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19. Abstract

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BIOLOGICAL SYNTHESIS OF A PROTEIN ANALOGUE OF ACETYLCHOLINESTERASE: MONOCLONAL ANTI-IDIOTYPE ANTIBODY ANALOGUE OF THE ESTERATIC SITE

FINAL REPORT

J. THOMAS AUGUST

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ABSTRACT

This study was designed to characterize the molecular structure of the active site of human acetylcholinesterase (AChE). Human erythrocyte acetylcholinesterase was purified to >98% homogeneity by monoclonal antibody affinity chromatography and size-exclusion HPLC, and amino acid sequence data for >100 residues were obtained from tryptic, chymotryptic, and V-8 peptide fragments of the enzyme. These sequence data were used to synthesize oligonucleotide probes with which to screen four human cDNA libraries for the gene encoding human acetylcholinesterase. Putative positive clones were detected in one of these libraries, a muscle cDNA library in pBR322. In addition, a number of monoclonal antibodies that bind to acetylcholinesterase were produced; however, only one of these antibodies, C1B7, inhibited the activity of the enzyme. C1B7 was extensively characterized and compared to the inhibitory antibody AE-2, produced by D. Fambrough et al. (Proc. Natl. Acad. Sci. U.S.A. 79, 1078, 1982). The two monoclonal antibodies were found to bind to two distinct sites on the enzyme, sites that differ not only from the active site but also from the peripheral binding sites on the enzyme for a number of anticholinergic drugs, including DFP, propidium, edrophonium, and neostigmine. A kinetic analysis of inhibition by C1B7 and AE-2 suggested that their inhibition of acetylcholinesterase is non-competitive and allosteric. Thus, these two antibodies were not suitable for studies of the active site of AChE; nevertheless, they identify two newly described sites on the enzyme that represent potential points of enzyme regulation.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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 Commission of Life Sciences, National Research Council (NIH Publication No. 86-23, Revised 1985).

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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RESEARCH PROBLEM

The goal of this research was to characterize biochemically and immunochemically the molecular structure of the active site of human acetylcholinesterase (AChE). The information gleaned from these studies can then be applied to the design and synthesis of chemically or immunologically derived analogues of the esteratic site.

The biochemical approach to this problem involved (a) isolation of preparative quantities of highly purified AChE from human erythrocytes; (b) determination of the amino acid sequence of peptide fragments derived from the intact AChE molecule; (c) generation of oligonucleotide probes based on these sequence data for use in screening cDNA libraries for the AChE gene; and (d) molecular cloning and expression of the gene encoding AChE.

The proposed immunochemical approach to characterization of the active site entailed (a) production of monoclonal antibodies capable of binding to human AChE and inhibiting its activity; (b) use of inhibitory monoclonal antibodies to characterize the binding and catalytic activities of the enzyme molecule and, ultimately, to generate anti-idiotypic analogues of the active site.

BACKGROUND

Acetylcholinesterase (AChE), the enzyme responsible for the rapid hydrolysis of acetylcholine, is an important modulator of neuronal function (1-5). In addition to its primary association with cholinergic neurons, AChE is also found in a few non-neuronal cells such as erythrocytes. The enzyme exists in multiple molecular forms (3), with a monomer unit of ~75,000 daltons (6-8).

A. PURIFICATION AND SEQUENCING OF HUMAN ERYTHROCYTE AChE

The erythrocyte is the most available source of human AChE. In this cell the enzyme occurs in dimeric form as an integral component of the plasma membrane (9-12). Purification of human erythrocyte AChE has been reported by several workers (11,13,14). Ott *et al*. (14) solubilized the enzyme with Triton X-100 and used acridinium affinity chromatography to obtain a single polypeptide of M_r 80,000. Rosenberry and Scoggin (11) used a similar procedure for a large-scale purification, which yielded ~5 mg of enzyme from 10 liters of packed erythrocytes. We have achieved a comparable yield of purified enzyme by use of monoclonal antibody affinity chromatography. Replacement of Triton X-100 with the dialyzable detergent ß-D-octylglucoside during the affinity chromatography step eliminated the aggregation observed in the presence of Triton and permitted us to use reversed phase HPLC to obtain a highly purified form of the enzyme that was suitable for amino acid sequence determination.

In the absence of amino acid sequence data for human erythrocyte AChE, other investigators had previously attempted to identify the gene for human AChE using probes derived from the *Torpedo californica* cDNA sequence (15). These studies resulted in the isolation of genes for pseudocholinesterase (16). We obtained amino acid sequence data for >100 residues of human erythrocyte AChE and have used this information to generate oligonucleotide probes in order to screen human cDNA libraries for the presence of the AChE gene. This contract was terminated before the cDNA clones could be isolated and characterized.

B. USE OF INHIBITORY MONOCLONAL ANTIBODIES IN STUDIES OF ENZYME FUNCTION

Pharmacological and biochemical studies of AChE have characterized it as a serine hydrolase with an active site composed of a substrate-binding (anionic) and an adjacent catalytic (esteratic) subsite. A number of peripheral sites have also been identified, at which cations, or acetylcholine itself, may regulate enzyme activity (17-22). No other endogenous modulators of the enzyme are known to exist (23).

Another approach to studying the structure and function of the AChE molecule has involved the use of monoclonal antibodies. In recent years several investigators have produced AChE-specific antibodies; however, only a small number of these antibodies inhibit the activity of the molecule (24-28). One such inhibitory monoclonal antibody is AE-2, produced by D. Fambrough et al. (24), which cross-reacts with AChE in the neuromuscular junction. We have prepared a large number of monoclonal antibodies that are reactive with human erythrocyte AChE and have identified and extensively characterized one of these antibodies, C1B7, which inhibits the activity of erythrocyte AChE by binding to a site that is distinct from the active site and from previously described peripheral sites on the enzyme. This antibody therefore identifies a new potential site at which the activity of AChE may be modified.

A principal goal in these studies was to obtain monoclonal antibodies that react with the active site(s) of the enzyme and to use these antibodies to prepare anti-idiotypic antibodies that mimic the active site in binding to substrate and inhibitors. However, none of the monoclonal antibodies developed in this or other laboratories appear to react with an enzyme catalytic site and thus these antibodies were not suitable candidates for preparing anti-idiotypic antibodies.

METHODS

A. PURIFICATION OF ERYTHROCYTE ACETYLCHOLINESTERASE

I. Acetylcholinesterase 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 acetylcholinesterase (AChE). Red cells were washed 3 times with equal volumes of isotonic buffer (5 mM sodium phosphate, pH 7, 0.9% NaCl). Cells were lysed by the addition of 20 volumes of hypotonic buffer (5 mM sodium phosphate, pH 7). Erythrocyte membranes were separated from hemoglobin and other soluble proteins by Millipore pellicon high-volume molecular filtration as described (29) with a Duripore filter (0.45 um) (Millipore Corp., Bedford, MA) in place of the PTKH 100,000 filter. Proteins of the concentrated, washed membranes were solubilized by adding Triton X-100, 1% final concentration, and the suspension was centrifuged at 100,000 x g. 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 immunoaffinity chromatography as described below.

II. Monoclonal antibody affinity column purification of acetylcholinesterase

a. Ascites preparation - Hybridoma cells secreting monoclonal antibody AE-4, specific for human acetylcholinesterase, were obtained from Dr. D. Fambrough, Carnegie Institute (24). Large amounts of immunoglobulin were obtained by growing cells as ascites tumors. Mice were injected intraperitoneally (i.p.) with 0.5 ml of pristane (2,6,10,14-tetra-methylpentadecane) 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 h.

b. Antibody preparation - Purification of the AE-4 antibody was carried out by ammonium sulfate precipitation and ion exchange chromatography (30,31). All procedures were carried out at 1 to 4°C. Solid ammonium sulfate was added to ascites fluid at a final concentration of 45%. 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 6 changes of dialysate. After

centrifugation at 100,000 x g for 1 h, 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/ml of ascites; the immunoglobulin was greater than 95% pure by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of disulfide reduced and non-reduced material.

c. Affinity column - The purified monoclonal antibody was dialyzed against a buffer solution containing 500 mM NaCl and 200 mM sodium citrate, pH 6.5, 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. The protein content of the filtrates indicated that 96% of the antibody preparation was coupled. A 1.5 cm x 10-cm column containing approximately 33 mg of AE-4 antibody coupled to 11 ml of Sepharose CL-4B beads was treated with (a) 50 ml of 50 mM diethylamine, pH 11.5; (b) 50 ml of 1 M Tris-HCl, pH 7.6, and (c) 50 ml of 5 mM Tris, pH 7.6 before applying the extract.

d. Affinity chromatography - The diluted 100,000 x g supernatant fraction of acetylcholinesterase was applied to the antibody column at a rate of 2 ml/min. Most of the protein in the extract passed through the column. After the protein sample was loaded, the column was washed successively with (a) 100 ml of buffer containing 20 mM $NaPO_4$ buffer, pH 7.5, and 0.2% Triton X-100; (b) 30 ml of 20 mM $NaPO_4$ buffer, pH 7.5, 0.5% octylglucoside; and (c) 30 ml of a borate-salt buffer (1 M NaCl, 100 mM boric acid, 25 mM sodium borate and 0.5% octylglucoside), pH 8.5. The column was then eluted at a rate of 0.5 ml/min with 100 ml of 50 mM diethylamine, pH 11.5, containing 0.5% octylglucoside. Fractions of 3 ml were collected in tubes containing 0.3 ml of neutralizing buffer (0.5 M NaH_2PO_4). The antigen eluted in 10 ml as a single, sharp peak of cholinesterase activity corresponding to a small protein peak. Analysis by PAGE of the individual fractions of the enzyme showed that the concentration of this eluted protein was proportional to enzyme activity. The active fractions were pooled and concentrated to 1.5 ml by use of a negative pressure dialysis-concentrating apparatus (Micro-Pro DiCon; Bio-Molecular Dynamics, Beaverton, OR) in 2000 ml of a buffer solution containing 100 mM sodium phosphate, 0.5 % octylglucoside, pH 8.0, at 4°C.

III. Size-exclusion HPLC

The monoclonal antibody affinity-purified AChE was subjected to final purification by size-exclusion HPLC.

The samples were loaded on a 7.5 mm X 30 cm TSK 3000 SW column and eluted with 50 mM sodium phosphate, pH 7.5, containing 150 mM NaCl and 0.3% octylglucoside at a flow rate of 1 ml/min. The fractions monitored at 280 nm were collected as 0.5 ml aliquots and analyzed for enzyme activity.

B. MONOCLONAL ANTIBODIES

I. Immunization and hybridoma production

a. Immunization with erythrocyte AChE - Twelve-week-old female Balb/c mice were injected with human erythrocyte AChE purified as described above. Three separate immunization schemes were followed:
(1) Conventional schedule as previously used to obtain anti-AChE monoclonal antibodies (24) but modified to include supplemental injections as follows: 10 ug AChE in complete Freund's adjuvant (0.1 ml) i.p.; 10 ug AChE in incomplete Freund's adjuvant i.p. one month later; 500 ug human erythrocyte ghost preparation subcutaneously (s.c) 1 month later, repeated after a second month; 50 ug AChE in PBS i.p. 2 months later; fusion on day 3 after boosting.
(2) High-specific-efficiency immunization as developed for other antigens by Cianfriglia et al. (32) but modified to allow a longer interval between priming and boosting as follows: 50 ug AChE in complete Freund's adjuvant (0.1 ml) i.p., repeated after 1 week; 50 ug AChE in PBS i.p. after 1 week; 50 ug AChE in PBS i.p. and i.v. after 3 months and repeated on the next 2 days, followed by fusion on the succeeding day.
(3) Splenic immunization as described by Thorpe et al. (33)
for other antigens: intra-splenic injection of 15 ug AChE in PBS and fusion 3 days later.
b. Immunization with active site peptide - A 13-amino acid peptide from the active site sequence for human butyrylcholinesterase, obtained from H. Soreq (Weizmann Institute, Rehovot, Israel) was used as antigen in two separate immunization schedules. An aliquot of this peptide was also labeled with ^{125}I by the keyhole limpet hemocyanin (KLH) procedure to a specific activity of 1.3 x 10^8 cpm/ug. For the first schedule the peptide was conjugated to KLH as described in Vergara et al. (34) for other peptides. Briefly, 1 mg of the peptide (mixed with 1 ug ^{125}I-peptide) in 0.3 ml of 0.1 M sodium phosphate buffer (pH 8.0) was added to 0.5 mg dialyzed KLH in 0.06 ml PBS and 5 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI) in 0.05 ml distilled water. The reaction mixture was incubated at room temperature overnight with stirring and then dialyzed overnight, with

two changes of PBS, at 4°C. The dialyzed conjugate contained about 34% of the trichloroacetic acid (TCA)-precipitable counts of labeled peptide, indicating that the peptide made up about 40% of the conjugate weight, representing about 3100 moles of peptide per mole of KLH. About 70 ug of conjugate in complete Freund's adjuvant was injected i.p. into mice and the injection repeated after 1 week. Two succeeding boosts, 1 month apart, each consisted of the same dose of conjugate in PBS i.p. The same peptide was conjugated to the polyclonal B cell activator lipopolysaccharide (LPS) from Salmonella typhimurium, according to the method of Lange et al. (35) for other antigens. Briefly, 1 mg of peptide in 0.3 ml of 0.1 M sodium phosphate buffer (pH 8.0) and 1 ug of ^{125}I-peptide were mixed with 1 mg activated LPS in 0.2 ml 0.001 N HCl and stirred for 18 h at 4°C. The mixture was then centrifuged at 15,000 x g for 15 min (RC-3 Sorvall ultracentrifuge) and washed twice with coupling buffer. The pellet contained about 1.4% of the TCA-precipitable original counts, indicating that the conjugate contained 14 ug of peptide/mg LPS or about 10 mmoles peptide/g of LPS. The pellet was sonicated for 15 sec after the addition of 1 ml buffer and 1.8 mg L-lysine to saturate residual active sites on LPS. Mice were injected with 10 ug of conjugate i.p. and fusion was performed on day 3.

c. Fusion protocol - Fusions were performed as described in Galfre et al. (36) with modifications as follows: About 10^8 spleen cells were fused with equal numbers of P3X63-Ag8-653 myeloma cells in the presence of 50% polyethylene glycol 1000 for 2 min. Cells were diluted with 5 ml Dulbecco's modified Eagle's medium over 1 min and then transferred to the same medium supplemented with 20% fetal calf serum, 10% 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 X 10^{-7} M), and thymidine (1.6 X 10^{-5} M) (HAT medium). Cells were plated on Falcon polystyrene flat-bottom 96-well plates containing 3000 Balb/c macrophages per well, and the medium was replaced on day 7 and subsequently at 3-day intervals.

II. Other monoclonal antibodies

Monoclonal antibodies AE-1, AE-2, AE-3, AE-4, AE-5, all specific for acetylcholinesterase (24), were kindly provided by Dr. D. Fambrough.

C. REDUCTION AND ALKYLATION OF AChE

In general, experiments were performed using the intact AChE dimer obtained following size-exclusion HPLC. In one series of experiments, AChE was dissociated into monomeric subunits by mild reductive alkylation (37). AChE (5 ug) was incubated for 1.5 h at 25°C in the presence or absence of 3 mM dithiothreitol (DTT) (Sigma Chemical Co., St. Louis, MO) in 50-ul volumes of 0.1 M Tris-HCl (pH 8.0). The reaction was terminated by the addition of iodoacetamide (to a final concentration of 20 mM) and activity assayed 1 h later. Reduced and alkylated AChE retained 95% of the activity of control enzyme incubated in the absence of DTT. Separation of the subunits was confirmed by SDS-PAGE (38).

D. ENZYME ASSAYS

Acetylcholinesterase activity was estimated by the method of Ellman *et al* (39). The reaction mixture, comprising 5 ul of 50 mM dithiobis(2-nitrobenzoic acid), 5 ul enzyme sample, 300 ul 100 mM sodium phosphate buffer (pH 7.5) and 2 ul of 75 mM acetylthiocholine substrate, was pipetted into wells of 96-well microtiter plates (Dynatech, Alexandria, VA). Absorbance at 405 nm was measured over a 30-min period using a TiterTek Multiskan-MC spectrophotometer (Flow Laboratories, Inc., McLean, VA) connected to an IBM PC XT. Data were analyzed using software developed by Nimbus, Inc., Baltimore, MD.

E. ASSAYS FOR ANTIBODY BINDING TO AChE

I. Screening of antibodies

Solid-phase radioimmunoassay (RIA) was used to assess the AChE-binding capacity of antibody-containing samples, including polyclonal mouse serum, hybridoma supernatants, ascites fluids and purified monoclonal antibodies. Antibodies were immobilized in wells of polystyrene 96-well microtiter plates (Dynatech Corp., Alexandria, VA) as follows: Five ug of goat anti-mouse IgG antibody (Cappel, Cooper Diagnostics, Cochranville, PA) in 0.05 ml PBS was added to each well and dried overnight at 37°C. The wells were then treated with 0.1 ml of 5% bovine serum albumin (BSA) in PBS for 30 minutes at 4°C to block excess non-specific binding sites. The antibody of interest (0.1-0.2 ug in 50 ul) was then added and incubated for 30 min at 4°C. The solution was then replaced for 30 min with 50 ul of a 4% normal mouse serum solution. After removal of normal serum, 1.2 ng of ^{125}I-AChE (10^5 cpm) in 50 ul of 0.2% BSA in PBS was added and incubated for 30 min at 4°C. Octylglucoside was omitted from the buffer because of

excessive non-specific binding by AChE in the presence of this compound. Unbound ^{125}I-AChE was removed by three washes with 0.1 ml of 1% BSA in PBS. Finally, 0.1 ml of 2 N NaOH was added to each well and incubated at 70°C for 15 min. The ^{125}I-AChE bound to specific antibody was measured with an LKB-Wallace RiaGamma 1271 gamma counter.

II. Activity of antibodies

A modification of the solid-phase RIA described above was used to determine the relative avidity of the antibodies. The antibody to be tested was immobilized in wells of microtiter plates previously treated with goat anti-mouse IgG. Increasing amounts (30 pg-1.6 ug) of unlabeled enzyme were added to 2 ng of ^{125}I-AChE (specific activity, 10^8 cpm/ug) per well in a final volume of 50 ul. After incubation for 20 h at 4°C, the unbound antibody was removed and washed several times and radioactivity bound was measured. Competition curves and Scatchard transformations were plotted to derive the apparent dissociation constants (Kd) for the antibodies.

III. Competitive binding assays

Cross-reactivity of antibodies was also analyzed by competition assays. In one set of experiments, a 10- or 100-fold molar excess of one monoclonal antibody was preincubated with 0.5 nmole ^{125}I-AChE in 0.5 ml PBS for 30 min at 4°C, and 50 ul of this mixture was then added to wells containing the competing antibody bound to goat anti-mouse IgG. Assays were also performed by incubating 2 ng of unlabeled AChE with antibody bound to the solid phase, followed by addition of 2 ng of ^{125}I-AE-2 antibody (1.7×10^8 cpm/ug).

Competitive binding assays using anticholinesterase drugs as inhibitors of antibody binding were also performed. AChE labeled with ^{125}I by the chloramine T procedure was incubated with varying concentrations of drug in PBS for 30 min at 25°C before use in RIA. In all cases, the anticholinesterase drug was also present during the antibody binding step of the RIA. Inhibition assays with propidium and gallamine were performed using 0.01 M Tris-HCl, with or without 0.1 M NaCl, as well as with PBS.

IV. Assay of inhibition by purified antibodies

The inhibition of enzyme by selected antibodies in solution was analyzed by incubating varying amounts of antibody at 25°C for 30 min with 0.05 pmoles of enzyme in 0.5 ml PBS with 0.2% BSA and 0.5% octylglucoside. The enzyme activity of 50 ul of this incubation mixture was then determined as described above.

Inhibition assays involving two antibodies were performed as follows: one antibody was incubated with enzyme for 30 min, followed by addition of the second antibody for 30 min, and enzyme activity was then assayed.

V. Kinetic assays

An initial Ackerman-Potter analysis of enzyme inhibition (40) was performed with varying final concentrations of enzyme (17-340 pM). Since irreversible binding was not demonstrated, a single low concentration (17 pM, or 5 fmoles/well) of enzyme was used in all subsequent analyses. These assays yielded a maximal velocity (V_m) of approximately 10 umoles/L/min; thus, the calculated turnover number (6 X 10^5 molecules/min/active site) corresponded to that reported by Bellhorn et al. for human erythrocyte AChE (41). Substrate concentrations were varied from 0.067 to 1 mM (suboptimal conditions) and from 0.5 to 20 mM (supraoptimal conditions). Analyses of inhibition patterns were made as outlined by Webb (42,43) for tightly bound inhibitors (mutual depletion systems). Estimates of the specific concentration of enzyme ($E_t' = E_t/K_i$) were 1.0 for C1B7 and 0.2 for AE-2, indicating lower boundary conditions of Zone B in mutual depletion system analysis. Mathematical models for partial competitive and non-competitive inhibition not presented in (42) were derived by C.E. Olson of this laboratory. Summation values of inhibition (42) were calculated by the formula $i_{1,2} = i_1 + i_2 - i_1 i_2$.

F. AChE SEQUENCE ANALYSIS

I. Protease digestion of purified AChE

a. Trypsin digestion - Size-exclusion HPLC-purified AChE (approximately 70 ug in 2 ml of 20 mM sodium phosphate buffer, pH 7.6, containing 0.2% octylglucoside) was incubated twice with 1.5 ug trypsin at room temperature for 4 h each.

b. Chymotrypsin digestion - AChE purified by monoclonal antibody affinity chromatography (150 ug in 350 ul of 100 mM sodium phosphate, pH 8.0, containing 0.5% octylglucoside) was incubated with 3 ug of chymotrypsin at 37°C for 2 h.

c. S. aureus V8 digestion - Size-exclusion HPLC-purified AChE (100 ug in 0.2 M sodium phosphate buffer, pH 8.2, containing 0.2% octylglucoside) was treated with 3 ug of S. aureus V8 for 18 h at 37°C.

II. Reversed phase HPLC separation of AChE peptides

Protease digests of the AChE were applied to a Vydac reversed phase C4 column (4.6 mm X 2.5 cm). The solvents were (A) 0.1% (TFA) and (B) acetonitrile:propanol (2:1) containing 0.1% TFA. The column temperature was maintained at 45°C. The peptides were eluted with a flow rate of 1 ml/min with the following gradient program: 0% B for 5 min, 0% B to 55% B for 75 min, 55% B to 100% B for 10 min and then 100% B for 10 more min. The fractions monitored at 280 nm were collected manually.

III. Amino acid sequence analysis

Individual peptide peaks from the reversed phase HPLC were dried under vacuum, redissolved in 100 ul of 50% acetic acid and analyzed with an Applied Biosystems model 470 A gas-phase sequencer and version 2 of the software (44). The liberated PTH-amino acids were identified by reversed phase HPLC on an Altex Ultrosphere C-18 column (4.6 mm X 25 cm) using an aqueous sodium acetate buffer with acetonitrile (45).

G. GENE CLONING

I. Oligonucleotide synthesis

AChE oligonucleotide probes 1 to 4 (see Results and Discussion section C.I., p. 23), derived from AChE tryptic peptide sequences (T7, T10, T11, as in Table 2), were synthesized on a DNA synthesizer (Model 380A, Applied Biosystems) and purified by reversed phase HPLC to remove failed sequences and breakdown products, using an N-ethylmorpholine/acetonitrile solvent system (46).

II. K562 cDNA libraries

cDNA libraries of the K562 human erythroleukemia cell line prepared in pBR322 and pUC8 (47) were obtained from Dr. Giovanni Rovera, Wistar Institute.

These cDNA libraries were screened using a standard protocol. In brief, E. coli DH5 competent cells were transformed with a pUC8 or pBR322 library and plated on the LB agar plates containing ampicillin and tetracycline, respectively. The colonies were duplicated on the nitrocellulose filter, amplified on chloramphenicol LB agar plates and screened with AChE probes 1 and 4 (Results and Discussion section C.I., p. 23) using the in situ hybridization procedure as described by Derek Woods (48). Approximately 4 X 10^5 ampicillin- and tetracycline-

resistant clones were screened for pUC8 and pBR322 libraries, respectively. The AChE oligonucleotide probes 1 and 4 were labeled with [lambda-^{32}P]-ATP by a standard protocol (49).

III. Human muscle cell cDNA library

A human muscle cDNA library made in pBR322 (50) was obtained from Dr. L. Kedes, Stanford University.

This library consists of approximately 5400 clones. It was used to transform E. coli DH5 competent cells and was screened with the same protocol as that used for screening K562 cDNA libraries. Each of the four AChE oligonucleotide probes derived from tryptic peptides was used individually to screen approximately 10,000 clones in duplicate. In the preliminary screening 10 positive clones were identified. The cDNA insert from these positive clones was isolated by the use of Pst I and Pvu II restriction endonucleases (49) and analyzed by Southern blot hybridization with AChE oligonucleotide probes.

IV. Human genomic library

A genomic library from human T cells in the bacteriophage vector was obtained from Dr. Giovanni Rovera, Wistar Institute, Philadelphia. This library was screened with AChE oligonucleotide probes (20-mers) synthesized with or without deoxyinosine. Two screening protocols were used for in situ hybridization: the standard protocol as described for K562 cDNA and human muscle cell cDNA libraries, and the protocol incorporating the use of tetraethyl ammonium chloride (51), which allows the stringency of hybridization to be controlled by probe length and not by the base composition.

RESULTS AND DISCUSSION

A. PURIFICATION OF HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE

I. Monoclonal antibody affinity chromatography

We have employed monoclonal antibody affinity chromatography for simple and efficient purification of the erythrocyte enzyme to near homogeneity with high yield (Table 1). Approximately 500 ug were obtained from 1000 ml of packed red blood cells. This yield is similar to that reported by Rosenberry and Scoggin for the acridinium ligand procedure (11). Recovery of enzyme activity was about 15% with a purification of more than 130,000-fold. The yield of AChE protein was about 60%, as determined by radioimmunoassay of enzyme protein, indicating that the overall purification of AChE was about 500,000-fold.

An important modification over earlier procedures was the replacement of the Triton X-100 used to solubilize the enzyme by ß-D-octylglucoside during affinity chromatography and elution from the column at pH 10.5 (see Methods for detailed protocol). Enzyme elution in the presence of Triton X-100 and higher pH apparently caused significant aggregation of the molecule. The change of detergent and pH resulted in greater yield and purity of material.

II. Size-exclusion HPLC

Final purification of the affinity-isolated material was achieved by size-exclusion HPLC (Fig. 1). AChE eluted as a single peak of enzymatic activity and protein. The enzyme was recovered as a high M_r fraction (>232,000 Da) rather than at the 140,000-Da position of the dimeric protein; we attribute this effect to the detergent bound to the protein.

The HPLC step was important to subsequent sequence studies, as it removed low molecular weight contaminants that might be present in high molar concentration, even when insignificant in mass or absorbance at 280 nm.

III. Purity

AChE purity was analyzed by SDS-PAGE (Fig. 2). The protein was stained by silver nitrate reduction. The AChE was apparently over 90% pure following affinity chromatography and appeared to be >98% pure after HPLC. The enzyme was present as a dimer before disulfide reduction and as a 70,000-Da monomer after reduction. When labeled with [^{3}H]diisappropylfluorophosphate ([^{3}H]DFP), a single band corresponding to the enzyme was observed.

TABLE 1: Purification of human erythrocyte acetylcholinesterase

Purification Step	Volume (ml)	Total Protein[b] (mg)	Total Activity[c] (A/min)	Activity Recovery (%)	Specific Activity (A/min/mg)	Purification (-fold)
HEMOLYSATE	21,217	488,286	292,971		0.6	1
RED CELL GHOSTS	1,132	5,040	262,080	89	52	486
100,000 x g SUPERNATANT	1,027	994	176,932	60	178	296
ANTIBODY AFFINITY	0.9	0.95	64,771	22	68,180	131,115

[a] Results are the mean value of four purifications.
[b] Protein was measured by the modified method of Lowry et al. (52).
[c] Enzyme activity was measured as described by Ellman et al. (39).

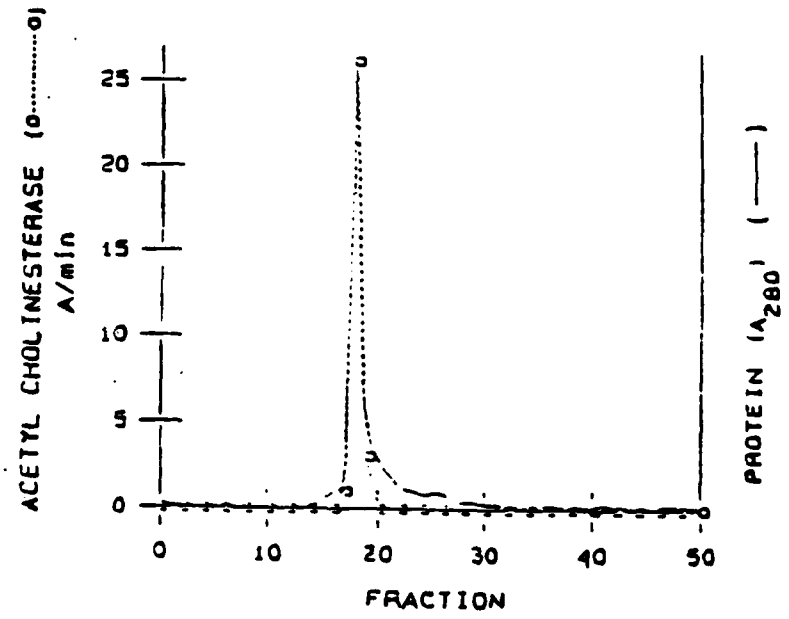

Figure 1: Size-exclusion HPLC of purified AChE. The concentrated affinity-purified fraction of AChE (20 ul, 10 ug) was placed in a 7.5 mm X 30 cm TSK-SW3000 column. The column was eluted with 50 mM $NaPO_4$, pH 7.5, containing 150 mM NaCl and 0.3% octylglucoside. The fraction volume was 0.5 ml. The tracing is of absorbance at 280 nm with a setting of 0.02 absorbance units as full scale. The peak fraction corresponded exactly to enzyme activity and to radioactivity of ^{125}I-AChE (0.01 ug, 1.3 X 10^6 cpm) added as an internal marker.

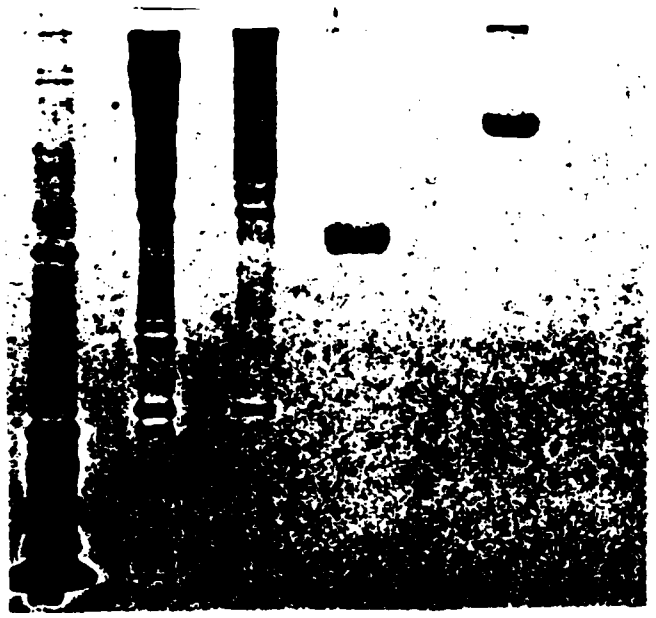

Figure 2: SDS-PAGE analysis of AChE purification. Lane 1, hemolysate, 60 ug; lane 2, red blood cell ghosts, 10 ug; lane 3, 100,000 xg supernatant, 3 ug; lane 4, affinity purified AChE, 1.5 ug after disulfide reduction; lane 5, affinity purified AChE, 1.5 ug protein, without disulfide reduction. The protein bands were visualized by silver staining. M_r standards are myosin heavy chain, 200 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa.

IV. Two-dimensional electrophoresis

The purified protein contained several isoelectric variants, as is typical of glycoproteins with charge heterogeneity in the oligosaccharides of the molecule (Fig. 3).

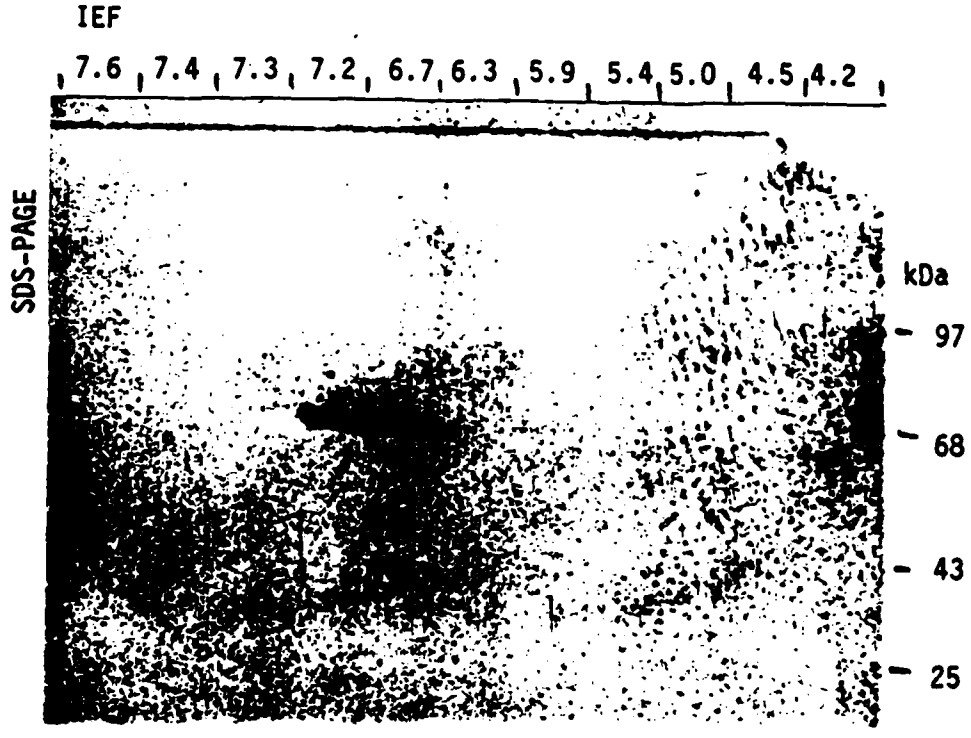

Figure 3: Two-dimensional gel analysis of purified AChE. HPLC-purified AChE, 7 ug, was analyzed as described by O'Farrell (52). The protein was stained with silver nitrate. M_r standards are phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and chymotrypsinogen, 25 kDa.

B. AMINO ACID SEQUENCE ANALYSIS

Sequence data for >100 residues were obtained from tryptic, chymotryptic and V8 peptides. These peptides represented approximately 20% of the intact enzyme molecule.

I. Tryptic peptides

Peptide mixtures generated by trypsin digestion were separated by reversed phase HPLC. Approximately 23 peptides were resolved (Fig. 4). Five have yielded satisfactory sequence data (Table 2).

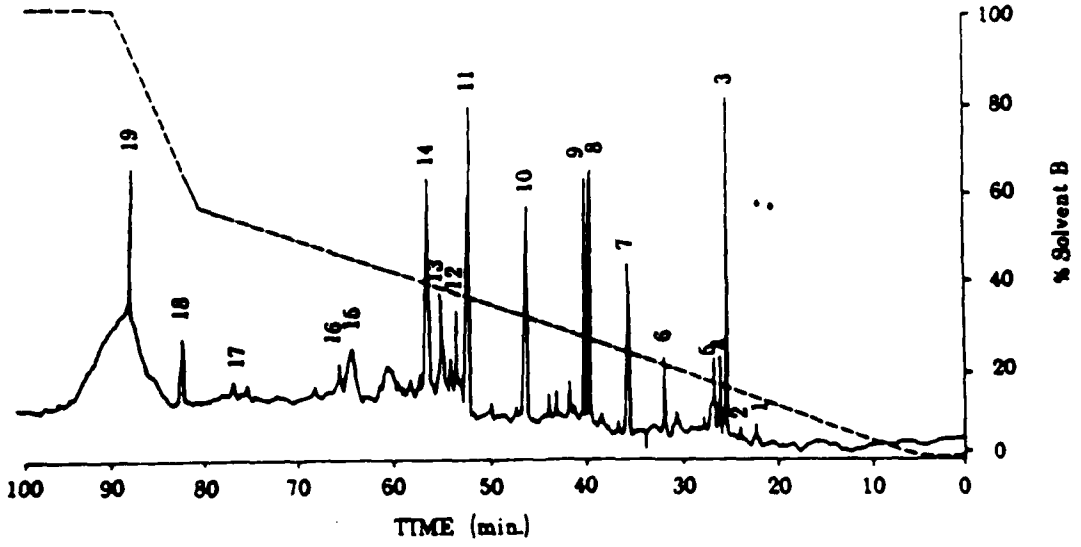

Figure 4: Preparation of tryptic peptides of AChE. AChE (70 ug) in 2 ml of 20 mM $NaPO_4$, pH 7.6, containing 0.2% octylglucoside was treated at room temperature for 8 h with a total of 3 ug of trypsin. The digest was applied to a Vydac C4 column (4.6 mm X 25 cm) maintained at 45°C. The solvents were (A) 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile:propanol (2:1) containing 0.1% TFA. The peptides were eluted at a flow rate of 1 ml/min with the following gradient program: 0% B for 5 min, 0% B to 55% B for 75 min, 55% B to 100% B for 10 min, 100% B for 10 min. The fractions were dried under vacuum and subjected to amino acid sequence analysis.

TABLE 2. Sequence analysis of AChE tryptic peptides

Cycle	T7 Amino acid	T7 Yield (1)	T7 Yield (2)[a]	T9 Amino acid	T9 Yield	T10 Amino acid	T10 Yield (1)	T10 Yield (2)[a]	T11 Amino acid	T11 Yield (1)	T11 Yield (2)[a]	T16 Amino acid	T16 Yield
		pmole	pmole		pmole		pmole	pmole		pmole	pmole		pmole
1	Ala	X[b]	80	Thr	99	X	X	X	Val	216	173	X	X
2	Val	224	46	Arg	50	Pro	276	298	Gly	142	214	X	X
3	Leu	265	62	Pro	210	Gln	116	108	Val	202	175	Thr	28
4	Gln	188	57	X	X	Try	156	24	Pro	296	189	Leu	75
5	Ser	56	X	Gln	73	Pro	218	237	Gln	119	64	Asp	32
6	Gly	129	20	Val	118	Pro	225	253	Val	182	101	Val	19
7	Ala	176	26	Leu	113	Tyr	144	148	Ser	40	12	Pro	49
8	Pro	190	57	Val	118	Thr	71	62	Asp	56	43	Leu	50
9	Asn	85	X	X	X	Ala	158	132	Leu	160	120	X	X
10	Gly	124	19	His	25	Gly	141	81	Ala	117	82	Met	36
11	Pro	131	35	Glu	71	Ala	101	99	Ala	126	90	Gly	27
12	Try	46	17	Asp	64	Gln	132	82	Glu	X	92	Val	33
13	Ala	103	12	His	25	Gln	122	105	Ala	106	64	Pro	37
14	Thr	35		Val	85	Tyr	116	95	Val	110	84	X	X
15	Val	82		Leu	94	Val	125	100	Val	92	108	Gly	25
16	Gly	101		Pro	98	Ser	26	18	Leu	115	108	Tyr	15
17	Met	74		Gln	48	Leu	93	116	Asp	X	22		
18	Gly	44		Glu	50	Asp	36	29	Tyr	73	59		
19				Asp	59	Leu	75	63	Thr	29	X		
20				Val	20	Arg	34	38	Asp	29	12		
21				Phe	45	Pro	85	94	Try	36	X		
22						Leu	63	42	Leu	64	47		
23						Glu	48	32	X	X	X		
24						Val	49	44	Pro	60	X		
25									X	X	X		
26									Asp	16	X		
27									Pro	46	X		
Avg. repetitive yield (%)		88.35	86.5		88		89.7	80.2		95.2	83		86

[a] repeated analysis
[b] unidentified residue

II. Chymotryptic peptides

Over 40 fractions have been obtained from AChE treated with chymotrypsin (Fig. 5). The amino acid sequence of three of these peptides in shown in Table 3.

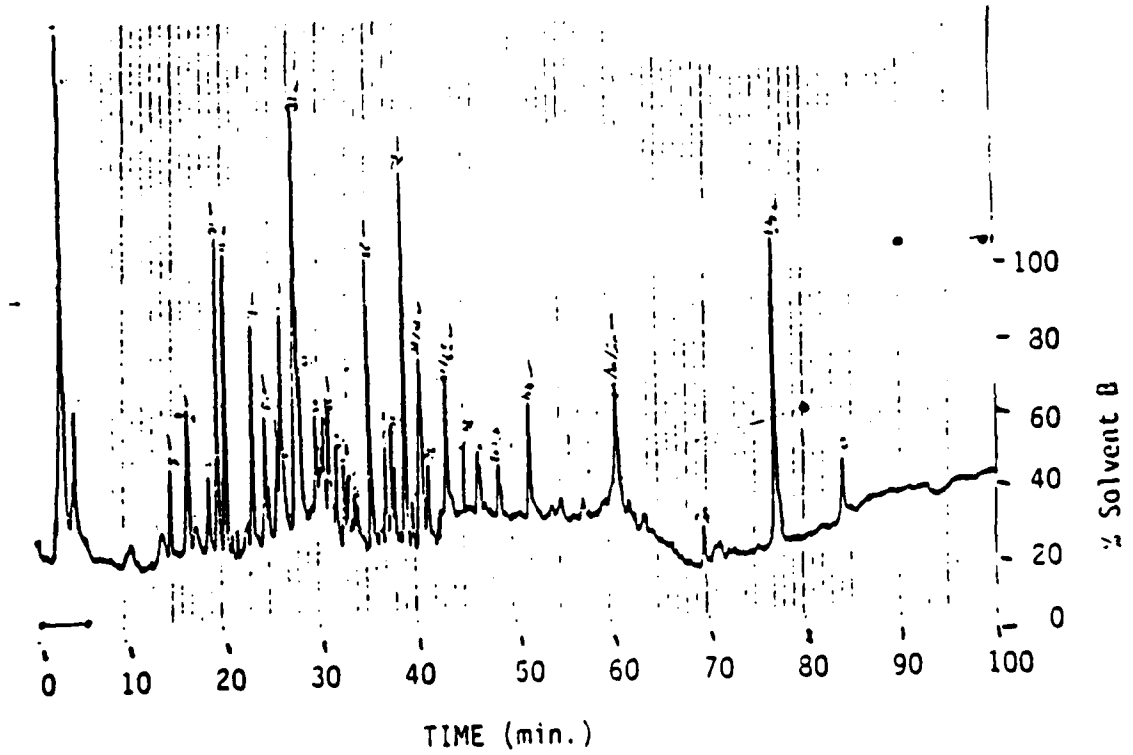

Figure 5: Preparation of chymotryptic peptides of AChE. AChE (150 ug in 350 ul 100 mM sodium phosphate, pH 8.0 containing 0.5 % octylglucoside) was treated at 37°C for 2 h with 3 ug of chymotrypsin. The peptides generated were separated on a C4 Vydac reversed-phase column as described for tryptic peptides.

TABLE 3

Amino acid sequence analysis of chymotryptic peptides of AChE

	CH_1		CH_2		CH_3	
Cycle	Amino acid	Yield	Amino acid	Yield	Amino acid	Yield
		pmole		pmole		pmole
1	Ala	494	X[a]	X	X	X
2	Ala	426	Asn	248	X	X
3	Gln	290	Gly	231	Try	93
4	Gly	368	Ala/Glu	148/137	Gly	36
5	Ala	346	Phe	106	Met	34
6	Agr	69			Gly	67
7	Val	355			Glu	17
8	Tyr	125				
9						
10						

[a]unidentified residue

III. V8 peptides

Digestion of AChE with S. aureus V8 protease yielded 9 peptide fractions upon resolution on size-exclusion HPLC (Fig. 6). Two of these peptides (V3 and V6) were analyzed (Table 4) and contained sequences highly similar to sequences within tryptic peptides T9 and T11 (Table 5). Repeated sequencing of these fragments is required to determine if the differences (residues 1 and 2 of V3 as compared to T9 and residues 5 and 7 of V6 as compared to T11) are significant.

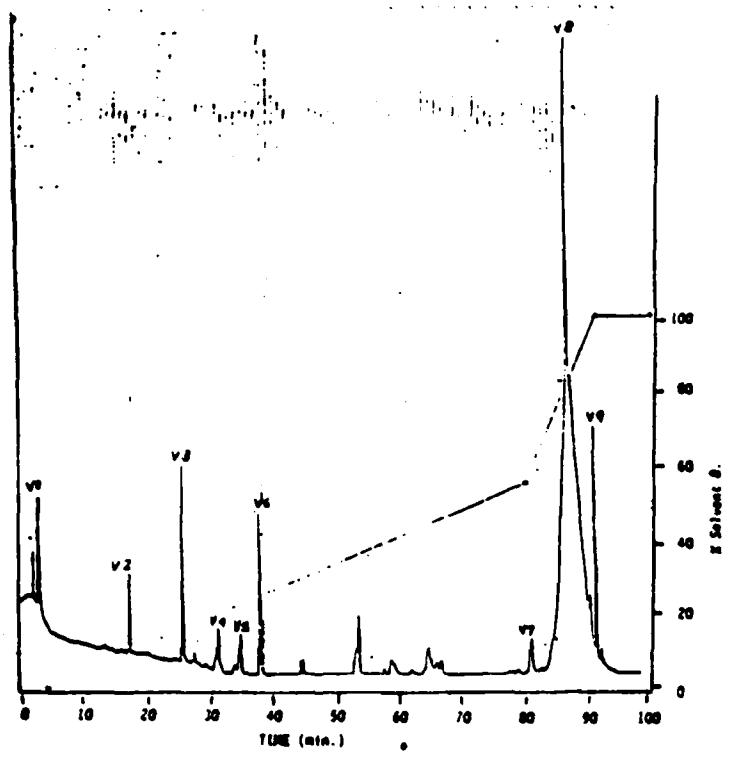

Figure 6: Preparation of V8 peptides of AChE. The AChE (100 ug) purified by size-exclusion HPLC was mixed with 3 ug of S. aureus V8 enzyme in 0.2 M sodium phosphate buffer, pH 8.2, containing 0.2% octylglucoside. The mixture was incubated at 37°C for 18 h. The peptides were then separated on a C4 Vydac reversed phase column under the conditions described for the separation of tryptic peptides.

TABLE 4. Sequence of analysis of AChE V8 peptides

	V3		V6	
Cycle	Amino	Yield acid	Amino	Yield acid
		(pmole)		(pmole)
1	Try	79	Ala	156
2	X[a]		Val	114
3	Val	53	Val	117
4	Leu	58	Leu	123
5	Pro	82	Ala	27
6	Gln	30	Tyr	67
7	Glu	13	Leu	31
8			X	
9			Try/Ala	27/24
10			Leu	47
11			Leu	44
12			Pro	21
13			Gly	11
14			Asp	5
15			Pro	44
16			Ala	21
17			Gly	7
18			Leu	50
19			Gly	9
Repetitive yield				66%

[a]Unidentified residue

TABLE 5. Comparison of tryptic acid and V8 peptides

	T9		V3		T11		V6
1	Thr				Val		
2	Arg				Gly		
3	Pro				Val		
4	X[a]				Pro		
5	Gln				Gln		
6	Val				Val		
7	Leu				Ser		
8	Val				Asp		
9	X				Leu		
10	His				Ala		
11	Glu				Ala		
12	Asp	1	Try		Glu		
13	His	2	X		Ala	1	Ala
14	Val	3	Val		Val	2	Val
15	Leu	4	Leu		Val	3	Val
16	Pro	5	Pro		Leu	4	Leu
17	Gln	6	Gln		Asp	5	Ala
18	Glu	7	Glu		Tyr	6	Tyr
19	Asp				Thr	7	Leu
20	Val				Asp	8	X
21	Phe				Try	9	Try/Ala
22					Leu	10	Leu
23					X	11	Leu
24					Pro	12	Pro
25					X	13	Gly
26					Asp	14	Asp
27					Pro	15	Pro
						16	Ala
						17	Gly
						18	Leu
						19	Gly

[a] Unidentified residue

IV. N-terminal sequence analysis

N-terminal sequence analysis of AChE yielded two different N-terminal sequence patterns, suggesting that the two 70-kDa subunits may be different. In two independent sequence determinations, the N-terminal amino acid residue was found to be either glutamic acid or arginine (170 pmole and 256 pmole, respectively, in the first run; 135 pmole and 455 pmole, respectively, in the second run). The succeeding 12 amino acid residues also yielded ambiguous sequence data, with the exception of valine at position 10. Data of Haas and Rosenberry (54), who have reported the presence of two N-terminal amino acids with stoichiometries of 0.66 Glu and 0.34 Arg per 70-kDa subunit, are consistent with this possibility. Their additional sequence was Glu-Gly-Ala-Glu-Asp-Ala. Their position 3 result differed from the analogous sequence determined by S.S. Taylor (personal communication cited in 54).

C. CLONING THE GENE FOR HUMAN AChE

Gene cloning was initiated by taking advantage of the sequence data and synthesizing oligonucleotide probes corresponding to the amino acid sequences. These probes were used to screen cDNA libraries by colony hybridization (55).

I. Synthesis of oligonucleotide probes

Several DNA probes containing a mixture of oligonucleotides that represent all possible codon combinations for small portions of the amino acid sequence of AChE were synthesized:

Probe 1: 17-mer corresponding to peptide T7:

```
Amino acid     Pro -- Asn -- Gly -- Pro -- Try -- Ala
                 A      A      A      A

Probe    3'    GGG -- TTG -- CCG -- GGG -- ACC -- CG   5'

                 C             C      C
                 T             T      T
```

Probe 2: 14-mer corresponding to peptide T11:

```
Amino acid     Gly -- Val -- Pro -- Gln -- Val
                 A      A      A      T

Probe    3'    CCG -- CAG -- GGG -- GTC -- CA   5'

                 C      C      C
                 T      T      T
```

Probe 3: 14-mer corresponding to peptide T7:

Amino acid		Thr	--	Val	--	Gly	--	Met	-- Gly
		A		A		A			
Probe	3'	TGG	--	CAG	--	CCG	--	TAC	-- CC 5'
		C		C		C			
		T		T		T			

Probe 4: 17-mer corresponding to peptide T10:

Amino acid		Gln	--	Try	--	Pro	--	Pro	--	Tyr	-- Thr
		C				A		A		A	
5' Probe	3'	GTT	--	ACC	--	GGG	--	GGG	--	ATG	-- TG
						C		C			
						T		T			

II. K562 cDNA libraries

The two K562 libraries were screened first with a positive control probe and subsequently with AChE probes 1 and 4.

c-myc positive control: A 17-base-long oligonucleotide probe (5' CGG-CAA-CGA-CGA-GAA-CA 3') was synthesized according to the nucleotide sequence reported for the c-myc gene by Watt et al. (47). Using this probe, strong positive signals were detected with both K562 cDNA libraries (K562-pUC8 and K562-pBR322), indicating that the experimental protocol used was appropriate for hybridization with a 17-base-long oligonucleotide probe. Based on this observation AChE probes 1 and 4 (both 17 bases long) were used to screen the cDNA libraries.

AChE probes: Approximately 400,000 clones of each library were screened twice. Positive clones were not detected with probes 1 and 4.

III. Human muscle cDNA library

This cDNA library was prepared using the procedure described by Okayama and Berg (56), which optimizes conditions for the reverse transcription of mRNA and results in cDNA covalently linked to the plasmid vector and a high yield of full-length cDNA clones. The library was tested with each of the 4 probes prepared from tryptic peptides, and 10 positive clones were identified. Southern blot analyses of the restriction endonuclease-isolated cDNA inserts of these clones suggested further that the clones might indeed contain AChE sequences.

D. STUDIES USING MONOCLONAL ANTIBODIES

I. Inhibitory monoclonal anti-AChE antibodies

Sixteen mouse monoclonal antibodies that bind to human red blood cell AChE were produced in four separate fusions. Most of the antibodies, as well as five others (AE-1, AE-2, AE-3, AE-4, and AE-5) previously described by Fambrough *et al*. (26), were of the IgG,k class with Ka values for binding to AChE in the range of 0.5 to 20 x $10^7/M^{-1}$ (Table 6). All of the antibodies were tested for their ability to inhibit AChE activity. Only two of the 21 antibodies, C1B7 and AE-2, significantly inhibited enzyme activity (Fig. 7). C1B7 was found to inhibit AChE activity by as much as 85-90% at relatively low concentrations. In contrast, higher concentrations of AE-2 were required to achieve maximum inhibition at about 75%. The two inhibitory antibodies had intermediate Ka values: $2.0 \times 10^7 M^{-1}$ for AE-2 and $5.0 \times 10^7 M^{-1}$ for C1B7.

II. Distinct binding sites for C1B7 and AE-2 on AChE

In order to determine whether C1B7 and AE-2 bound to similar or different regions of the AChE molecule, competition assays were performed in which ^{125}I-labeled AChE was preincubated with one of the inhibitory antibodies and then tested for subsequent binding to the second inhibitory antibody. These experiments, summarized in Table 7, demonstrate that C1B7 and AE-2 antibodies bind to different sites on AChE. Preincubation of labeled AChE with AE-2 had no effect on subsequent binding of AChE to C1B7 but almost completely blocked subsequent binding to AE-2. Similarly, preincubation of AChE with C1B7 did not inhibit subsequent binding to AE-2 but did block binding of AChE to C1B7. Among the non-inhibitory antibodies tested, only AE-1 showed any effect. Preincubation with C1B7 or AE-1 inhibited subsequent binding of AChE to the other. AE-1 did not, however, significantly affect AE-2 binding.

III. Inhibition of monomeric and dimeric AChE by C1B7 and AE-2

Since erythrocyte AChE is composed of two subunits, it was of interest to determine whether the antibodies bind to and inhibit the activity of the individual subunits as well as the intact dimer. Conditions for mild reduction and alkylation were established that resulted in complete dissociation of the AChE subunits with retention of >90% of the enzymatic activity. Subunit dissociation was verified by SDS-PAGE analysis of the enzyme. Following

TABLE 6

ANTI-ACETYLCHOLINESTERASE ANTIBODIES

Isotypes and binding constants of 16 monoclonal antibodies prepared from four separate fusions in this laboratory, and of antibodies AD-1, -2, -3, -4, and -5 which have been previously reported by Fambrough et al. (24).

Ab Source	Ab Name	Isotype[a]	Kd[b] (10^{-1} M^{-1})
Fusion 1	SA5	IgM,k	2.5
Fusion 2	F3F2	IgM,k	1.7
	F3G10	IgM,k	1.1
Fusion 3	C1B7	IgG1,k	5.0
	C1C7	IgG1,k	ND
	C1C8	IgG1,k	1.8
	C1D7	IgG1,k	0.7
	C1F9	IgG1,k	ND
	C1G4	IgG1,k	0.8
	C1H7	IgG1,k	ND
	C1H9	IgG1,k	11.4
	C1H11	IgG1,k	ND
Fusion 4	C2A7	IgG1,k	ND
	C2B8	IgG1,k	ND
	C2C4	IgG1,k	0.5
	C2H7	IgG1,k	6.9
Fambrough (26)	AE-1	IgG1,k	7.0
	AE-2	IgG1,k	2.0
	AE-3	IgG1,k	20.0
	AE-4	IgG1,k	6.0
	AE-5	IgG1,k	16.0

[a] Isotypes were determined using a Zymed immuoassay kit.
[b] Binding constants were calculated from Scatchard plots of antibody binding to AChE, as described (32).

TABLE 7: Competition of antibodies for binding to AChE

Immobilized[a]	Inhibition of ^{125}I-AChE Binding by Competing Antibody (%)[b]		
	AE-2	C1B7	AE-1
C1B7	4.4	89.9	97.7
AE-2	88.7	0.0	8.7
AE-1	12.8	66.7	97.9

[a] A 100-fold molar excess of one monoclonal antibody was preincubated with 0.26 pmole [^{125}I] in 0.5 ml PBS for 30 min at 4°C, and 50 ul of this mixture was added to wells containing antibody immobilized on goat anti-mouse IgG.

[b]Control binding: C1B7 19,500 cpm
AE-2 11,500 cpm
AE-1 19,500 cpm

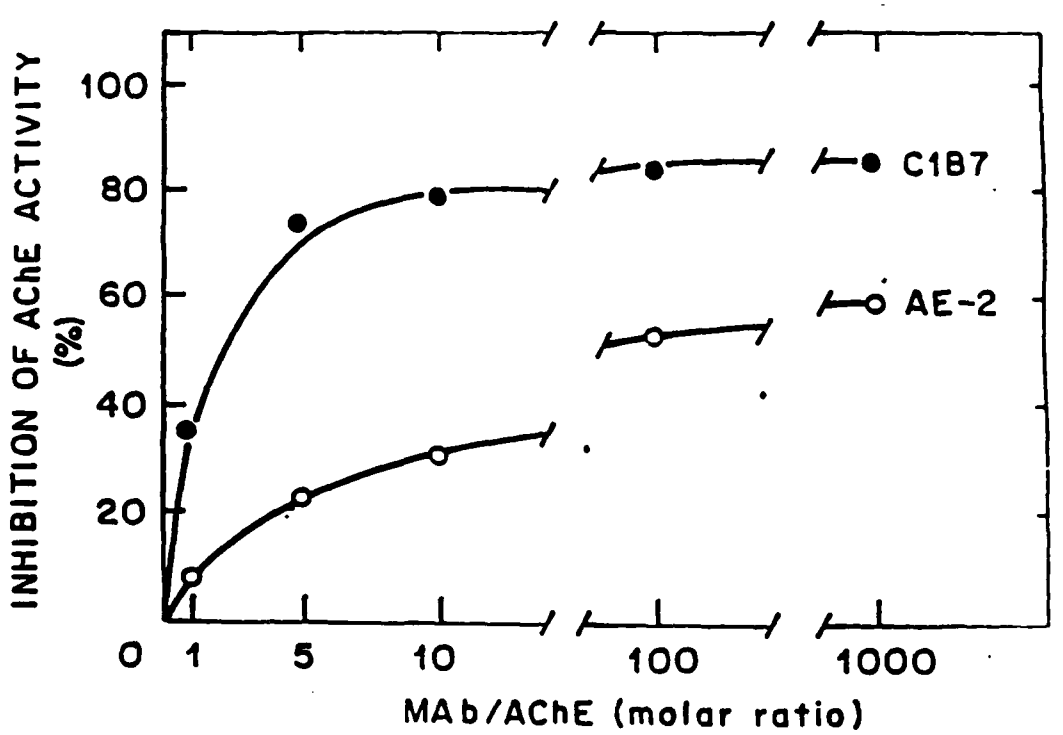

Figure 7: Antibody inhibition of AChE activity. AChE (17 pM) was preincubated with the indicated amounts of each monoclonal antibody. After 30 min, AChE activity remaining in each sample was measured using the Ellman assay. Inhibition by C1B7 (●) and AE-2 (O). Inhibition by all other monoclonal antibodies, as well as by PBS and P3 controls, was <5%.

reductive alkylation AChE migrated as a single ~75 kDa band under both reducing and non-reducing conditions, indicating complete reduction of the disulfide linkage and formation of monomers. When these fully active AChE monomers were treated with C1B7 or AE-2 for 30 min at 25°C, inhibition was essentially identical to that observed for the native AChE dimer (73.9% inhibition for monomeric vs. 79.8% for dimeric C1B7; 55.2% inhibition for monomeric vs. 58.0% for dimeric AE-2).

Two mechanisms are possible to account for the finding that the maximal inhibition of enzyme activity by AE-2 under these conditions was about 50%. (a) AE-2 recognizes and binds only to half or more of the monomers and inhibits these totally; or (b) AE-2 binds all of the monomers and only partially inhibits the activity of each. The results of two further experiments indicated that the antibody binds both monomers. First, AChE monomers and dimers were quantitatively absorbed by AE-2 bound to Sepharose beads, as determined by monitoring the disappearance of enzyme activity. Second, when binding of AChE monomers and dimers was monitored by directly assaying enzyme activity bound to AE-2 immobilized on microtiter plates, weight-equivalent activity was detected.

IV. Distinct binding sites for anticholinesterase compounds and inhibitory antibodies on AChE

The possibility that C1B7 and AE-2 inhibit enzyme activity by binding directly to the active site or to previously identified allosteric sites was investigated. Competition assays in which AChE was pretreated with well-defined anticholinesterase compounds revealed no specific inhibitory effect on antibody binding to the pretreated AChE. The following anticholinesterase compounds were tested: the irreversible esteratic site-directed inhibitor DFP; the reversible site-directed inhibitors neostigmine, carbachol, edrophonium, and BW284c51; and the allosteric site-directed inhibitors gallamine and propidium (Table 8). Pharmacologic levels (i.e., concentrations of drug that are just sufficient to inhibit enzyme activity completely) had no effect at all on antibody binding. In subsequent experiments, much higher levels (at least 1000-fold greater than the Kd) of each drug were also tested for inhibition of antibody binding, and no specific inhibition of antibody binding was observed. That is, 10-100 mM levels of the various anticholinesterase drugs prevented the binding of non-inhibitory antibodies in addition to the binding of the inhibitory antibodies (i.e., AE-2 and C1B7). Acetylthiocholine, which is used as the substrate for AChE in the Ellman assay (39), also had no effect on C1B7 or

TABLE 8: Anticholinesterase compounds that do not compete with inhibitory antibodies for binding to AChE

Drug	ACHE Site	Kd of Drug for AChE[a]	Concentrations showing no competition with C1B7 or AE-2 in binding assay[b]
		M	M
DFP	Esteratic	10^{-6}	10^{-7} - 10^{-2}
Neostigmine	Esteratic Anionic P1	10^{-7}	10^{-6} - 10^{-5}
Carbachol	Esteratic Anionic P1	10^{-4}	10^{-3} - 10^{-1}
Edrophonium	Anionic P1	10^{-5} 10^{-4}	10^{-3} - 10^{-1}
BW	Anionic P1	10^{-6}	10^{-3} - 10^{-2}
Gallamine	Anionic P2	10^{-5}	10^{-12} - 10^{-2}
Propidium	P1	10^{-5}	10^{-12} - 10^{-2}

[a]Kd represents the concentration of drug at half-maximal inhibition (esteratic or anionic sites) or dissociation of propidium (peripheral sites), as determined Ellman assays (39).

[b]For the binding assay, 0.26 pmole [^{125}]AChE in 0.5 ml PBS was preincubated with varying concentrations of drug for 30 min at 25°C, and 50 ul of this mixture was then added to wells containing immobilized antibody.

AE-2 binding to AChE in concentrations up to 20 mM.

Inhibition experiments with the active center subsite-directed drugs DFP and edrophonium were performed in two different ways. First, enzyme was totally inactivated by preincubation with various levels of DFP or edrophonium. Both the pretreated and the untreated enzymes were found to bind to AE-2 and C1B7 equally well. In a second series of experiments, AChE was preincubated with AE-2 or AE-4 and then treated with drug. In this case the kinetics of drug inactivation of residual AChE activity were unaffected by pretreatment with antibody. Thus the binding of drug to enzyme did not affect antibody binding, nor did antibody binding to enzyme affect drug action on residual AChE activity.

V. Kinetic analysis of AChE inhibition

Since binding of the antibodies was not blocked by site-specific ligands, inhibition must be considered allosteric. In order to analyze the mechanism of allosteric inhibition, assays of enzyme activity were performed at varying concentrations of antibody and substrate. Kinetic parameters characterizing antibody inhibition were derived from Eadie-Hofstee (Fig. 8) and Webb plots (Fig. 9) as described by Webb (56). The data are summarized in Table 9.

V_m fell as antibody concentration increased; it was reduced by 50% ($V_{im}/V_m = 0.50$) at an antibody concentration, $(I_t)_{50}$, of slightly >1 (molar ratio of antibody/enzyme) for C1B7 and about 10 for AE-2. In contrast, K_m changed very little as substrate concentration increased: Nearly parallel lines were observed in the Eadie-Hofstee plots, and the ratio of their slopes remained close to 1. Thus the affinity of substrate for the enzyme was unaffected.

It should also be noted that inhibition by either antibody was not total. This effect was measured in the Webb plot, where residual activity (β) in the presence of an infinite concentration of antibody is estimated from the ordinate intercept ($1/1-\beta$). Using the values of alpha and β obtained in these plots, reasonable estimates of K_i were obtained.

In order to confirm the non-competitive nature of inhibition by C1B7 and AE-2, and to compare it to the known allosteric nature of substrate inhibition, kinetic assays were performed at very high and at supraoptimal substrate concentrations (Fig. 10). In this case, activity-substrate plots (V vs. S) were chosen as most illustrative of the patterns which result. For both C1B7 and AE-2, little if any change in K_m was observed at low antibody:enzyme ratios. At ratios above 10, shifts in K_m (i.e., substrate concentration at Vim/2 for a particular

TABLE 9

Kinetic Parameters of Inhibitory Antibodies[a]

Inhibitor		Kinetic Parameter					
mAb[b]	Concentration	v_m	v_{im}/v_m	k_m			k_i
	mAb/AChE	umoles		mM			mAb/AChE
C1B7	0	11.2		0.11			
	1	6.9	0.62	0.12	1.0		0.8
	2	5.2	0.47	0.15	1.3		1.0
	5	3.1	0.28	0.13	1.2		1.1
	10	2.5	0.22	0.13	1.2		1.5
	100	1.8	0.16	0.13	1.2	0.09	8.2
AE-2	0	12.7		0.12			
	2	10.3	0.81	0.15	1.2		5.0
	5	8.4	0.66	0.16	1.3		5.3
	10	6.5	0.51	0.16	1.3		4.8
	100	3.6	0.28	0.22	1.8	0.26	2.8

[a] Kinetic parameters were determined from analysis of Eadie-Hofstee and Webb plots
[b] mAb: monoclonal antibody

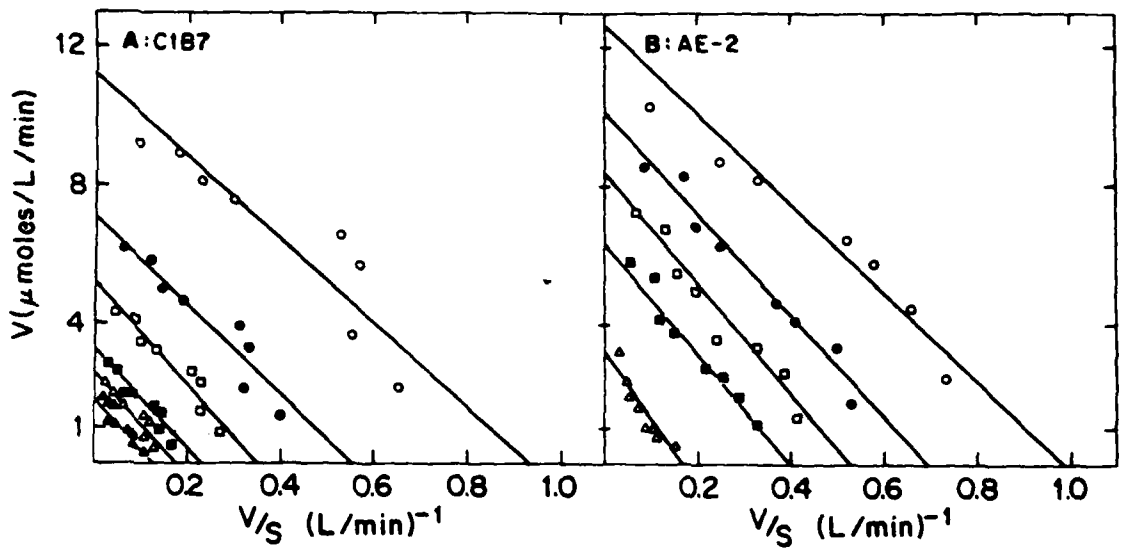

Figure 8: <u>Antibody inhibition of AChE activity: Eadie-Hofstee plot</u>. AChE (17 pM) was preincubated with varying levels of C1B7 and AE-2. After 30 min AChE activity was determined in the presence of varying levels of substrate. Panel A, inhibition by C1B7: C1B7/AChE molar ratio = 0 (○), 1 (●), 2 (□), 5 (■), 10 (△), and 100 (▲). Panel B, inhibition by AE-2: AE-2/AChE molar ratio = 0 (○), 2 (●), 5 (□), 10 (■), and 100 (△).

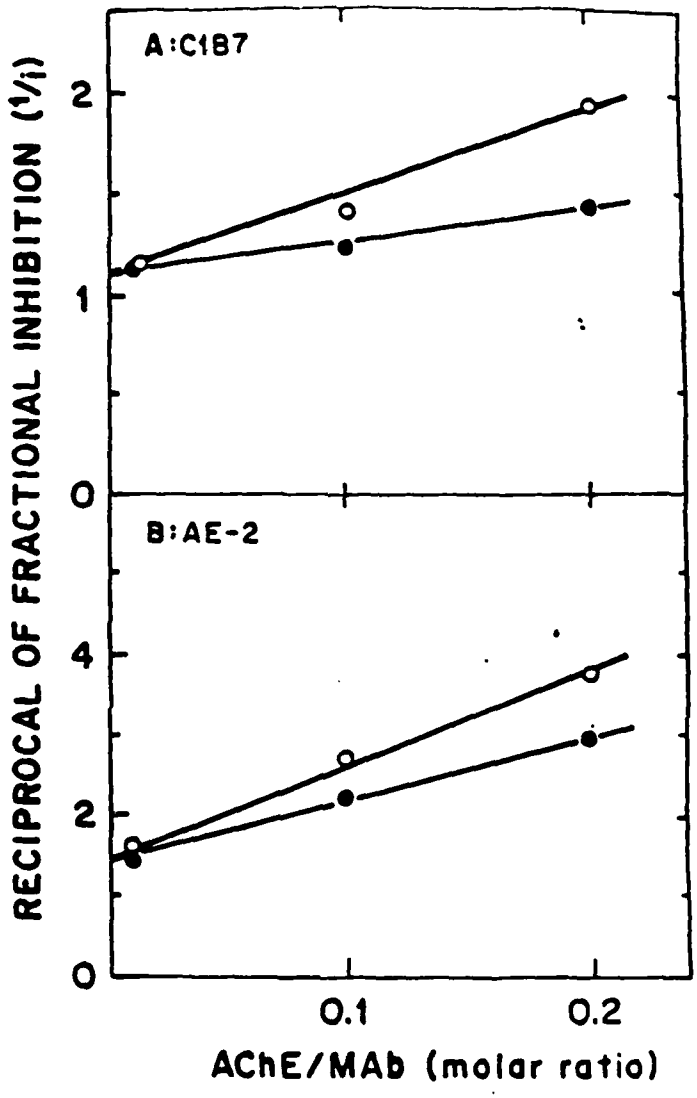

Figure 9: Antibody inhibition of AChE Activity: Webb plot. AChE (17pM) was preincubated with varying amounts of both C1B7 and AE-2. After 30 min AChE activity was determined in the presence of varying substrate concentrations. Panel A: Inhibition by C187. Panel B: Inhibition by AE-2. Substrate concentration: 0.33 mM (O) and 1.0 mM (●).

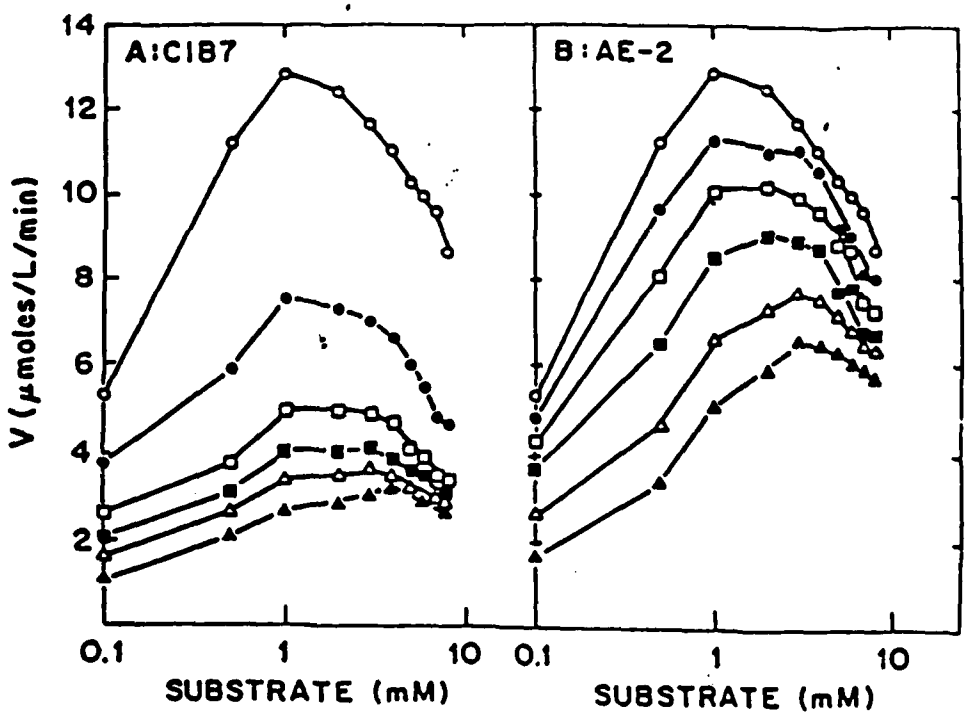

Figure 10: Antibody inhibition of AChE activity: Effects of high and supraoptimal substrate concentrations. AChE (17 pM) was preincubated with varying amounts of both C1B7 and AE-2. After 30 min AChE activity remaining was determined. Panel A, inhibition by C1B7: Antibody/AChE molar ratio = 0 (○), 1 (●), 2 (□), 5 (■), 10 (△), and 100 (▲). Panel B, inhibition by AE-2: Antibody/AChE = 0 (○), 2 (●), 5 (□), 10 (■), 25 (△), and 100 (▲).

curve) were observed. The inhibition produced by C1B7 and AE-2, however, could not be fully reversed by increasing the substrate level, even though the optimal substrate concentration was shifted at least three-fold, from 1.0 to 3.0 mM, in the presence of high antibody concentration.

The data in Fig. 10 also demonstrate typical substrate inhibition of AChE activity at mM concentrations. This substrate inhibition was also observed in the presence of antibody. Substrate inhibition and antibody inhibition exhibited summation; i.e., they were found to be essentially additive.

VI. Summation of inhibition of AChE activity by C1B7 and AE-2

As shown in Fig. 11, the inhibitory effects of the two antibodies were also found to be additive. In these experiments, AChE was treated sequentially with the two antibodies and AChE activity was subsequently determined. When AChE was pretreated with AE-2, subsequent addition of varying amounts of C1B7 resulted in increased levels of inhibition that were similar to the theoretical sum of the levels of inhibition for the two antibodies acting separately. Similarly, when enzyme was pretreated with C1B7, subsequent treatment with AE-2 resulted in inhibition that was also essentially additive.

Taken together, the binding and the enzyme kinetic data suggest the presence of additional sites or structural conformations of the enzyme that affect the catalytic process, other than those defined by traditional pharmacological agents. Five different inhibitors (DFP, neostigmine, carbachol, edrophonium, and BW284c51), which collectively span the critical structures of the active site, did not interfere with antibody binding. Nor did the antibodies interfere with substrate binding to the active site. Two different peripheral site-specific ligands (propidium and gallamine) also failed to inhibit antibody binding, and the antibodies failed to prevent substrate-dependent inhibition of enzyme activity. Finally, neither of the two inhibitory antibodies blocked binding by the other. We conclude that two different sites on the enzyme, previously not known to exist but discovered or created by these antibodies, are potential points of enzyme regulation by virtue of their effects on the rate of substrate catalysis.

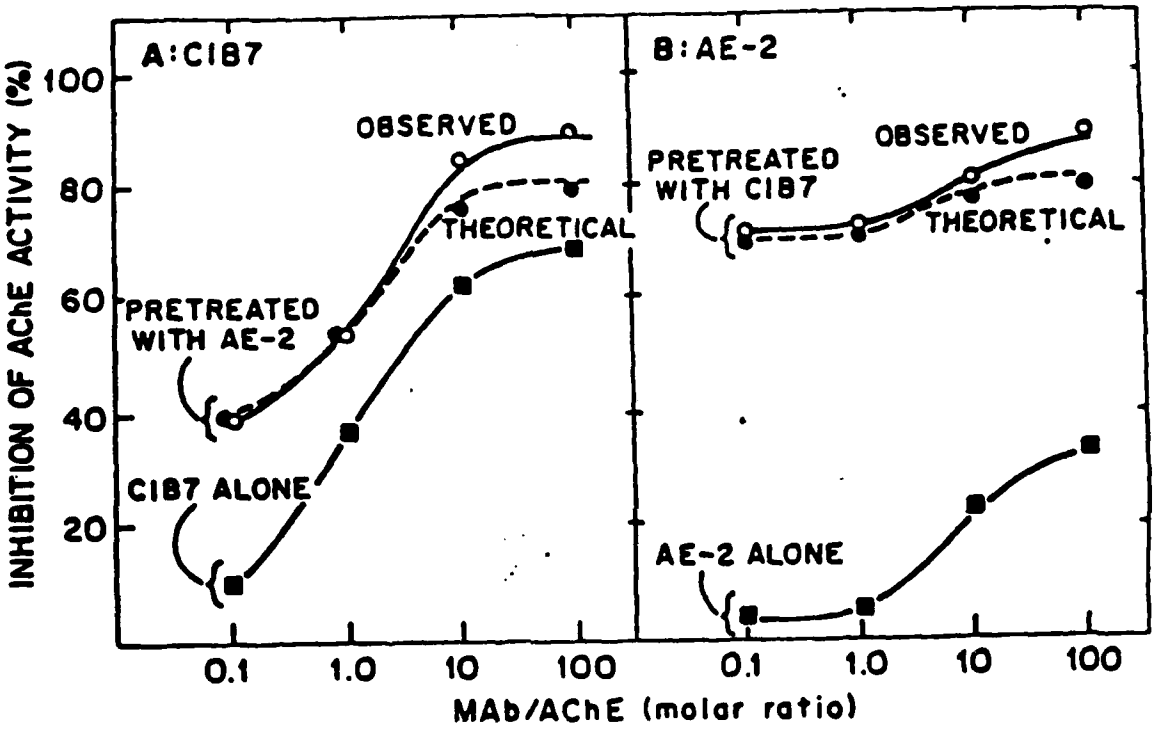

Figure 11: Antibody inhibition of AChE activity: observed vs. theoretical summation of C1B7 and AE-2 effects. AChE (17 pM) was pretreated for 30 min with a fixed amount of one antibody. Following a 30 min treatment with the other antibody, enzyme activity remaining was measured. Panel A: AChE pretreated with 100-fold molar excess of AE-2 followed by C1B7 (○); inhibition by C1B7 alone, with no AE-3 pretreatment (■). Panel B: AChE pretreated with 100-fold molar excess of C1B7, followed by AE-2 (○); inhibition by AE-2 alone, with no C1B7 pretreatment (■). Theoretical values were calculated as described in Materials and Methods.

CONCLUSIONS AND RECOMMENDATIONS

Use of monoclonal antibody affinity chromatography combined with size-exclusion HPLC has permitted us to purify human erythrocyte AChE in preparative quantities to >98% homogeneity. We have subsequently obtained amino acid sequence data from peptide fragments of the purified enzyme, and we have used this information to generate oligonucleotide probes in order to screen appropriate human cDNA libraries as a first step in the cloning and sequencing of the gene encoding erythrocyte AChE. Using probes containing all the possible base sequences corresponding to several of the AChE tryptic peptides, we identified putative positive clones in one of the four human cDNA libraries examined. Termination of the funding for these studies prevented the characterization of these clones.

We have used three different immunization techniques to generate mouse monoclonal antibodies that bind to human erythrocyte AChE. Of the 16 AChE-specific monoclonal antibodies identified, only one (C1B7) is capable of inhibiting the activity of the enzyme. This finding is consistent with the observation by other investigators that inhibitory monoclonal antibodies against AChE are relatively uncommon. Extensive characterization of the binding and inhibitory properties of C1B7 and of an inhibitory antibody produced by Dr. D. Fambrough, AE-2, has shown that the two antibodies bind to distinct sites on the enzyme, both of which are different from the active center and also from the known peripheral sites of binding by anti-cholinesterase drugs. These two antibodies are, therefore, not appropriate for use in generating anti-idiotypic monoclonal antibodies as described in our proposed plan of research. However, the fact that C1B7 recognizes a novel site on AChE has considerable significance for studies of AChE modification by exogenous modulators of enzyme function. It will be necessary to generate large numbers of additional monoclonal antibodies against human erythrocyte AChE in order to identify appropriate active site-specific inhibitory monoclonal antibodies for use in generating anti-idiotype antibody analogues of the active site of AChE.

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Sue Penno, Ph.D.	Investigator
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