and S. J. Singer. The preparation and properties of some tritiated diazonium salts and related compounds. *Biochemistry* **6**:881–87 (1967).
10. Ott, P., B. Jenny, and U. Brodbeck. Multiple molecular forms of purified human erythrocyte acetylcholinesterase. *Eur. J. Biochem.* **57**:469–480 (1975).
11. Ellman, G. L., K. D. Courtney, V. Andres, Jr., and R. M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**:88–95 (1961).
12. Johnson, C. D., and R. L. Russell. A rapid simple radiometric assay for cholinesterase, suitable for multiple determinations. *Anal. Biochem.* **64**:229–238 (1975).
13. Kalow, W., and K. Genest. A method for the detection of atypical forms of human serum cholinesterase: determination of Dibucaine numbers. *Can. J. Biochem. Physiol.* **35**:339–346 (1957).
14. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **92**:248–254 (1976).
15. Fazekas de St. Groth, S., and D. Scheidegger. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods* **35**:1–21 (1980).
16. Köhler, G., and C. Milstein. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.)* **256**:495–497 (1975).
17. Tracy, R. P., R. M. Currie, R. A. Kyle, and D. S. Young. Two-dimensional gel electrophoresis of serum specimens from patients with monoclonal gammopathies. *Clin. Chem.* **28**:900–907 (1982).
18. Peck, C. C., and B. B. Barrett. Nonlinear least squares regression programs for microcomputers. *J. Pharmacokinet. Biopharm.* **5**:537–541 (1979).
19. Lockridge, O., H. W. Eckerson, and B. N. LaDu. Interchain disulfide bonds and subunit organization in human serum cholinesterase. *J. Biol. Chem.* **254**:8324–8330 (1979).
20. Niday, E., C. S. Wang, and P. Alaupovic. Studies on the characterization of human erythrocyte acetylcholinesterase and its interaction with antibodies. *Biochim. Biophys. Acta* **459**:180–193 (1977).
21. Dunnette, J. H., and R. M. Weinshilboum. Serum dopamine-β-hydroxylase activity in non-human primates: phylogenetic and genetic implications. *Comp. Biochem. Physiol.* **75**:85–91 (1983).
22. Muensch, H., A. Yoshida, K. Altland, W. Jensen, and H. W. Goedde. Structural difference at the active site of dibucaine resistant variant of human plasma cholinesterase. *Am. J. Hum. Genet.* **30**:302–307 (1978).
23. Lockridge, O., and B. N. LaDu. Comparison of typical and usual human serum cholinesterase. *J. Biol. Chem.* **253**:361–366 (1978).
24. Sutcliffe, J. G., T. M. Shinnick, N. Green, and R. A. Lerner. Antibodies that react with predetermined sites on proteins. *Science (Wash. D. C.)* **219**:660–666 (1983).
25. Massoulie, J., and S. Bon. The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Annu. Rev. Neurosci.* **5**:57–106 (1982).
26. Koelle, W. A., G B. Koelle, and E. G. Smyrl. Effects of persistent selective

suppression of ganglionic butyrylcholinesterase on steady state and regenerating levels of acetylcholinesterase: implications regarding function of butyrylcholinesterase and regulation of protein synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **73**:2396–2398 (1976).

27. Augustinsson, K.-B. The evolution of esterases in vertebrates, in *Homologous Enzymes and Biochemical Evolution* (N. van Thoai and J. Roche, eds.). Gordon and Breach, New York. 299–311 (1968).

28. Muensch, H., H. W. Goedde, and A. Yoshida. Human serum cholinesterase subunits and number of active sites of the major component. *Eur. J. Biochem.* **70**:217–223 (1976).

Send reprint requests to: Dr. Stephen Brimijoin, Department of Pharmacology, Mayo Clinic, Rochester, Minn. 55905.