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FOR THE COMMANDER:

CORNELIUS R. FAY III
Lieutenant Colonel, MS
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Biological Synthesis of a Protein Analogue of Acetylcholinesterase: Monoclonal Anti-Idiotype Antibody Analogue of the Esteratic Site

Annual Report

J. Thomas August

July 10, 1984

Supported by

U. S. Army Medical Research and Development Command
Fort Detrick, Frederick, MD 21701-5012

Contract No. DAMD17-83-C-3135

Johns Hopkins University School of Medicine
725 N. Wolfe Street
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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

acetylcholinesterase; monoclonal antibodies; active site; diisopropylfluorophosphate

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The goal of this research during the first year of the contract was to develop a method for the purification of human erythrocyte acetylcholinesterase and to initiate the preparation and analysis of monoclonal antibodies. This was accomplished by the preparation of red blood cell membrane ghosts, enzyme solubilization with a non-ionic detergent, and enzyme purification by monoclonal antibody affinity chromatography. Sixty ml of packed red blood cells yielded a final fraction of 750 ug, approximately

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20. ABSTRACT (continued)

20,000-fold purified. The purified fraction contained a single protein of about 75,000 daltons that was labeled with ^{3}H-diisopropylfluorophosphate and gave a single peak during high-performance liquid chromatography on a TSK-SW3000 silica-supported hydrophilic polymer column. Mice were injected with the purified enzyme for the preparation of monoclonal antibodies, and assay systems to screen for anti-acetylcholinesterase, anti-active site, and anti-idiotype monoclonal antibodies have been developed.

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SUMMARY

The goal of this research during the first year of the contract was to develop a method for the purification of human erythrocyte acetylcholinesterase and to initiate the preparation and analysis of monoclonal antibodies. This was accomplished by the preparation of red blood cell membrane ghosts, enzyme solubilization with a non-ionic detergent, and enzyme purification by monoclonal antibody affinity chromatography. Sixty ml of packed red blood cells yielded a final fraction of 750 ug, approximately 20,000-fold purified. The purified fraction contained a single protein of about 75,000 daltons that was labeled with ^{3}H-diisopropylfluorophosphate and gave a single peak during high-performance liquid chromatography on a TSK-SW3000 silica-supported hydrophilic polymer column. Mice were injected with the purified enzyme for the preparation of monoclonal antibodies, and assay systems to screen for anti-acetylcholinesterase, anti-active site, and anti-idiotype monoclonal antibodies have been developed.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Problem:

The goals of this research are to biochemically and immunochemically characterize the molecular structure of the active site of human acetylcholinesterase (AChE). This information should lead to the capability of chemically or immunologically synthesizing analogues of the esteratic site. The first step or problem in this program is the purification of the enzyme from human erythrocytes in preparative quantities. Our research during the first year has been directed primarily at this purification procedure.

Background:

AChE is associated chiefly with cells involved in cholinergic synaptic transmission and is also found in a few non-neuronal cells like erythrocytes. The enzyme exists in a variety of aggregation states with a monomer unit of about 75,000 Mr (1,2,3). Two general classes have been distinguished: asymmetric forms, which contain catalytic units covalently linked to a collagenous tail, and globular forms, which are assemblies of catalytic units devoid of the collagenous component (4). In the plasma membrane of the erythrocyte, the enzyme exists as a globular dimer of 180,000 Mr. In the central nervous system, a membrane-bound tetrameric form predominates. In skeletal muscle, globular forms (nomomeric, dimeric, and tetrameric) occur as both soluble and tightly membrane-bound molecules, and some globular forms are secreted. In addition, skeletal muscle contains complex asymmetric forms in which up to three tetramers are covalently linked to a collagenous tail. The occurrence of these asymmetric forms often correlates with innervation, and in most species asymmetric forms are located primarily in the area of innervation and constitute a major fraction of the esterase at neuromuscular junctions. This localization appears to be mediated by the interaction of the collagen-like tail structure with the extracellular basement membrane matrix (5,6,7).

All of the molecular species of AChE appear to have the same enzymatic sites. Fambrough, et al. (8), prepared five monoclonal antibodies that bound to purified human erythrocyte AChE, each reacting with different antigenic sites on the AChE molecule. All of these antibodies cross-reacted with human and monkey neuromuscular junctions. It was concluded that a high degree of homology exists between the AChE of erythrocytes and neuromuscular junctions.

The most available source of human AChE is the erythrocyte. AChE is an integral part of the red blood cell membrane (9,10) and is one of the most active of known catalytic agents, with a turnover number of about 6×10^5 moles/min/active site. Purification of human erythrocyte AChE has been reported by several workers (11,12,13). For example, Ott et al. (12) solubilized the human erythrocyte enzyme with Triton X-100 and used acridinium affinity chromatography to obtain a fraction with 3800 IU per mg protein specific activity. The purified enzyme appeared as a single polypeptide of 80,000 Mr on SDS-gel electrophoresis under reducing condition. Rosenberry and Scoggin (13) used a similar procedure for a large-scale purification yielding about 5 mg of enzyme from 10 liters of erythrocytes.

Herein we report the purification of human erythrocyte AChE by monoclonal antibody affinity chromatography.

Enzyme Preparation:

AChE Extraction From Human Erythrocytes - Packed human erythrocytes from 10- to 20-day-old collections of blood obtained from the central blood bank of the Johns Hopkins Hospital were used as the starting material for the purification of AChE. Red cells were washed three times with equal volumes of isotonic buffer (5 mM sodium phosphate, pH 7, 0.9 percent NaCl). Cells were lysed in 40 volumes of hypotonic buffer (5 mM sodium phosphate, pH 7). Ghosts were prepared according to the procedure of Rosenberry et al. (14), using a Millipore pellicon high-volume molecular filtration apparatus. A single modification of the procedure was the use of a Durapore filter (0.45 μm) in place of the PTKH 100,000 filter. Triton X-100, 1 percent final concentration, was added to solubilize the membrane proteins, and the suspension was centrifuged at 100,000 x g for 60 min. The 100,000 x g supernatant was diluted 1:5 with 5 mM sodium phosphate buffer, pH 7, and passed through a 30 ml Sepharose-4B column to remove any non-specifically absorbed proteins. The soluble enzyme was then purified by immuno-affinity chromatography as described below.

Monoclonal Antibody Affinity Column Purification of AChE - Hybridoma cells secreting monoclonal antibody AE-4, specific for human AChE, were obtained from Dr. D. Fambrough, Carnegie Institute (8). Large amounts of immunoglobulin were obtained by growing cells as ascites tumors. Mice were injected intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane), and 1 week later were injected with 1 to 2 x 10^7 hybridoma cells. Ascites fluids were inactivated at 56°C for 30 min, and clarified by centrifugation at 100,000 x g for 1 hr.

Purification of the AE-4 antibody was carried out by ammonium sulfate precipitation and ion exchange chromatography (15,16). All procedures were carried out at 1 to 4°C. Solid ammonium sulfate was added to ascites fluid at a final concentration of 45 percent. The precipitated protein was collected by centrifugation at 20,000 x g for 20 min, suspended in 10 mM sodium phosphate buffer, pH 8.0, and dialyzed for 36 h in 2,000 ml of the same buffer with six changes of dialysate. After centrifugation at 100,000 x g for 1 hr, the protein solution was applied to a 2.5 x 9-cm DEAE-cellulose (Whatman DE-23) column equilibrated in 10 mM sodium phosphate buffer, pH 8.0. The unabsorbed material contained 5 to 10 mg of immunoglobulin per ml of ascites; the immunoglobulin was shown to be greater than 95 percent pure by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis (SDS-PAGE) of reduced and non-reduced material.

The purified monoclonal antibody was dialyzed against a buffer solution containing 500 mM NaCl and 100 mM sodium carbonate buffer, pH 8.3, and coupled to cyanogen bromide-activated Sepharose CL-4B (200 mg of cyanogen bromide/ml of packed beads) at a ratio of 3 mg of protein/ml of packed beads, as previously described (15,16). The protein content of the filtrates indicated that 96 percent of the antibody preparation was coupled. A 1.5 cm x 10 cm column containing approximately 30 mg of AE-4 antibody coupled to 10 ml of Sepharose CL-4B beads was treated sequentially with 50 ml of 100 mM diethylamine, pH 11.5; 50 ml of 1 M Tris-HCl, pH 7.6; and 50 ml of 5 mM Tris, pH 7.6, before the erythrocyte extract was applied.

Approximately 2300 ml of the diluted 100,000 x g supernatant was applied to the antibody column at a rate of 2 ml/min. Most of the protein in the extract was not retained by the column. After the protein sample was loaded, the column was washed with 150 ml of the column buffer, followed by 150 ml of

a borate-salt buffer, pH 8.5 (1 M NaCl, 100 mM boric acid, 15 mM sodium borate, 0.2 percent (w/v) Triton X-100) to remove components adsorbed non-specifically to the column. The column was then eluted at a rate of 0.5 ml/min with 100 ml of 100 mM diethylamine, pH 11.5, containing 0.2 percent (w/v) Triton X-100, and 20 percent glycerol. Fractions of 2 ml were collected in tubes containing 0.5 ml of neutralizing buffer (2 M Tris-HCl, pH 7.6). The antigen eluted in 32 ml as a single, sharp peak of antigenic activity corresponding to a small protein peak. Analysis by polyacrylamide gel electrophoresis of the individual fractions of the enzyme showed that the concentration of this eluted protein was proportional to enzyme activity (data not shown). The active fractions were pooled and concentrated to 1.5 ml by use of a negative pressure dialysis-concentrating apparatus (Micro-Pro Di Con; Bio-Molecular Dynamics, Beaverton, OR) in 2000 ml of a buffer solution containing 20 mM sodium phosphate, 100 mM NaCl, 0.1 percent (w/v) Triton X-100, pH 8.0, and 20 percent glycerol at 4°C.

The summary of a typical procedure starting with ten units of red blood cells is shown in Table 1. An apparent 44-fold purification and recovery of 40 percent were obtained by preparing the red cell ghost fraction. The crude hemolyzate activity assay is ambiguous, however, since the cells likely contain other contaminating esterase activities. Thus, there is probably greater recovery of activity and greater purification than is shown. Antibody affinity chromatography resulted in 500-fold purification with 30 percent recovery of enzyme activity. The final yield was 750 ug with an overall purification of at least 20,000-fold.

Enzyme Characterization:

[^{3}H]-DFP Labeling and PAGE - The purified enzyme analyzed by SDS-PAGE showed a single major polypeptide stained by the silver reduction procedure or with Coomassie brilliant blue. The apparent molecular weight was 75,000.

This polypeptide was proven to be the AChE by DFP labeling. The conditions for DFP labeling were established by treating the purified AChE (5×10^{-2} pmoles) with varying amounts of DFP (0-20 pmoles) in 0.1 M Tris-HCl pH 7.5 containing 2 percent propylene glycol in a final volume of 10 ul. After 20 min at room temperature, enzyme activity was estimated by the Ellman procedure (17).

The enzyme was almost totally inhibited (90 percent) by a 400:1 molar ratio of DFP:AChE. Based on these results, enzyme was labeled by reacting 7.5 pmoles of AChE with 3000 pmoles of ^{3}H-DFP (specific activity = 5.2 Ci/mm). After 30 min, the [^{3}H]-DFP-AChE adduct was precipitated with 10 volumes of acetone at -20°C for 15 hr. After centrifugation at 12,000 x g for 30 min, the pelleted protein was solubilized and analyzed by SDS-PAGE (Figure 1). The gel, analyzed both by silver stain and autoradiography, revealed a single radiolabeled polypeptide corresponding to the purified protein of 75,000 daltons.

High-performance Liquid Chromatography (HPLC) - The purity of the fraction was also demonstrated by size-exclusion on a TSK-SW3000 silica-supported hydrophilic polymer column. A single major protein peak detected by ultra-violet absorbance at 280 nm was present (Figure 2). This peak corresponded perfectly to the enzyme activity of the fractions and to the elution of ^{125}I-labeled AChE.

TABLE 1

Purification of human erythrocyte acetylcholinesterase[a]

Purification Step	Volume (ml)	Total Protein[b] (mg)	Total Activity[c] (ΔA/min)	Activity Recovery (%)	Specific Activity (ΔA/min/mg)	Fold Purification
HEMOLYSATE	24,000	264,000	233,000		0.9	1
RED CELL GHOSTS	540	2,100	94,000	40	44	49
100,000 x g SUPERNATANT	465	1,023	94,000	40	91	100
ANTIBODY AFFINITY	1.5	0.75	17,000	7	23,000	26,000

[a] Purification was performed as described in the text.
[b] Protein in the hemolysate, red cell ghosts and 100,000 x g supernatant preparations measured by the modified method of Lowry (18), and in the purified fraction by fluorometric assay (19).
[c] Enzyme activity was measured as described by Ellman et al. (17).

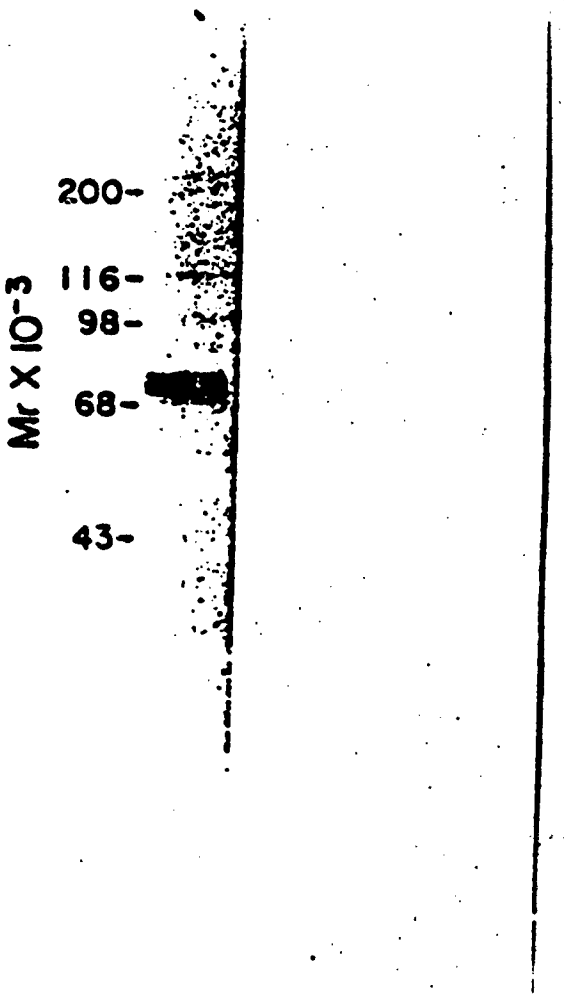

Figure 1. Purified acetylcholinesterase labeled with [^{3}H]-DFP. Purified AChE was labeled with [^{3}H]-DFP, as described in the text, treated with a buffer containing 62.5 mm tris-Cl pH 6.8, SDS 2 percent; β-mercaptoethanol 5 percent and electrophoresed on a 10 percent acrylamide gel according to the method of Laemmli (20).

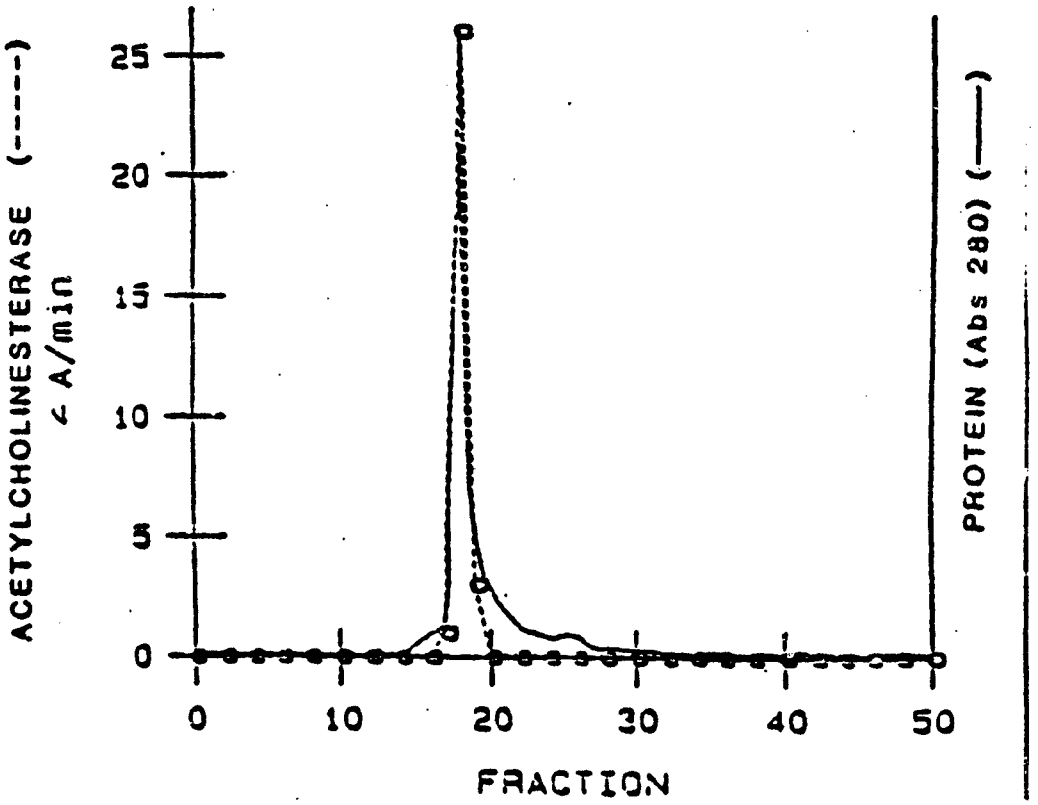

Figure 2. Size-exclusion HPLC of purified acetylcholinesterase. The concentrated affinity-purified fraction of AChE (20 µl, 10 µg) was placed in a 7.5 mm X 30 cm TSK-SW3000 column. The colum was eluted with 5 mM Tris-HCl, pH 7.5, at 0.5 ml/min. The fraction volume was 0.5 ml. The tracing is absorbance at 280 nm with a setting of 0.02 absorbance units full scale. The peak fraction corresponded exactly to enzyme attivity and to radioactivity of [^{125}I]-AChE (0.01 µg, 1.3 X 10^6 cpm), an aliquot of the purified fraction iodinated by the chloramine-T procedure and added as an internal marker.

Monoclonal Antibodies:

Research on the major procedures and assay systems for production of monoclonal antibodies and for detection of anti-active site and anti-idiotype monoclonal antibodies had been initiated during the first year of the contract.

Immunization and Hybridoma Production - Twelve week old female Balb/c mice were injected with human erythrocyte AChE purified as described above. A rapid immunization schedule was adapted for use with the purified enzyme: seven total injections were given at intervals of three days. Ten micrograms protein in complete Freund's adjuvant were given initially, followed by an identical dose in incomplete Freund's in subsequent booster injections. The initial subcutaneous injections included footpad, inguinal, and axillary sites; secondary injections were in the inguinal region (21).

Two days after the last injection the lymph nodes and spleen were removed; 2.5×10^7 lymphocytes and 1.5×10^8 splenocytes were fused with equal numbers of P3X63-Ag8-653 myeloma cells in the presence of 50 percent polyethylene glycol 1000 for two min. Cells were diluted with 5 ml Dulbecco's modified Eagle's medium over one min and then transferred to the same medium supplemented with 20 percent fetal calf serum, 10 percent NCTC 135, Hepes (10 mM), insulin (0.20 U/ml), pyruvate (50 ng/ml), oxalacetic acid (0.15 mg/ml), hypoxanthine (10^{-4} M), aminopterin (4×10^{-7} M), thymidine (1.6×10^{-5} M) (HAT medium). Lymph node cells were distributed in 0.5 ml into 48 wells and spleen cells into 72 wells of Linbro 24 mm well plates, in which 2.5×10^4 Balb/c macrophages in 1 ml HAT medium had previously been placed. Replacement of 0.5 ml HAT medium was made on day 7 after fusion.

Screening for anti-acetylcholinesterase, anti-active site, and anti-idiotype antibodies - Wells are screened at two weeks after fusion by solid phase radio-immunoassay techniques. Anti-AChE antibodies are assayed in the following manner. Culture supernatant, 50 µl, is dispensed into the wells of Dynatech polystyrene 96 well microtiter plates containing 5 µg of goat anti-mouse IgG antibody absorbed to the plate and incubated with 5 percent bovine serum albumin to block excess charge. The wells are washed with 1 percent bovine serum albumin after which 1 ng of AChE labeled with ^{125}I (10^9 cpm per µg) is dispensed into the wells and incubated for 30 minutes at 4°C. Excess AChE is removed by washing and the labeled AChE bound to specific antibody is taken up in 0.1 ml of 2 M NaOH after incubation at 70°C for 15 min. Radioactivity was measured with a gamma counter.

Antibodies detected by the enzyme binding assay are all screened for possible recognition of the enzyme active site by analyzing the effect of the antibody on enzyme activity. Red blood cell ghosts (40 µg protein), containing approximately 16 ng of AChE, are immobilized onto the bottom of microtiter plates and incubated with antibody for 1 h at 40°C. After removal of unbound antibody, AChE activity is measured by the Ellman procedure (17). With this procedure, the Fambrough antibody 2A8D-6 (8) appears to inhibit enzyme activity (Fig. 3). A search for additional antibodies and further analysis of the 2A8D-6 antibody will be continued in the second year of the contract.

A variation of the same assay system is used to detect anti-idiotype antibodies directed against the combining site of anti-acetylcholinesterase antibodies (22). Supernatants or purified antibodies are plated as before on goat anti-mouse IgG-covered wells, at or near saturation of the goat antibody combining sites. This is followed by incubation with nonspecific IgG to block any further available sites. The candidate anti-idiotype immunoglobulin, 50 ul, is then plated, followed by ^{125}I-AChE. Significant inhibition of radiolabeled ligand binding suggests the presence of a relevant anti-idiotype antibody.

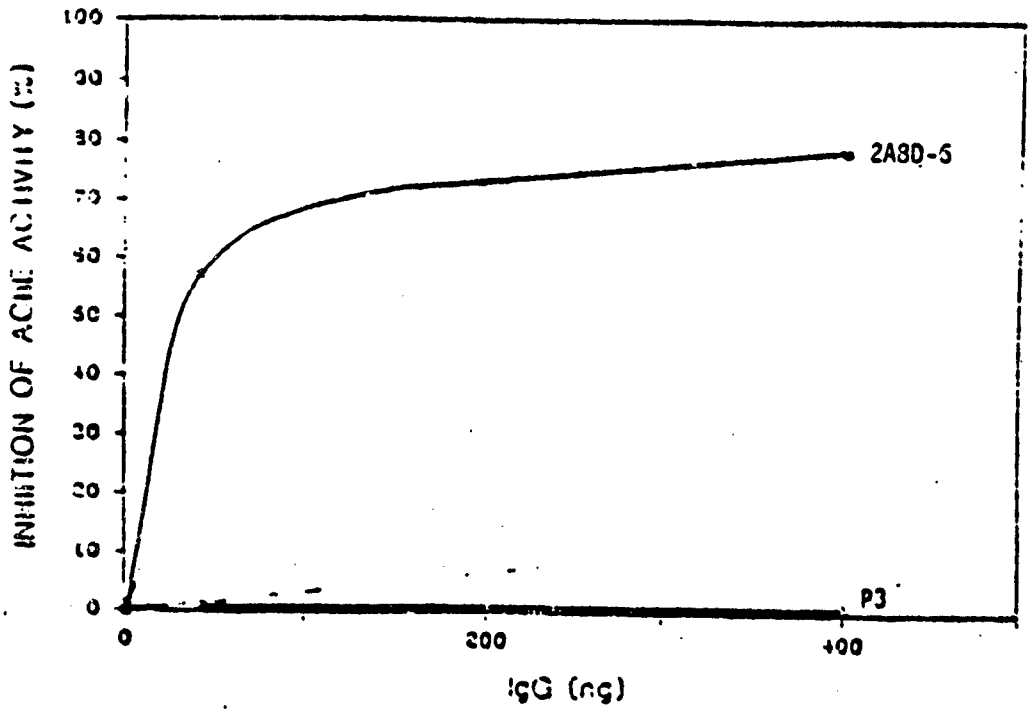

Figure 3. Monoclonal antibody inhibition of acetylcholinesterase activity. Red blood cell ghosts (40 ug protein), containing approximately 16 ng of AChE, were immobilized onto the bottom of microtiter plates. Purified monoclonal antibodies in varying concentrations were incubated with the membrane protein for 1 hr at 4°C. After removal of unbound antibody, AChE activity was determined by the Ellman procedure (17).

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FOR THE COMMANDER:

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