HUMAN BRAIN ACETYLCHOLINESTERASE

A THESIS SUBMITTED FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

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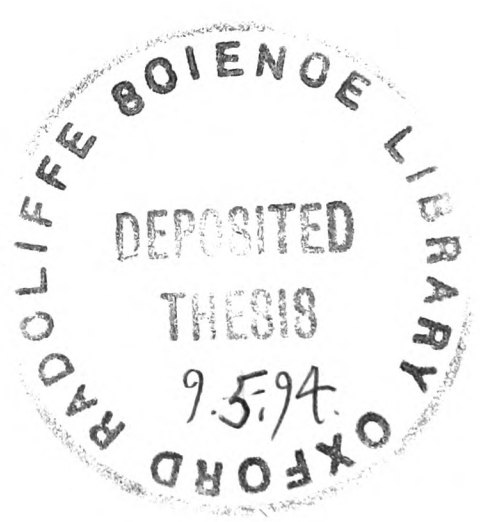
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ABSTRACT

HUMAN BRAIN ACETYLCHOLINESTERASE

Abstract of a thesis submitted for the degree of Doctor of Philosophy by **PHILIPP NOVALES-LI,** ***St. Hugh's College, University of Oxford, Michaelmas Term 1993.***

Procedures for the preparative purification of human acetylcholinesterase (AChE; EC 3.1.1.7) have been examined. Chromatofocussing showed caudate nucleus and cerebrospinal fluid (CSF) AChE eluting between isoelectric points (pI) of 5.2 - 5.57, whilst isoelectric focussing (IEF) revealed heterogeneous AChE isoforms migrating between pI 4.5 - 6. Affinity chromatography proved to be better in yielding highly pure samples. The ligands used were either acridinium or procainamide, which yielded brain AChE recoveries of 10.1% and 42.8%, respectively. Various other methods were used such as gel filtration and ion-exchange chromatography on a Pharmacia SMART System.

Purified brain AChE was used as antigen to generate anti-AChE monoclonal antibodies (mAbs) by in vivo immunization. BMS-3E4, -7G10, and -9F4 reacted with erythrocyte (G_2) AChE, whilst BMS-6D6 bound to brain (G_4) AChE in dot blot, titration, and sedimentation experiments. Conversely, the four mAbs recognized only the G_4 form and not the G_2 form on immunoblotted IEF and non-denaturing Clarke gels. Deglycosylation studies suggest that the four mAbs recognize a carbohydrate epitope linked to AChE.

As a preparative step, tissue culture media containing these mAbs were ammonium sulphate precipitated and purified on a gel filtration column. Under reducing conditions, two bands migrating at 80 and 25 kDa were seen, corresponding to the heavy and light chains of Immunoglobulin M (IgM).

Four additional mAbs were raised by the novel method of in vitro immunization, using a synthetically-produced C-terminus peptide as antigen. BMS-5, -6, -7, and -8 are all IgMs which recognize soluble brain and not erythrocyte nor membrane-bound AChE in dot blots, IEF, and sedimentation analyses.

Although immunocytochemistry of the above-mentioned mAbs did not demonstrate any positive cholinergic binding, the present mAbs may still offer potential clinical and biochemical applications.

THIS THESIS IS LOVINGLY DEDICATED TO:

The Rt. Hon. Angelita Novales-Li
Dr. Erlinda T. Novales
The Hon. Maryann Novales-Li

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LIST OF ABBREVIATIONS

ACh	*acetylcholine*
AChE	*acetylcholinesterase*
AD	*Alzheimer's disease*
APS	*ammonium persulphate*
ASF	*antigen specific factor*
BMS	*Bristol-Myer Squibb*
BSA	*bovine serum albumin*
BuChE	*butyrylcholinesterase*
ChAT	*choline acetyltransferase*
CNS	*central nervous system*
CSF	*cerebrospinal fluid*
DAB	*diaminobenzidine tetrachloride*
DEAE	*diethylaminoethyl*
DMF	*N,N-dimethylformamide*
DMSO	*dimethylsulfoxide*
DPA	*(3H)N,N-dimethyl-2-phenyl-aziridinium*
ELISA	*enzyme-linked immunoassay*
FBS	*foetal bovine serum*
FCS	*foetal calf serum*
FPLC	*fast performance liquid chromatography*
HAT	*hypoxanthine, aminopterine, and thymidine*
HPLC	*high performance liquid chromatography*
HT	*hypoxanthine and aminopterine*
IEF	*isoelectric focussing*
Ig	*immunoglobulin*
IgM	*immunoglobulin M*
IMDM	*Iscove's Modified Dulbecco's Medium*
kDa	*kilodalton*
LPS	*lipopolysaccharide*
mAb	*monoclonal antibody*
NMR	*nuclear magnetic resonance*
NSF	*antigen nonspecific factor*
OA	*ovalbumin*
PAGE	*polyacrylamide gel electrophoresis*
PBS	*phosphate buffered saline*
PEG	*poly(ethylene) glycol*
pI	*isoelectric point*
R.T.	*room temperature*
S	*Svedberg unit*
SEM	*standard error of the mean*
SDS	*sodium dodecylsulphate*
TEA	*tetraethyl ammonium*
TEMED	*N,N,N',N'-tetramethyl-1,2-diaminoethane*

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Chapter I.

INTRODUCTION

GENERAL INTRODUCTION

General classes of cholinesterase

It is a generally held view that choline esters are hydrolyzed by cholinesterases (Stedman and Stedman, 1935). Their role in cholinergic neurotransmission was first described by Dale (1914). Although several types of cholinesterases may exist, only two classes have been well defined to date. One is the "true" or "specific" cholinesterase under which acetylcholinesterase (AChE) is classified (Augustinsson, 1948). The other is the "pseudo" or "nonspecific" cholinesterase of which butyrylcholinesterase (BuChE) is an example (Mendel and Rudney, 1943). These two classes are basically distinguished by their specificities for their respective substrates, i.e. acetylcholine and butyrylcholine for AChE and BuChE, respectively. In some cases however, substrate specificity is not well-defined, as in the case of propionylcholine of which hydrolysis by BuChE is carried out faster than the hydrolysis of butyrylcholine. From a historical point of view, AChE was referred to as an "erythrocyte enzyme", whilst BuChE was termed "serum enzyme" to describe their initial sites of isolation. Recently, however, with the plethora of research being carried out on the biology and chemistry of both representative classes, a more straightforward terminology, i.e. acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8) has been adopted.

AChE and BuChE belong to a family of enzymes which are known to exist in multiple molecular forms (i.e. polymorphic), and may either occur in soluble or membrane-bound states. In spite of AChE and BuChE being distinct structural entities, their identification may oftentimes be confusing. Therefore, the following criteria, taken from Cooper *et al.* (1986), should be taken into consideration: (1) Optimum substrate- Faster rates of enzyme activity are achieved when acetylcholine and butyrylcholine are hydrolyzed by AChE and BuChE, respectively. But this should not be taken as the general rule. As a case in point, chick brain AChE hydrolyzes propionylcholine faster than acetylcholine. Furthermore, AChE from a bee's head does not hydrolyze acetylcholine nor butyrylcholine, but will hydrolyze methacol (acetyl-ß-methylcholine). (2) Dose-response relation- At super-saturating concentrations of acetylcholine, butyrylcholine, or propionylcholine, AChE activity is inhibited, thus displaying a bell-shaped substrate-velocity curve (Mendel and Rudney, 1943). Conversely, BuChE is not inhibited at high substrate concentrations, giving a normal Michaelis-Menten curve. (3) Enzyme localization- AChE is widely distributed in neural tissue whereas BuChE may be found in non-neural tissue. Again as an exemption to the rule, neural tissues like the autonomic ganglia and non-neural tissues like the liver and lungs all contain AChE and BuChE. Further, although the plasma contains BuChE, erythrocytes contain only AChE. (4) Response to inhibitors- The use of

inhibitors proved useful tools to specifically distinguish one enzyme from the other. AChE may be inhibited by specific inhibitors like BW284C51 and WIN 8077 (Ambenonium). Other reversible inhibitors include edrophonium and the carbamoylating drugs, like neostigmine and physostigmine, which all bind to the active site of AChE. Conversely, agents like gallamine and propidium bind to a peripheral site of the enzyme. With respect to BuChE, this can be inhibited by diisopropyl phosphorofluoridate.

The above-mentioned criteria, although not exhaustive, proved sufficient basis for the clear delineation of AChE from other cholinesterases. In the remaining part of this introduction, emphasis will be made on the biology, chemistry and structure of AChE, which is the main protein of interest in this thesis.

Molecular forms of AChE

AChE exist as six molecular forms, based on studies of its hydrodynamic properties. Chemical analysis of AChE from the electric organs of *Electrophorus electricus* (electric eel) and *Torpedo marmorata* (marine electric ray) have shown that the variation in molecular forms is mainly in their solubility and mode of attachment to plasma membranes, rather than differences in catalytic rates. Studies on AChE extracts solubilized by homogenization and subjected to sedimentation analysis in sucrose gradients provided the basis for classifying the six molecular forms into two main classes-- one existing as an assembly of

homologous subunits, the other as an assembly of dimensionally heterologous subunits. For purposes of nomenclature, these forms have been termed G_1, G_2, G_4 and A_4, A_8, A_{12}, respectively (Bon *et al.*, 1979).

The G forms are composed of globular catalytic subunits which appears as monomers (G_1), dimers (G_2), or tetramers (G_4). Their sedimentation coefficients (in Svedberg units) are 5.35S (G_1), 7.7S (G_2), 11.8S (G_4) for *Electrophorus* AChE (Bon *et al.*, 1976), and 5.0S (G_1), 7.8S (G_2), 11.1S (G_4) for *Torpedo* AChE (Bon and Massoulie, 1980). Using the products of sedimentation coefficients and Stokes radius, the molecular mass of these forms were calculated as 70 kDa (G_1), 155 kDa (G_2), and 331 kDa (G_4).

G_1 AChE is a monomer composed of a globular catalytic subunit which represents the simplest active form of this enzyme. The rest of the G forms differ in the degree of association of the identical subunits. In G_2 AChE, the subunits are associated as dimers linked together by a direct intersubunit disulfide bond (Bon and Massoulie, 1976). This form is mainly present in *Torpedo* electric organs, insect CNS, and mammalian erythrocytes. G_4 AChE is a tetramer composed of two pairs of dimers, of which the catalytic subunits are covalently associated by disulfide bonds into dimers. The two dimers are probably linked together by van der Waals forces. G_4 AChE is mainly found in mammalian CNS, where it has been demonstrated that two of the four catalytic subunits of G_4 are disulfide bonded

to a 20 kDa membrane-binding, noncatalytic subunit (Inestrosa *et al.*, 1987; Gennari *et al.*, 1987).

Globular AChE forms differ not only in their degree of assembly, but also in their hydrophobicity. These exist both as hydrophobic molecules, interacting with detergents; or as hydrophilic molecules, which are unaffected by non-ionic detergents after extraction. For example, G_1 is mainly hydrophilic because it lacks a short strand of hydrophobic peptide in its chain (Lazar and Vigny, 1980). Alternatively, hydrophobicity (as in the case of G_2 and G_4) may result from post-translational modification by the covalent attachment of a glycoinositol phospholipid or hydrophobic chain, respectively to the C-terminal carboxyl groups.

The A forms are composed of catalytic subunits arranged in single (A_4), double (A_8), and triple (A_{12}) tetrameric groups, in which two catalytic subunits are linked by an intersubunit disulfide bond, whilst the other two are disulfide-linked to a 24 kDa non-catalytic, collagen-like tail. The latter forms a rod-like fibrous tail of 50 nm length and 2 nm width, which is composed of three polypeptides linked by intersubunit disulfide bonds and arranged as a triple-helical structure (Rosenberry *et al.*, 1980). This triple helix domain is referred to as a collagen-like structure, due to the presence of marked amounts of hydroxyproline and hydroxylysine, components which are characteristic of collagen, as based on amino acid sequence analysis (Rosenberry and Richardson, 1977).

It is actually this collagen-like tail which gives some degree of asymmetrical dimension to this enzyme form. Likewise, this tail provides a structural framework to allow the "A" forms to be tethered to the basal lamina of synapses.

Their sedimentation coefficients are 9.1S (A_4), 14.2S (A_8), 18.4S (A_{12}) for *Electrophorus* (Bon *et al.*, 1976), and 9.4S (A_4), 13.4S (A_8), 17.4S (A_{12}) for *Torpedo* (Bon and Massoulie, 1980). Their calculated molecular weights are 410 kDa, 796 kDa, and 1150 kDa, respectively. A_{12}, which represents the principal molecular form of this class is found mostly in neuromuscular synapses.

Cell surface association of AChE molecular forms

The multiple molecular forms of AChE differ in their modes of anchoring to the cell surface membrane. One such mechanism, possessed by A_{12} AChE, involves interaction via ionic forces of a collagen-like fibrous tail to a heparin-like proteoglycan matrix. Another, as in the case of G_2 AChE, involves a phosphatidylinositol moiety. And finally, G_4 AChE possesses a 20 kDa hydrophobic polypeptide anchor (Silman and Futerman, 1987). The details of these modes of attachments are herewith presented.

(1) Asymmetric A_{12} AChE

Earlier studies on asymmetric A_{12} AChE have designated the collagenous subunit of this enzyme as responsible for its attachment to basement membrane components. The A_{12}

AChE form binds best to basement membrane heparan sulphate proteoglycan and type V collagen, and to a lesser extent to laminin, fibronectin, and type I collagen, but not to type IV collagen (Vigny *et al.*, 1983). Intense interest on the role of heparan sulphate proteoglycans on the anchorage of asymmetric AChE to the synaptic basal lamina was heightened after the discovery that heparin, a sulfated glycosaminoglycan, was capable of solubilizing AChE from the endplate regions of the rat diaphragm muscle. It was observed that heparin, at an ED_{50} dose of 50 μg/ml, specifically released the A_{12} and A_8 forms of the enzyme (Torres and Inestrosa, 1983). Work on rat sciatic nerve preparations also showed that heparin released asymmetric AChE from these ligated nerves. Application of other glycosaminoglygans, such as chondroitin sulphate and hyaluronic acid, did not demonstrate any visible solubilizing effect. Further, application of protamine seemed to have abolished the solubilizing effect of heparin (Torres *et al.*, 1983). Solubilization was done under high-ionic strengths (i.e. 100 mM NaCl) to release the asymmetric A_{12} form, sedimenting at 16S. Under low-ionic strengths however, heparin releases synaptic AChE not as individual 16S forms, but as multi-molecular aggregates sedimenting at 30S, which may represent the physiologically important state of aggregation of the 16S form in the basal lamina (Torres and Inestrosa, 1985). From these findings, it was postulated that "heparin-like binding domains", possibly located on A_{12} AChE's collagen-like tail, are

involved in the immobilization of asymmetric AChE to the basal lamina of neuromuscular junctions. Likewise, it is possible that heparin may be involved in the intra-axonal localization and transport of the A_{12} form.

In order to prove the interaction between asymmetric AChE and heparan sulphate proteoglycans, several experiments were devised. In one study, specific glycosaminoglycan hydrolases, such as heparitinase (an enzyme that catalyzes heparan sulphate) and chondroitinase ABC (catalyzes both chondrotin-4 and -6 sulphates and dermatan sulphate) were incubated with electric organs of *Discopyge tschudii*. Heparitinase treatment, but not chondroitinase ABC, was able to extract asymmetric A_{12} AChE from the extracellular matrix (Brandan *et al.*, 1985). In another experiment, the binding of exogenous asymmetric A_{12} AChE to intact cells that are rich in cell-surface heparan sulfate proteoglycans was examined. A_{12} AChE was shown to bind specifically to intact endothelial cells in a time-dependent and saturable manner. This binding was reversed by a high concentration of exogenous heparin, but not by chondroitin sulphate or hyaluronic acid (Brandan and Inestrosa, 1986). These findings provide sufficient evidence to suggest that a heparan sulphate proteoglycan is involved in the anchorage of the collagen-tailed A_{12} AChE to the basal lamina at the neuromuscular junction.

In a further study, the distribution of asymmetric AChE was analysed in a stable variant (F3) of a rat pheochromocytoma cell line (PC12) which lacks a heparan

sulphate proteoglycan on its cell surface. Following treatment with membrane-permeable and membrane-impermeable inhibitors of AChE, it was discovered that about 60% of asymmetric AChE was located in the internal cytoplasm of the variant F3 cells, whilst in normal cells, about 90% of the enzyme was located externally. Moreover, when all forms of AChE from the F3 cells were irreversibly blocked, newly-synthesized AChE first appeared in the internal pool and then was detected on the cell surface (Inestrosa *et al.*, 1985). This finding may suggest that A_{12} AChE is first assembled within the cytoplasm and then brought externally following synthesis and distribution of proteoglycans.

(2) Dimeric Globular (G_2) AChE

Recently, glycosylated forms of phosphatidylinositol have been implicated to subserve biological membrane functions in eukaryotic organisms. It has been shown that these molecules play a role in anchoring particular cell-surface proteins to the membrane (Low and Saltiel, 1988). The amphiphilic globular G_2 AChE form is one example of a molecule which is anchored to a cell membrane by a covalently-linked phosphatidylinositol-containing glycophospholipid (Futerman *et al.*, 1985c). This was supported by the observation that phosphatidylinositol-specific phospholipase C (PIPLC) from *Staphylococcus aureus* was capable of extracting AChE from synaptic plasma membranes and intact synaptosomes of *Torpedo ocellata*

electric organs. The effect of other phospholipases, such as a nonspecific phospholipase C from *Clostridium welchii*, failed to solubilize AChE from *Torpedo*. The solubilized AChE sedimented at 7S, corresponding to the G_2 AChE form (Futerman *et al.*, 1985a). Direct biochemical analysis of the membrane-anchoring chain was carried out by the use of a photoactive reagent, 3-trifluoromethyl-3-(m-[125I]iodophenyl) diazirine ([^{125}I]TID), which has a high affinity for hydrophobic domains in proteins. Its mode of action is by attachment into the hydrophobic phase of membranes or into detergent micelles, and upon photoactivation, it reacts covalently with protein sequences in this phase. The labeled fragment was shown, after proteolysis of the G_2 AChE form, to have a molecular size of 3 kDa (Stieger *et al.*, 1984).

Based upon a nitrous acid deamination reaction, the attachment of the phosphatidylinositol moiety to the globular catalytic subunit of AChE was shown to be through a glycosidic linkage to a sugar (glucosamine) residue bearing a free primary amino group. The glucosamine is attached to a partially-characterized glycolipid, and the latter is attached to the COOH-terminus of the enzyme subunits by an ethanolamine residue (Low *et al.*, 1987; Rosenberry *et al.*, 1986). Recently, it has been shown that the glycan connecting AChE and the phosphatidylinositol moiety has been conserved during evolution. This moiety is present in other surface proteins such as alkaline phosphatase, Thy-1 glycoprotein, and *Trypanosoma*

variant surface glycoproteins (Low *et al.*, 1986). Attachment of the glycosyl-phosphatidylinositol moiety to AChE is directed by a signal sequence at the C-terminus of the protein which is itself removed during the attachment process. It is suggested that this anchor may play a versatile role in regulating cell-surface protein expression (Low, 1989).

Identification of this covalently-bound phosphatidylinositol moiety has been made for G_2 AChE from a variety of sources. These include the following:

Torpedo electric organ	(Futerman *et al.*, 1985b),
Xenopus laevis skeletal muscle	(Inestrosa *et al.*, 1988),
Schistosoma mansoni tegumental membrane	(Espinoza *et al.*, 1991),
Bovine adrenal medulla chromaffin cells	(Prieto *et al.*, 1989),
Bovine erythrocyte	(Roberts *et al.*, 1988),
Rat liver hepatocytes	(Perelman and Brandan, 1989).

All of these enzymes are solubilized by PIPLC, with the exception of human and mouse G_2 AChE which are only partially solubilized. This is so because the glycoinositol phospholipid membrane anchor of human G_2 AChE is novel, in that it is palmitoylated on the inositol ring. It is the existence of this additional fatty acyl chain that is responsible for the resistance to PIPLC solubilization (Roberts *et al.*, 1988). Recently however, a serum anchor-specific phospholipase D was shown to specifically cleave this anchor moiety (Toutant *et al.*, 1989).

(3) Tetrameric Globular (G_4) AChE

The detergent-soluble G_4 AChE form is most commonly found in mammalian brain tissues. Initially, it was speculated that both the G_2 and G_4 forms share a common form of anchorage. However, incubation of G_4 AChE with PIPLC did not yield any traces of solubilization (Futerman *et al.*, 1987).

Extraction of G_4 AChE by detergent from bovine caudate nucleus, followed by affinity purification was then attempted. When the purified material was subjected to non-reducing conditions, five bands corresponding to monomers and disulfide-linked oligomers of individual 70 kDa subunits were seen (Inestrosa *et al.*, 1987). When the enzyme was labeled with [^{125}I]TID, which was mentioned above as a photoactivated reagent selective for the hydrophobic membrane-binding domains of proteins, reducing conditions show silver-staining of a single band at 70 kDa, although the [^{125}I]TID-label corresponded to a 20 kDa band. It is proposed that this 20 kDa hydrophobic subunit contains the membrane attachment site of the enzyme (Fuentes and Inestrosa, 1988).

The presence of this 20 kDa subunit was also confirmed in human caudate nucleus AChE (Gennari and Brodbeck, 1985). In a related study, analysis of G_4 AChE from human caudate nucleus revealed that under limited digestion with proteinase K, an enzyme was isolated which was catalytically active and yet hydrophilic and incapable of aggregation (Gennari *et al.*, 1987). Using bovine brain G_4

AChE which was treated with proteinase K and labeled with [^{125}I]TID, a 13 kDa hydrophobic fragment was identified. It was suggested that this 13 kDa fragment, which comes from a previously labeled 20 kDa species, may be directly involved in anchoring G_4 AChE to neuronal plasma membrane (Fuentes *et al.*, 1988). More recently, analysis of this 13 kDa fragment revealed that upon digestion with pronase, a protease-resistant fragment of 7 kDa, containing fatty acids, could be identified (Inestrosa and Perelman, 1990).

From biochemical studies of the 20 kDa subunit, it can be deduced that three different subdomains exist: a proximal subdomain of 7 kDa having disulfide linkages to the catalytic subunit, a central 6 kDa subdomain which is sensitive to proteinase K, and a distal 7 kDa pronase-resistant subdomain which is directly bound to the neuronal membrane.

Binding Sites in the AChE Molecule

AChE is an allosteric enzyme possessing a highly efficient catalytic mechanism. With the advent of studies on the primary/quaternary structure and molecular biology of this enzyme, it was then possible to assign roles to specific amino acid residues in relation to substrate-binding kinetics.

(1) Esteratic Active Subsite

Although AChE belongs to a global family of serine hydrolases, it does not show any similarity in sequence to

other serine hydrolases such as trypsin or serine protease. Nonetheless, they all share a common mode of catalysis which involves a process of acylation-deacylation at a serine residue in the catalytic site (MacPhee-Quigley *et al.*, 1985). This serine residue has a functional -OH group which is hydrogen bonded to a "catalytic triad", i.e. Ser-OH-His-HOOC-Asp. Indeed, it has been shown that AChE from diverse sources display functional similarities in reaction dynamics and transition state structures (Acheson and Quinn, 1990).

To elucidate the residues involved in enzyme catalysis, serines were labeled with [3H]isopropyl fluorophosphate and then site-directed mutation of serine residues was made. Mutation of *Torpedo* AChE ser-200 to cysteine brings about reduced activity, whilst its mutation to valine abolishes catalytic activity (Gibney *et al.*, 1990). In comparison with other species, the active site serine is at position 198 in human serum AChE (Lockridge *et al.*, 1987), position 195 in rabbit liver microsome AChE (Korza and Ozols, 1988), and at position 201 in the moth *Heliothis* (Hanzlik *et al.*, 1989). In position 440, mutation of two conserved histidines to glutamine led to abolition of catalytic activity, whilst for mutation at position 425, only a partial reduction was noted. Thus, His-440 plays the role of an intermediary charge relay residue. As for the third residue in the "catalytic triad", this was previously thought to be glutamate at position 199. Recently however, x-ray crystallography

revealed this to be a glutamate at position 327 (Sussman *et al.*, 1991). This is quite unusual since in other serine hydrolases, Asp is usually the third residue in the triad. The importance of this "catalytic triad" as the active esteratic site is in optimizing the nucleophilicity of the serine-OH group, and to allow proton transfer during catalysis. This could then influence substrate association and the ability of a second substrate to modify catalysis (Hucho *et al.*, 1991). Structurally, this catalytic triad is contained in a deep and narrow gorge which forms the binding pocket for acetylcholine (Maelicke, 1991).

(2) Central anionic subsite

The observation that charged substrates promoted greater hydrolysis by AChE led to the possibility of the presence of an "anionic subsite" near the active site (Tougu *et al.*, 1987). This is further supported by the observation that highly methylated ammonium inhibitors of AChE exert greater blocking effect. This may suggest that such an anionic subsite possesses a negative charge around its surrounding and is also hydrophobic.

Identification of the anionic subsite in the catalytic centre of AChE was made possible by affinity labelling of peptide sequences with the alkylating agent [3H]N,N-dimethyl-2-phenyl-aziridinium (DPA). Coupled with the use of specific ligands, it was observed that in the presence of edrophonium (a specific AChE inhibitor which binds to the anionic subsite), labelling with DPA was precluded

(Weise *et al.*, 1990). Using this approach, the peptide sequence of the anionic site for Torpedo AChE was found at positions 79-87, corresponding to Ser-Gly-Ser-Glu-Met-Trp-Asn-Pro-Asn. Binding of the label was at Trp-84 (Kreienkamp *et al.*, 1991).

(3) Peripheral anionic subsite

The ability of some cationic substances to noncompetitively inhibit AChE activity has opened the possibility of the existence of additional anionic binding subsites. The ability of acetylcholine, haloxon, and a coumarin derivative to dissociate propidium from the "peripheral" anionic subsite was demonstrated by direct fluorescent titrations and inhibition kinetics. It was then suggested that propidium acts as a selective ligand for the peripheral anionic site of AChE (Radic *et al.*, 1991).

Using affinity labeling with DPA, the peripheral subsite was located at positions 270-278, corresponding to a sequence of Lys-Pro-Gln-Glu-Leu-Ile-Asp-Val-Glu (Weise *et al.*, 1990).

The peripheral anionic site is said to possess allosteric properties. Binding of a substrate to this site brings about carbamoylation at the esteratic active site. Further, conformational changes in the active site are also brought about, as deduced from polarometric and NMR studies (Hucho *et al.*, 1991). Glutamate-199, which is next to the active site serine-200, may be involved in this conformational change (Gibney *et al.*, 1990).

Drug Inhibitors to AChE

To effectively analyze the structure-activity relationship of AChE, there was a need to develop potent inhibitors to the enzyme. Several of these inhibitors are organophosphorus compounds which show non-reversible inhibition of AChE. Soman is a powerful organophosphorus AChE inhibitor demonstrating an immediate and potent effect which generally differed from one AChE structure to another. The time course of recovery was at least 96 hrs, depending on the structure. In general, the effect lasted even after 8 days (Lemercier *et al.*, 1983). On a physiological level, soman-induced AChE inhibition brought about convulsion in rats (Petrali *et al.*, 1991). In another study, it was observed that the degree of in vivo inhibition by O-pinacolyl-methylfluorophosphonate increased in proportion to the molecular size of AChE (Bajgar and Mate, 1989). In a similar manner, in vivo injection of diisopropyl fluorophosphate (DFP) on rat brain showed more pronounced inhibition of the G_4 form vis-à-vis G_1. It was suggested that in vivo inhibition was dependent on variations in accessibility of the inhibitor, possibly due to the difference in subcellular localization of G_4 and G_1. The possible role of intrinsic sensitivity of each form was precluded since in vitro effects did not show any remarkable inhibitory differences between the two AChE forms (Volpe *et al.*, 1990). Other organophosphorus compounds, which are commonly used as systemic insecticides, have been shown to exert in vivo inhibitory

effects on AChE. These include O,O-dimethyl O-(2,2-dichlorovinyl)phosphate (DDVP), fenitrothion, formothion, and phorate (Singh and Agarwal, 1983; Kobayashi *et al.*, 1983).

In a recent study, the effect of several inhibitors on AChE molecular forms was comparatively investigated on rat brain. Heptylphysostigmine (HEP) was more selective for soluble G_1 AChE than the soluble G_4 form. On the other hand, neostigmine showed selective inhibition for both the soluble and detergent-extracted G_1 forms. Other inhibitors such as physostigmine, echothiophate, BW284c51 [1,5-bis-(4-allyldimethylammoniumphenyl) penta-3-1 dibromide], tetrahydroaminoacridine, and metrifonate showed similar inhibitory potencies for both soluble and detergent-extractable forms (Ogane *et al.*, 1992a). In another study done in human brain AChE, a similar preferential inhibition of the soluble G_1 form by heptylphysostigmine was noted. It was also discovered that edrophonium inhibited the soluble G_4 form more potently than G_1 (Ogane *et al.*, 1992b). These results suggest that the differential sensitivity of AChE molecular forms to these inhibitors are mediated through an allosteric mechanism. Further, an explanation on the mode of drug inhibitory action on AChE may be somewhat difficult since both the esteratic active and the peripheral anionic sites are implicated. Nonetheless, for inhibitors which directly act on the esteratic active site, it is likely that an acylation or

deacylation step brings about the inhibitory action (Taylor, 1991).

Genetic Basis of AChE Structure

The complete amino acid sequence of AChE from *Torpedo californica* has been deduced from the nucleotide sequence of a cloned complementary DNA (cDNA). *Torpedo* AChE is a 575-residue protein which displays striking homology to the C-terminal portion of thyroglobulin (Schumacher *et al.*, 1986). The sequence of foetal bovine serum (FBS) AChE has a high degree of similarity with that of other cholinesterases, liver carboxyesterases, esterase-6, lysophospholipase, and also, thyroglobulin (Doctor et al., 1990). In comparing this sequence with AChE from other sources, the amino acid sequence of FBS AChE shows homologies of 90% with human AChE, 88% with mouse AChE, 60% with *Torpedo* AChE, 50% with human serum BuChE, and 39% with *Drosophila* AChE (Doctor *et al.*, 1990; Rachinsky *et al.*, 1990). More recently, the sequence of human AChE has been demonstrated (Soreq *et al.*, 1990).

Identification of the intersubunit and intrasubunit disulfide bonds for *Torpedo* AChE, based upon tryptic digestion, gel filtration, reverse-phase HPLC, and sequencing, revealed 8 cysteine residues per catalytic subunit. Three pairs of intrasubunit disulfide bonds were identified, to wit: Cys-67 and Cys-94, Cys-254 and Cys-265,

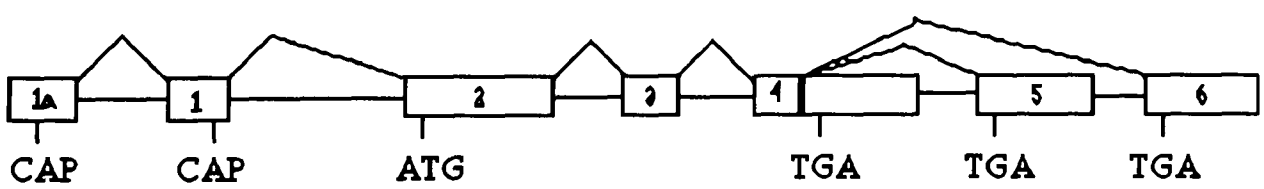
and Cys-402 and Cys-521. Towards the C-terminal, Cys-572 forms an intersubunit disulfide bond with Cys-572 in another subunit. A potential free sulfhydryl residue was identified as Cys-231. The *Torpedo* intrasubunit disulfide bonds are conserved in the same positions as in thyroglobulin, whereas Cys-231 and Cys-572 are not (MacPhee-Quigley *et al.*, 1986). In comparison, intrasubunit disulfide bonds for human serum AChE were located between Cys-65 and Cys-92, Cys-252 and Cys-263, and Cys-400 and Cys-519. The intersubunit disulfide bond was made at Cys-571, whilst the single sulfhydryl residue was at Cys-66. In general, disulfide bonds in human serum AChE are almost similar in location to those of *Torpedo* AChE. However, AChE's disulfide bonds seem to differ from those of other serine esterases such as trypsin and subtilisin (Lockridge *et al.*, 1987).

Studies on cDNA and amino acid sequence of AChE have revealed that the multiple molecular forms of AChE are most likely encoded within one single gene (Gibney *et al.*, 1988). In the AChE gene, two exons, corresponding to bases -22 to 1502 and 1503 to 1669, are believed to encode a leader peptide and the first 535 amino acids of the catalytic subunit. A third exon (III) encodes a hydrophobic c-terminal region (Maulet *et al.*, 1990).

Results obtained from Northern blot hybridization of AChE RNA revealed different messenger RNA species which may account for the structural diversity of AChE. In general, polymorphism may be brought about by oligomerization and

cross-linking of catalytic subunits, limited proteolysis, attachment to unrelated proteins, or post-translational modifications such as sugar moieties of glycoproteins. In the case of AChE, alternative mRNA splicing of a single gene giving rise to globular and asymmetric forms, may be a more plausible explanation for polymorphism (Sikorav *et al.*, 1988). Based on RNase protection experiments, three points of divergence in the mRNA species have been demonstrated. The divergence corresponding to exon III of the open reading frame, namely within the C-terminal region just 5' to the TGA termination codon, gives rise to two classes of AChE (Schumacher *et al.*, 1988).

In one experiment, expression of exons I, II and IIIH from the cDNA by transient transfection in COS-1 cells brought about assembly of G_2 AChE on the outer surface of the cell membrane. Application of phosphatidylinositol-specific phospholipase C released this enzyme into the medium. Further, a cDNA encoding a mutant truncated AChE which lacks the C-terminus sequence encoded by exon IIIH



Genomic structure of the mammalian AChE gene (taken from Massoulie *et al.*, 1993). Exon 4 continues into the genomic sequence as a 'read-through' (R) transcript or spliced alternatively to exon 5 (H subunit) or 6 (A subunit). The R transcript is present in mouse but not in human erythrocytes.

brought about a protein which was secreted directly into the medium. Conversely, an exon I and IIIH fusion mutant when transfected in COS-1 cells expressed a protein on the cell surface (Gibney and Taylor, 1990). *Ergo*, the C-terminus domains expressed by either of the two coding exons seem to determine the cellular disposition of the enzyme. Exons I, II and IIIH encode the globular phosphatidylinositol-containing AChE (G_2 form), whilst exons I, II and IIIA encode for the asymmetric collagen-tailed AChE (A_{12} form). These conditions are consistent with the existence of a single gene coding for the different catalytic subunits of *Torpedo* AChE (Sikorav *et al.*, 1987). Such is also the case for variants of avian AChE which are encoded by alleles of a single gene and where differences in assembly and localization are brought about by postranslational modifications (Rotundo *et al.*, 1988). Evidence for a single gene has also been shown for human serum and brain BuChE (McTiernan *et al.*, 1987; Arpagaus *et al.*, 1990).

Assembly and processing of AChE

The assembly and processing of AChE molecular forms have been studied in neuronal and muscle cells in culture. It is postulated that a single transport pathway is responsible for deposition of both soluble and membrane-bound AChE.

In cultured chick embryo muscle cells, asymmetric A_{12} AChE is assembled in the Golgi apparatus (Rotundo, 1984).

Only catalytically active subunits are processed by the cell. About 70-80% of the newly-synthesized AChE is catalytically inactive and therefore do not transit the Golgi apparatus, being degraded intracellularly by an unknown mechanism. This mode of degradation is thought to function as a post-translational regulatory step in controlling abundance of exportable proteins (Rotundo, 1988). The processed enzyme is then transported intracellularly through isolated clathrin-coated vesicles, being brought to the plasma membrane and then anchored to an extracellular heparan sulphate proteoglycan basal lamina (Benson *et al.*, 1985; Rotundo *et al.*, 1989).

Non-cholinergic functions of AChE

It is a generally held view that the main role of AChE would be in hydrolysing ACh, and hence terminating its transmitter action in neuromuscular junctions. Its presence, however, in non-neuronal tissues such as erythrocytes (Ott *et al.*, 1975), platelets (Smith *et al.*, 1980), and lymphocytes (Szelenyi *et al.*, 1982) has led to speculations that AChE may subserve functions other than the classical role in cholinergic transmission.

(1) AChE secretion from non-cholinergic cells

In some areas of the brain like the cerebellum, globus pallidus, hypothalamus, and substantia nigra, the level of AChE is somewhat high vis-à-vis that of choline acetyltransferase (ChAT), which is the enzyme responsible

for synthesizing acetylcholine (ACh). This may imply that AChE subserves a function other than hydrolysing and hence terminating the action of ACh.

AChE may exist as a secretory protein and such is the case in the hippocampus where secreted AChE originates from nerve terminals of the cholinergic septohippocampal pathway. It has been shown that AChE in the hippocampus carries out a cholinergic function (Appleyard and Glozier, 1990). In the cerebellum, however, AChE is secreted, but no electrophysiological evidence of cholinergic function has been established. It was suggested that AChE originates from the climbing fibre system which projects from the inferior olive onto Purkinje cells (Appleyard *et al.*, 1988). In a similar manner, AChE is secreted in the substantia nigra, independent of cholinergic transmission (Greenfield *et al.*, 1984). This secretion in the substantia nigra results in an increase in dopaminergic activity in the ipsilateral striatum, suggesting that AChE may be associated with dopaminergic rather than cholinergic activity in this region (Weston and Greenfield, 1985). The region of the dopaminergic neurones which are particularly sensitive to AChE include functionally active apical dendrites (Hajos and Greenfield, 1992). Behavioural studies have demonstrated that release of AChE in the substantia nigra can induce movement and stereotypy (Greenfield, 1991). Recently, it has been suggested that AChE can enhance the release of dopamine from the nigrostriatal pathway, thus modifying locomotor activity (Hawkins and

Greenfield, 1992b). It has also been postulated that varying subpopulations of nigrostriatal neurones display different behavioural effects to AChE (Hawkins and Greenfield, 1992a).

Indeed, a neuromodulatory action on the activity of nigrostriatal cells can be assigned to AChE (Last and Greenfield, 1987). Based on electrophysiological studies in the guinea pig substantia nigra, AChE was shown to induce a reversible hyperpolarization of the membrane, accompanied by a decrease in input resistance which is sometimes preceded by a brief burst of spontaneous action potentials (Greenfield *et al.*, 1988). It was suggested that this phenomenon is mediated by an ATP-sensitive K+ channel, although a clear mechanism of action has not yet been established (Webb and Greenfield, 1992).

(2) AChE as proteases

Affinity purified samples of AChE from *Torpedo* electric organs and foetal bovine serum have been shown to possess trypsin-like endopeptidase and metalloexopeptidase-like activities (Chubb *et al.*, 1983). This finding has opened the possibility of AChE being involved in the hydrolysis of polypeptides in neuronal tissues. Recently, the effect of AChE on the breakdown of a synthetic hexapeptide LWMRFA (leu-trp-met-arg-phe-ala) was tested. AChE cleaved this into LWMR and FA. In the presence of a trypsin inhibitor (diisopropylfluorophosphate), cleavage of the hexapeptide was precluded. In some cases, cleavage of

the C-terminal alanine was also observed, and this was made more rapid in the presence of Zn^{++} or Co^{++} (Small *et al.*, 1987). Further, AChE has been noted to hydrolyse leucine-enkephalin as well as neurotensin (Checler and Vincent, 1989). It is speculated that the two peptidase activities may be associated with two distinct active sites in the AChE molecule. This suggestion was recently supported by the observation that the trypsin-like endopeptidase activity of *Torpedo* AChE was associated with a serine residue which was distinct from the ser-200 of the catalytic triad, although the exact position has not been determined (Small and Chubb, 1988).

The assignment of these functions with AChE have proffered the suggestion that AChE may be zymogens (inactive precursors) of proteases (Small, 1990). In one experiment, AChE underwent autolysis to yield a 25 kDa fragment (from the 75 kDa AChE monomer) which possessed trypsin-like activity. This 25 kDa polypeptide shares a similar N-terminal sequence to pancreatic trypsin (Small and Simpson, 1988). The other fragment, a 50 kDa polypeptide demonstrated carboxypeptidase activity. Hence, autolysis of AChE monomers may possibly give rise to neuropeptide processing enzymes. Based on this finding, it could be speculated that AChE in its native state is a molecule possessing esteratic sans protease activity. Proteolysis of a catalytic subunit yields two fragments with cryptic endogenous activities (Small, 1990).

(3) AChE as regulators of cellular proliferation

The gene locus of AChE has been assigned to CHE-1 of chromosome 3q, more specifically, 3q21-q26. This 3q region is commonly mutated when associated with abnormal megakaryocyte proliferation in acute myelodysplastic anomalies (Soreq *et al.*, 1987). Analysis of peripheral blood DNA from patients with leukemia showed amplifications of both AChE and BuChE genes, as well as alterations in their structure. Coamplification of these two related but distinct genes in pathological blood cell production may suggest that AChE and BuChE are involved in regulating the differentiation of megakaryocytes (Lapidot-Lifson *et al.*, 1989).

The role of AChE in neuronal growth in the peripheral and central nervous systems has been suggested. In chick brain embryo, AChE was detected at very high concentrations during early development of the diencephalon, telencephalon and retina. This is a stage when cholinergic neurotransmission is still nonexistent (Layer *et al.*, 1987). In tissue-cultured sympathetic neurones, AChE is distributed as clusters on the surface of neurite growth cones, but is absent from cell bodies. Neurones synthesize several forms of AChE, but only the G_4 form is preferentially transported along the neurites. The specific localization of this form may indicate its distinct functional role in neuronal development (Rotundo and Carbonetto, 1987). In a related observation, G_4 AChE was also noted to be expressed during postnatal development

of the rat somatosensory cortex (Inestrosa and Ruiz, 1985). Further, AChE has been noted to be transiently present in the developing central nervous system during stages of neuronal migration and axonal outgrowth. In one experiment, dorsal root ganglion neurones were cultured on substrata composed of sciatic nerve and spinal cord frozen sections. It was observed that AChE levels were modulated by the substratum, in proportion to the degree of neuritic outgrowth (Gupta and Bigbee, 1992).

Finally, changes in AChE activity during postnatal development of the cat brain have been examined in order to assign a role for AChE in CNS maturation. Surprisingly, it was suggested that AChE may play a part in the maturation of the cat brain cortex, possibly in cholinergic development and/or as a cell growth regulatory factor (de Gandarias *et al.*, 1992).

Implications of AChE in Disease States

(1) Hirschsprung's disease

This is also known as congenital megacolon or total colonic aganglionosis and is a specific disorder of intestinal motility usually observed in infancy, resulting in severe constipation. The main lesion is a congenital absence of myenteric ganglion cells in the distal portion of the colon, with an accompanying increase in number and size of nerve fibers.

Two forms of AChE (G_1 and G_4) have been identified in bowel samples of control and Hirschsprung's disease

patients. In pathological states, the ratio of G_4/G_1 AChE is elevated, maybe as a result of increased nerve fiber proliferation in the aganglionic megacolon (Rakonczay and Nemeth, 1984; Sun *et al.*, 1987). Biochemical assay of erythrocyte AChE also showed a high level of activity, correlating well with AChE activity in rectal biopsy samples (Yanagihara *et al.*, 1983). In serum samples, BuChE did not display any significant difference between control and Hirschsprung's disease patients (She *et al.*, 1984). Histochemical analysis revealed an increase in AChE positive nerve fibers around the lamina propia and muscularis mucosa of the rectum (Goto *et al.*, 1984a). It was observed that AChE first appears at high levels in the lamina propia within three weeks postnatally. Thereafter, AChE levels in the lamina propia are reduced, but are increased in the muscularis mucosa (Goto *et al.*, 1984b). Hence, histochemical diagnosis of Hirschsprung's disease is reliable up to three weeks of age, but not thereafter since suction biopsy specimens preclude the muscularis mucosa due to weak suction pressure. However, AChE assay in blood samples may act as an adjuvant in reliable diagnosis of this disease. An estimation of the ratio AChE/BuChE activity in resected tissue may even prove to be of discriminatory diagnostic value (Causse *et al.*, 1987).

(2) Neural tube defects

Neural tube defects include anencephaly, spina bifida, and encephalocele.

Analysis of amniotic fluid from pregnancies with open neural tube defects revealed the presence of high levels of AChE, and it was suggested that this soluble AChE form reached the amniotic fluid from cerebrospinal fluid leaking from the open neural tube (Smith et al., 1979). As a corollary, a monoclonal antibody (mAb) was raised against G_2 AChE which had the capacity to bind to human brain AChE from pathological samples. This mAb is currently being used to detect AChE levels in amniotic fluid (Rasmussen *et al.*, 1987) and has proved useful for the detection of neural tube defects in early pregnancy, with a very high specificity (Loft, 1990).

(3) Amyotrophic lateral sclerosis (ALS)

ALS is a pathology of the skeletal muscular neurones, affecting both the upper motor neurones (from the brain to the spinal cord) and the lower motor neurones (from the spinal cord to muscle tissues). This disease leads to spasticity, exaggerated reflexes, and atrophy of muscles.

Biochemical studies of phrenic nerves from post-mortem ALS samples revealed decreased axonal transport of AChE, as a result of distal axonal atrophy (Bradley *et al.*, 1983). Analysis of AChE activity from muscle biopsies of ALS patients showed complete disappearance of G_4 and A_4 AChE, as well as marked reduction in G_1, A_8 and A_{12} forms. Histochemical studies showed large reductions of AChE in synaptic infoldings and sarcoplasmic reticulum of motor endplate sections (Fernandez *et al.*, 1986). It is

suggested that alterations in levels of AChE molecular forms may be related to the alterations in normal trophic neuromuscular interactions, as seen in ALS (Schoenen, 1988).

(4) Alzheimer's Disease (AD)

Senile dementia of the Alzheimer type is a disease which initially manifests as loss in recent memory with preservation of remote memory. The pathology is insidious, leading to global memory loss, aphasia, apraxia, and impairments in levels of consciousness. A straightforward diagnosis of AD can only be obtained by post-mortem identification of plaques and tangles, which are two hallmarks of AD that distinguish it from other forms of dementia. Various etiological factors have been postulated such as viral infection, aluminium toxicity, angiopathy and blood-brain barrier incompetence, amyloid beta-protein deposition, and neurotransmitter/receptor deficiencies (reviewed by Glenner, 1989).

Histochemical studies of post-mortem AD brain tissues revealed marked reduction of AChE in the neocortex, possibly due to a primary degeneration of cholinergic axons to the cortex, and subsequent loss of perikarya from the subcortical nucleus (Candy *et al.*, 1983). A similar reduction in AChE has been observed in the upper brain stem (Shortridge *et al.*, 1985) and nucleus basalis of Meynert (Rasool *et al.*, 1986). Likewise, in the latter two regions, an extensive formation of neurofibrillary tangles was seen.

It is postulated that death of cholinergic neurones may be associated with tangle formation. In contrast, no significant correlation between neuritic plaques and AChE reduction in AD was reported (Brashear *et al.*, 1988). In the plasma and erythrocyte of AD patients, however, no significant reduction in AChE was recorded (Sirvio *et al.*, 1987). Cerebrospinal fluid (CSF) from AD patients has revealed AChE reduction in ventricular samples (Appleyard *et al.*, 1987), but not of those taken from lumbar punctures (Lal *et al.*, 1984; Appleyard *et al.*, 1987). The lower level of AChE in ventricular CSF may be correlated with cholinergic changes in the forebrain of AD patients. Although lumbar CSF does not display any marked reduction in AChE, its use in the diagnosis of AD may still be feasible. Recently, my colleague (Navaratnam *et al.*, 1991) demonstrated the presence of an anomalous AChE band in isoelectric focussing electrophoresis of lumbar CSF from histologically-diagnosed AD patients. This finding may prove useful for the ante-mortem diagnosis of AD.

(5) Other neurological conditions

Reports on alterations of AChE levels have also been reported for other pathological states, although extensive studies on these have not been carried out. Reduction in AChE activity was seen in Pick's disease (Meier-Ruge *et al.*, 1984), Huntington's chorea (Ruberg *et al.*, 1987), skeletal muscle of host infected with *Trypanosoma cruzi* (Brennessel *et al.*, 1985), and erythrocyte membrane

abnormalities (Godin *et al.*, 1988). In experimental animals, allergic neuritis showed a decrease in the velocity of AChE orthograde transport (Oikarinen and Kalimo, 1984), whilst in homozygous dysgenic mouse where skeletal musculature development is blocked, A_{12} AChE is reduced (Rieger *et al.*, 1984). In other cases, a rise in AChE levels was noted such as in neuronal intestinal dysplasia (Fadda *et al.*, 1983), aquagenic pruritus (Bircher *et al.*, 1988), chicken muscular dystrophy (Sketelj *et al.*, 1983), and in CSF of patients with Parkinson's disease (Direnfeld *et al.*, 1984). As for the latter however, some cases have not shown any positive correlation between AChE level alterations and Parkinson's (Sirvio *et al.*, 1987).

A Final Remark

In the past ten years, tremendous progress has been made in elucidating the structure and biology of AChE. Although questions pertaining to the nature of membrane attachment, genetic basis of origin, and structure-activity relationship of AChE have been partly resolved, there are still queries that remain to be addressed. For example: Does the gene that encodes for both A_{12} and G_2 also encode for G_4 AChE? Are A_4 and A_8 AChE degradation products of A_{12} AChE? Does the polymorphism of AChE have any correlation with the reported non-cholinergic functions of this enzyme? Indeed, with the state-of-the-art techniques available to us in biochemistry and molecular biology, it won't be too long before such mysteries will be unravelled.

In the meantime, however, the present thesis hopes to contribute a humble soupçon of knowledge to the current research on AChE. In particular, my research focussed on establishing an optimal procedure for the purification of AChE to be used for the purpose of raising monoclonal antibodies (mAbs) which can distinguish the erythrocyte and brain forms of this enzyme. The potential applications of such mAbs in histochemical research and clinical diagnostics are limitless.

Chapter 2.

MATERIALS AND METHODS

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals were obtained from either Sigma Chemical Co. or BDH Chemicals Ltd. and were of analytical grade.

Tissue Sample Preparation

All tissue samples were obtained courtesy of the Department of Neuropathology (Radcliffe Infirmary, Oxford University). Permission for the ethical conduct in obtaining these samples has been given to the Oxford Project to Investigate Memory and Ageing (OPTIMA).

Post-mortem lumbar cerebrospinal fluid (CSF) was obtained from patients by needle aspiration through the dura after removal of the L_4-L_5 vertebral bodies. All samples were proportioned into 150 µl aliquots and kept in a -70° C freezer until used. On the day prior to experimentation, CSF samples were thawed and centrifuged at 50,000 g for 2 hrs to remove contaminating debris. The supernatant was collected and stored at -20° C.

Caudate nucleus from post-mortem brain tissue was homogenized at a ratio of 5:1 (v/w) in 10 mM $Na/KHPO_4$ buffer (pH 7.0). Homogenization was carried out by fifteen passes in a Potter glass homogenizer with a teflon pestle. The prepared homogenates were frozen at -20° C until further use. To prepare supernatants containing "soluble" acetylcholinesterase (AChE), the homogenates were thawed and centrifuged at 50,000 g for 2 hrs, and the supernatants

were collected and stored at -20° C. The remaining pellet was used to collect the "membrane-bound" form of AChE. This pellet was suspended in an equal starting volume of 144 mM NaCl + 10 mM $Na/KHPO_4$ buffer (pH 7.0) with 1% Triton X-100 (Boehringer Mannheim). This was mixed at 4° C for 2 hrs on a rotary mixer. Thereafter, the suspension was centrifuged at 50,000 g for 2 hrs and the supernatant was collected and stored at -20° C.

Acetylcholinesterase Activity Assay

The method used for determining AChE activity, with acetylthiocholine as substrate, was based on that described by Ellman *et al.* (1961) with modifications done by colleagues in our laboratory. Supernatants at 25 µl were aliquoted into 96-well microtitre plates and topped with 175 µl of Ellman buffer (1 mM acetylthiocholine iodide + 0.33 mM dithionitrobenzoate in 50 mM Na/KH_2PO_4). Incubation was at 25° C. Absorbance was measured at 405 nm on a microtitre plate reader (Titertek Multiscan). Kinetic changes in absorbance due to spontaneous hydrolysis of acetylthiocholine was measured every 10 secs for 1 min. The average kinetic absorbance change per min was used in the following equation:

$$\text{Rate} = \frac{\text{Abs min}^{-1}}{(\varepsilon)\ (L)}$$

ε = Extinction coefficient at 405 nm of 5-thio-2-nitro-benzoic acid (1.344×10^4)

L = Path length of sample (0.58 cm)

Rate = Expressed in U mol l^{-1} min^{-1}

To obtain a value in U l^{-1}, the rate was multiplied by a dilution factor of 8×10^6, taking into account that 1 U of AChE = 1 μmole acetylthiocholine cleaved per min.

Chromatofocussing

Pooled samples of cerebrospinal fluid (CSF) or caudate nucleus homogenates from Alzheimer's disease-diagnosed patients were obtained from the Department of Neuropathology (Radcliffe Infirmary, Oxford). Caudate nucleus homogenates were separated into supernatants (previously described) and this, as well as the CSF were separately freeze-dried in 10 mM NH_4HCO_3. The freeze-dried powder was dissolved in 1 ml of distilled H_20 and desalted on a 10 ml Sephadex G 25 column (PD10; Pharmacia). For chromatofocussing, a Mono P HR 5/20 (Pharmacia) column was equilibrated with start buffer (25 mM Tris-HCl titrated to pH 7.25 by iminodiacetic acid), and the desalted sample was loaded into the column. After complete penetration of the sample, the column was then eluted by forming a gradient with the elution buffer made up by a 1:8 dilution of Polybuffer 74 (Pharmacia), pH 4.5. Protein elution was monitored by an absorbance of 275 nm on a UV monitor (2138 Uvicord S; LKB Bromma) and recorded on a pen recorder (Model 2210; LKB Bromma). All samples and buffers were

filtered on a 0.2 μm nylon membrane filter (Whatman) and applied using a peristaltic pump (2132 Microperpex; LKB Bromma) at a flow rate of 350 μl/min. A total of 80 fractions of 350 μl each were collected. The formed pH gradient was tested by checking the pH of each collected fraction using a portable pH meter (PHA 270; Whatman). For a narrower pH gradient, the column was equilibrated with 25 mM piperazine titrated to pH 6.3, and eluted using the elution buffer mentioned previously. All experiments were carried out in a cold room (4-6° C). Aliquots from the collected fractions were assayed for AChE activity using a modification of the method of Ellman *et al.* (1961) described earlier.

Affinity Chromatography for the Purification of AChE

For the preparation of an acridinium affinity column, 3 g of freeze-dried AH-Sepharose 4B (Pharmacia) was washed and reswollen with 600 mls of 500 mM NaCl. This yielded about 12 mls of final gel volume. In the meantime, 100 mg of acridinium carboxylic acid (Aldrich) was dissolved in 10 mls of ethylene glycol (Fluka) and its pH adjusted to 6.5 with NaOH. Thereafter, 100 mgs of N-(Dimethylaminopropyl)-N'-ethylcarbodiimide-HCl was mixed to the ligand solution and then with the gel suspension. This was left mixing overnight on a rotary mixer at room temperature. The next day, the slurry was washed first with 60 mls of 50% ethylene glycol solution, then with 120 mls of 50% ethylene glycol + 100 mM Na borate solution (pH 9.25), and finally

with 500 mM NaCl + 100 mM Na borate solution (pH 9). This washing step was necessary to wash away excess ligand, urea derivative, and unreacted carbodiimide. The coupled gel was kept in PBS (120 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4) + 0.1% Na azide.

For preparation of the procainamide affinity column, 10 mls of ECH-Sepharose 4B (Pharmacia) was washed with 100 mls of distilled water. The ligand was weighed to give a 100-fold molar excess over the sepharose active groups (i.e. 2.7 g procainamide), dissolved in 10 mls of distilled H_20, and adjusted to pH 4.8. The gel and the ligand were mixed and 100 mg of N-(Dimethylaminopropyl)-N'-ethylcarbodiimide-HCl was added to this mixture and left overnight at room temperature on a rotary mixer. The next day, the slurry was washed first with 100 mls of distilled H_20, then with 400 mM $NaHC0_3$ (pH 7.2), and finally with PBS + 0.1% Na azide.

In purifying AChE, tissue supernatant was mixed with equal volumes of PBS and applied to either affinity column at a flow rate of 200 µl/min at 4-6° C. The gel was then washed with ten column volumes of low salt buffer (20 mM Na_2HP0_4 + 5 mM EDTA + 0.1% Triton X-100, titrated to pH 7.2), and then with ten column volumes of high salt buffer (400 mM NaCl in low salt buffer, pH 7.2). For elution, 400 mM tetraethyl ammonium (TEA) and 10 mM decamethonium in low salt buffer was used at a flow rate of 50 µl/min. To get rid of the TEA and decamethonium, the eluted fractions were subjected to gel filtration on a Sephadex G 25 column

(PD10; Pharmacia). Fractions collected were then assayed for AChE activity using the earlier mentioned method of Ellman *et al.* (1961).

Analytical Gel Filtration Chromatography of Purified AChE

The elution volumes of affinity purified AChE were determined on a 9.4 mm x 250 mm gel filtration column (GF-450; Zorbax) which was equilibrated with 50 mM Na_2HPO_4 + 50 mM NaCl (pH 7.2). Affinity purified samples at 100 µl were applied and eluted at a flow rate of 340 µl/min at 4-6° C. Each fraction collected was assayed for AChE activity using the method of Ellman *et al.* (1961). In order to determine the approximate molecular size of AChE, its elution profile was plotted against the logarithmic elution plot of markers such as thyroglobulin 660 kDa; ferritin 445 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; β-lactoglobulin, 54 kDa; carbonic anhydrase, 29 kDa; trasylol, 6.5 kDa; and cobalamin, 1.35 kDa.

Ion-Exchange Chromatography on a SMART (Pharmacia) System

A 350 µl sample of procainamide affinity purified caudate nucleus material was loaded into a Mono S-PC 1.6/5 (Pharmacia) ion-exchange column, which was earlier equilibrated with 50 mM succinate (pH 5.35). The material was eluted with 50 mM succinate + 300 mM NaCl (pH 5.35) at 4-6° C and the collected fractions were assayed for AChE activity. Flow rates were set at 100 µl/min.

Polyacrylamide Gel Electrophoresis

(1) Isoelectric focussing (IEF)

The method that I used was a modification of that described by Giulian *et al.* (1984), all procedures being carried out at room temperature. A stock solution of 58.4 g acrylamide and 1.6 g bisacrylamide made up to 200 mls in distilled H_20 was prepared. For the gel mixture, 1.1 mls of the acryl/bisacryl stock solution was mixed together with 420 µls ampholine pH 5-7 (Pharmacia) and 600 µls glycerol in 3.8 mls of deionized H_20. This was degassed for about 15-20 mins. In the meantime, an 80 mm x 100 mm glass containing a sheet of GelBond PAG (FMC BioProducts) and an alumina plate separated by a 0.75 mm spacer were assembled on an electrophoresis unit (2050 Midget; LKB Bromma). (N.B. The GelBond was omitted when gels were to be used for Western blotting). This "sandwich" was sealed at the bottom with a heated 2% agarose solution. The degassed solution was then polymerized by the addition of 11.5 µl TEMED and 12.5 µl of 10% ammonium persulphate. This solution was poured into the sandwich and sealed at the top with a 10-well comb. This was allowed to polymerize for about 1 hr. In the meantime, 200 mls each of catholyte (10 mM histidine) and anolyte (10 mM glutamic acid) buffers were prepared and extensively degassed for about 1 hr. After the gel has polymerized, the upper buffer chamber was filled with catholyte whilst the lower buffer chamber was filled with anolyte. Pre-focussing was commenced at a constant voltage of 200 V (15 mins), 300 V (15 mins), and

400 V (1 hr). During this time, a volume of AChE sample containing 2.5 mU activity was concentrated to 10 µl in an ultrafilter unit (Ultrafree-MC; Millipore) by centrifuging (Centra-4B; International Equipment Co.) at 8000 g. The concentrated material was mixed with 10 µl of sample buffer (30 µl ampholine pH 5-7, 75 µl glycerol, 10 µl Tween-20 in 345 µl deionized H_2O). After pre-focussing, solutions from the upper and lower chambers were discarded and refilled with freshly degassed buffers. A total of 2.5 mU of AChE activity was loaded into each well and focussing was commenced at a constant voltage of 300 V (25 mins) and 1000 V (3.5 hrs). During the run, the gel system was cooled by running tap water. After focussing, the gel was incubated overnight at 30° C with 17 mg acetylthiocholine in 30 mls of incubation medium (90 g Na_2SO_4, 7.29 g Na maleate, 0.9 g Glycine, 0.6 g $CuSO_4$, 0.444 g $MgSO_4$ in 500 mls distilled H_2O). The following day, specific AChE bands became visible as precipitates of copperthiocholine and the gel was extensively washed with five changes of distilled water. To heighten staining of bands, the gels were incubated with a filtered solution of saturated dithiooxamide.

(2) Sodium dodecylsulfate (SDS) electrophoresis

The method used was a modification of that described by Laemlli (1970). Stock solutions were prepared as such: Acrylamide Stock (30 g acrylamide, 0.8 g bisacrylamide in 100 mls deionized H_2O); Running gel Buffer (18.17 g Tris,

0.4 g SDS in 100 mls deionized H_2O, titrated to pH 8.8); and Stacking Gel Buffer (6.06 g Tris, 0.4 g SDS in 100 mls deionized H_2O, titrated to pH 6.8). A "sandwich" was assembled onto an electrophoresis apparatus, in a manner similar to the one mentioned above. A 12% running gel solution was prepared (3 mls acrylamide stock, 1.85 mls running gel buffer, 2.55 mls deionized H_2O, 5µl TEMED, 75 µl 10% ammonium persulfate) and gently poured into the cast until about 2 cm from the top. This was then overlaid by overlapping buffer (1 ml running gel buffer and 3 mls deionized H_2O) and left until the bottom layer polymerizes. In the meantime, electrode buffer (0.303 g Tris, 1.44 g glycine, 0.1 g SDS in 100 mls deionized H_2O) and sample buffer (2.5 mls stacking gel buffer, 360 mgs SDS, 2 mls glycerol and 1 ml mercaptoethanol made up to 10 mls with deionized H_2O, and then added with a soupçon of bromophenol dye) were prepared. When the running gel has polymerized, the topmost overlapping buffer was discarded and replaced with 5% stacking gel solution (0.825 mls acrylamide stock, 1.25 mls stacking gel buffer, 2.875 mls deionized H_2O, 3.5 µl TEMED, 50 µl ammonium persulphate) and sealed with a 10-well comb. Samples were then concentrated (when necessary) to about 10 µl, mixed with 10 µl of sample buffer, and then boiled for about 5 mins. Marker proteins (β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa) were prepared in a similar manner. When the stacking gel has polymerized, electrode buffer was poured into the top and

bottom buffer chambers, then the samples were applied and subjected to electrophoresis for about 1 hr (or until the dye has migrated to the bottom) at constant voltage of 150 V at room temperature. Bands were detected by fixing the gel in 10% w/v trichloroacetic acid for 10 mins, followed by a rinse in distilled H_20, and overnight incubation with Electrophoresis Gel Stain (Gradipore Ltd.). Destaining was done with 6% acetic acid to enhance contrast.

(3) Non-denaturing electrophoresis

The method used was essentially a modification of that developed by Clarke (1964). The following stock solutions were prepared: Acrylamide stock (30 g acrylamide, 1 g bisacrylamide in 123 mls deionized H_20); Gel buffer solution (2.9 g glycine, 0.6 g Tris in 98 mls deionized H_20); Electrode buffer (2.9 g glycine, 0.6 g Tris, 0.5 mls 1N HCl in 97.5 mls deionized H_20); and APS solution (35 mgs ammonium persulphate in 2.5 mls deionized H_20). A 7% Clarke gel was prepared (2.926 acrylamide stock, 1.25 mls gel buffer solution, 5.324 mls deionized H_20, 0.5 mls APS solution and 3.5 µl TEMED) and poured into the cast. This was allowed to polymerize and then pre-ran at a constant voltage of 75 V for 30 mins with a 1:7 dilution of gel buffer solution in deionized H_20. In the meantime, sample was prepared and mixed with equal amounts of sample buffer (5 g sucrose and 1 ml of 1% bromophenol blue solution made up to 10 mls of deionized H_20). When the pre-run is over, the gel buffer solution was discarded and replaced with a

1:10 dilution of electrode buffer in deionized H_20. The samples were applied and the gel was subjected to electrophoresis at a constant voltage of 200 V at room temperature. The gel was then stained for AChE activity using the incubation medium described in the section on IEF.

In vivo immunization

(1) Priming of mouse

Female BALB/c mice (3-4 months old) were intraperitoneally implanted with a nitrocellulose strip (BA 83; Schleicher and Schuell) which was previously blotted with affinity-purified human erythrocyte AChE. Two months later, a booster immunization of 3.5 µg affinity-purified AChE from human caudate nucleus was injected intravenously through the tail vein.

(2) Dissection of spleen from mouse

The following procedures were based on methods learned after an apprenticeship with Dr. Nigel Groom (Oxford Brookes University) and from a European Community Sponsored Workshop on "Applications of Advanced Immunological Techniques" held at the University of Oporto (Portugal). On the day of fusion, SP2/0-Ag14 myeloma cells (kindly provided by Dr. N. Groom), which were earlier grown to confluency and subcultured on the previous day, were counted to yield a population of 5 x 10^5 cells/ml in peak condition. Counting of SP2s was done on a Coulter counter.

The collected cells were aseptically transferred into a 75 cm^2 flask and kept in a humidified incubator (5% $C0_2$ and 95% air/gas phase) (IR 1500; ICN Flow Ltd.) until further use.

In the meantime, the immunised mouse was taken and killed by a quick blow to the head and then by cervical dislocation. The dead mouse was then immersed in 70% alcohol and laid on a flat board, ready for dissection. An incision was made on the skin covering the area of the spleen, thereby exposing the peritoneum, which was then lifted to expose the spleen. Every now and then, 70% alcohol was squirted around the spleen and dissecting instruments were flamed to achieve optimal aseptic conditions. The spleen was isolated, cleared of its fascia, and transferred from one petri dish to another containing 10 mls of Iscove's Modified Dulbecco's Medium (IMDM; Gibco). From this point on, all procedures were done in a laminar flowhood (Gelair BSB48; ICN Flow Ltd.). The spleen was washed in a petri dish containing 10 mls of fresh IMDM. On the third petri dish, the spleen was teased apart and crushed using sterile forceps. Leaving all visible clumps behind, the spleen suspension was aspirated and transferred to a Falcon tube (Sterilin). The spleen cells were counted on a haemocytometer, but before this, 170 mM NH_4Cl was added to an aliquot of the spleen cell suspension in order to lyse the erythrocytes in suspension, so that only the lymphocytes are counted. For fusion, a

total of 1 x 10^8 spleen cells and 3 x 10^7 SP2 myeloma cells were required.

Hybridization Procedure

(1) Preparation of cells

The required amount of spleen cells and SP2 myeloma cells were aseptically transferred into a Falcon tube. The ratio of spleen cells to SP2s is usually 2:1, but if there are more spleen cells than this, a higher ratio can be used. The cell mixture was centrifuged (BR 401; Denley) at 1600 rpm for 10 min. After the spin, the supernatant was gingerly aspirated with a pipette so as not to disturb the pellet. Fresh IMDM (without serum) was transferred into this tube and the pellet was resuspended by sucking the pipette up and down. This was then spun again at 1600 rpm for 10 min. At the end of the spin, all the supernatant was completely removed to leave a dry pellet and then the tube was left in a warm H_20 bath (37° C).

(2) Fusion Procedure

Hybridization was performed essentially according to the procedure devised by Kohler and Milstein (1975). PEG 1500 (Boehringer) was asceptically transferred to a sterile Falcon tube and incubated in a warm (37° C) H_20 bath. Over 1 min, 1 ml PEG 1500 (warmed to 37° C) was gently added into the tube, while the suspension was gently stirred with the pipette tip. At the end of the 1 min, the pellet was stirred gently for another 90 sec. While keeping the tube

in the H_20 bath, 40 mls of IMDM (without serum) was gently added in a manner whereby 1 ml was added over the first 30 sec, 3 ml over the next 30 sec, then 5 mls over the next 30 sec. The speed of addition was progressively increased so that the remainder IMDM was added over the last 30 sec. The fused cells were allowed to stand for 8 min at ambient temperature followed by 2 min in the H_20 bath at 37^{o} C. Thereafter, the fused cells were spun down at 1000 rpm for 10 min. When this was over, the supernatant was carefully removed and the pellet was resuspended in 50 ml single strength HAT medium. This was prepared as 11% Foetal calf serum (Myoclone Plus, Gibco), 1% HAT (50x concentrate, Flow Laboratories), and 2.2% Penicillin-Streptomycin (1000x concentrate, Gibco) in 100 mls IMDM. The solution containing the hybridomas were then transferred into a 75 cm^2 flask and allowed to stand for 5 hrs in a CO_2 incubator. Thereafter, the remaining 50 mls of single strength HAT medium was added to the flask, together with 10 mls of thymocyte feeder layer. The latter was prepared by killing a Balb/c mouse (3-4 weeks old) and isolating its thymus. This was then made into a thymocyte cell suspension, in a manner similar to that done for preparing a spleen cell suspension. Using a multi-channel pipetter (Transferpette-8; Brand), the hybridoma suspension was then dispensed (100 µl/well) into ten 96-well plates (Falcon 3072; Becton Dickinson) and placed in a humidified incubator.

(3) Spotting and Preventing Contamination

On day 2, signs of contamination were checked for and any contaminated plates were discarded. In some cases, it was possible to keep a contamination localised (especially so with fungus at the hyphal stage) without having to discard the entire plate. Media from contaminated wells were removed by suction and in its place, a saturated solution of sterile $CuSO_4$ was dispensed. If contamination at the sporing stage is spotted, it is best to discard the plate altogether since there is very little chance to localize the contamination.

(4) Feeding of wells

Plates were fed with fresh HAT medium every 2-3 days. This was done with a multi-channel pipetter by removing 100 µl medium and replacing with 100 µl of fresh HAT medium. Feeding was done slowly in order that the colonies were kept intact and that cells maintain close to one another so that the local concentrations of growth factors were higher. By day 10-14, the wells started to turn acid (orange-yellow in colour) and hybridoma colonies could be seen with the naked eye. At this point, the supernatants were ready for screening to determine which wells were secreting antibodies of interest. (N.B. It is advantageous to carry out three feedings before screening so that antibodies produced by non-fused spleen cells, which would die eventually, may be diluted out.)

Enzyme antigen capture assay

This procedure is a modification of that described by Norgaard-Pedersen *et al.* (1983) and Rasmussen *et al.* (1987). Microtitre plates (96-well; Alpha) were coated with a 1:500 dilution of rabbit anti-mouse immunoglobulin (Ig) (Z259; Dako) in 50 mM $NaHCO_3$ buffer titrated to pH 9.5 and containing 0.1% Na azide preservative. These were kept in a moist polystyrene box for at least 24 hrs at 4° C. Upon use, the Z259 Ig solution was aspirated and transferred to a new microtitre plate. This Ig solution can be used up to five times. Unbound antibody was rinsed away (usually five washings) with wash solution (1.2% NaCl + 0.05% Tween 80 in distilled water), and residual sites were blocked with an ELISA Blocking Reagent (Boehringer Mannheim) and then left incubating for 1 hr at 25° C on a shaker (DSG Titertek; Flow Laboratories). In between each step, the wells were not be allowed to dry out. Wells were rinsed again five times with wash solution before addition of 100 µl hybridoma supernatant and 100 µl of conjugate buffer (50 mM Na_2PO_4, 1.2% NaCl, 0.5% Tween 80 in 100 mls H_20, titrated to pH 7.0). The plates were then left incubating overnight at 4° C on a shaker. During this stage, the captured Z259 Ig bound to mouse Ig present in the hybridoma supernatant. In most cases, however, not all the Ig will be bound since there may be insufficient captured Z259 Ig to do this. After another rinsing step, wells were incubated with 100 µl brain or erythrocyte supernatants in 100 µl conjugate buffer. Control wells contained tissue culture supernatant

in place of tissue extract. After 5-6 hrs at 37° C on a shaker, wells were washed five times to remove unbound enzyme. The activity of "captured" AChE was measured at room temperature by the previously outlined colorimetric method of Ellman *et al.* (1961) applied directly to the microtitre plate. Absorbance readings done at intervals were carried out at 405 nm on a Titertek Multiscan.

Cloning by Limiting Dilution

From the wells which displayed strong signals (i.e. containing hybridomas secreting the antibody of interest) on the enzyme capture assay, the hybridoma colonies were disrupted and 50 µl aliquots were transferred to 24-well plates (25820; Corning) containing 1 ml of HAT medium. Once growth has reached a mid log phase, limiting dilution was carried out. Cells from each hybridoma cell line were counted on a haemostat. These cells were then plated at a density of 80, 40, 20, 10, 5, and 1 into each row, respectively, of a 96-well microtitre plate filled with 200 µl HAT medium. In wells plated with 10, 5, and 1 cells, 10% feeder supplement (Immune Systems) was usually added to sustain cell growth. The plates were kept in a polystyrene box and placed in a humidified incubator. By day 10-13, cell growth has normally reached confluency and the supernatants were again assayed for its antigen specificity. Positive signals from wells seeded with 1 cell/well were noted and these were grown and subjected to a second limiting dilution. In total, three successive

limiting dilution steps were carried out to achieve a cell line with definite monoclonality. After the third limiting dilution step, selected hybridoma clones were expanded in culture, first in 24-well microtitre plates (Sterilin), then to 25 cm^2 flasks (Corning), and finally to 75 cm^2 flasks (Corning). Cell lines were grown and subcultured until sufficient amounts of spent tissue culture supernatant could be collected. At this time, cells were grown in two passes of HT medium [11% foetal calf serum (Myoclone Plus, Gibco), 1% HT (50x concentrate, Flow Laboratories), 2.2% Penicillin-Streptomycin (1000x concentrate, Gibco) in 100 mls IMDM (Gibco)], until finally, the cells were subcultured in IMDM growth medium.

Preparation of Spent Tissue Culture Supernatant

The hybridomas were allowed to grow to confluency until metabolism ceased. The hybridoma supernatants were transferred into Falcon tubes and spun at 1600 rpm for 10 mins to remove dead cellular debris. The supernatants were collected and a 1:9 (v/v) ratio of 1 M Tris-HCl (pH 7.5) was added to each supernatant. Sodium azide at 0.1% was added as a preservative. Aliquots were stored at -20° C and a working solution was kept at 4° C.

Cryopreservation of Cells in Liquid Nitrogen

Hybridomas were allowed to grow until mid log phase was reached. In the meantime, freezing medium was prepared and stored at -20° C. This was made up of a 1:9 (v/v)

ratio of dimethylsulfoxide (DMSO) and filter-sterilised foetal bovine serum (Myoclone Plus; Gibco). The hybridomas were resuspended by banging the base of the culture flask. These were aspirated into a Falcon tube and spun at 1600 rpm for 10 min. The supernatants were collected and the pellets were resuspended in 5 mls of freezing medium. This was then transferred to 5 cryogen ampules (Nunc) at 1 ml each. The ampules were slow cooled by placing these in the middle of a covered styrofoam box. This was placed in a -20° C freezer for 30 min and then left in a -80° C freezer overnight. The next day, the ampules were transferred into liquid nitrogen storage tanks and kept until future use.

In vitro immunization

In vitro immunization is a tissue culture technique which produces primed splenocytes which in turn may be applied for hybridoma production. For our purpose, we made use of a recently-patented "Cel-Prime In Vitro Immunization Kit" (Immune Systems) to facilitate raising mAbs via this novel approach. The procedure that we employed was a modification of the method recommended by Reading (1982). In brief, the three main stages are:

(1) Preparation of antigen and establishment of support cell monolayer

The antigen used for this in vitro immunization procedure was a synthetic peptide of a sequence identical to the 16 carboxy-terminal amino acids of human brain AChE.

This peptide (courtesy of K. Giles) was conjugated to a carrier molecule, which in this case was bovine serum albumin (BSA). The latter was rendered more soluble by reacting its amino groups with succinic anhydride thus transforming them into carboxyl groups. Conjugation was fairly straightforward, requiring no addition of any conjugating agents. The peptide was conjugated to the carrier by a disulfide bond between the cysteine residues of the peptide and the free thiol group in BSA. The configuration formed by this complex more or less mimics that of the tertiary structure of one catalytic subunit of brain AChE.

Two separate batches of a mixture of 15 μg of the peptide per se and 15 μg of the peptide conjugate were dissolved in 300 μl each of splenocyte medium (Immune Systems). These were then made sterile by gamma irradiation at 50 Gy (500 rads). Three days before priming of splenocytes, a 25-ml bottle of support cell medium (Immune Systems) was thawed at room temperature. Under aseptic conditions, 5 ml of support cell medium was pipetted into a 25 cm^2 flask. A vial of support cells (Immune Systems) was rapidly thawed by constant shaking under hot running water. The support cells were pipetted and transferred into the 25 cm^2 flask. This was then sealed with a cap and placed for 24 hrs in a humidified incubator (Automatic CO_2 Incubator IR 1500; ICN Flow Ltd.) with a 5% CO_2 and 95% air gas phase. The next day, medium from the 25 cm^2 flask was pipetted out and discarded. A

total of 5 mls of support cell medium was gently applied into the flask together with 30 μg of the erstwhile sterilised antigen. The flask was capped, returned to the CO_2 incubator and left undisturbed for 48 hrs.

(2) Preparation of splenocytes

One 20-ml bottle of splenocyte medium (Immune Systems) and a flask of immunization medium (Immune Systems) were thawed in room temperature. In the meantime, one Balb/c mouse (5 weeks old) was taken and killed by a laboratory approved method. The dissection of the spleen is similar to that mentioned in a previous section. Once the spleen has been isolated, this was ruptured and teased in 10 mls of splenocyte medium into a crude splenocyte suspension. With a syringe, the splenocyte suspension was drawn up leaving all visible lumps behind. This was applied into a Falcon tube and centrifuged at 1800 rpm for 5 min.

(3) Immunization

The flask of support cells was taken from the CO_2 incubator and transferred to a laminar flow hood. The medium was pipetted and discarded. A total of 5 mls of fresh support cell medium was gently applied to rinse the cell monolayer. This was then pipetted and discarded. The medium from the centrifuged splenocyte suspension was pipetted out and the splenocyte pellet was resuspended in 5 mls of immunization medium. This was then transferred into the 25 cm^2 flask and added with the remaining immunization

medium. The second batch of sterilized antigen preparation was also applied to this immunization medium. The flask was capped, placed in a CO_2 incubator, and left undisturbed for 4 days. After this time, the medium started turning yellow-orange in colour, indicating that cell growth and metabolism has ensued during the immunization stage. The primed splenocytes were now ready for fusion with myeloma cells, following the procedure mentioned in a previous section.

Antibody Screening ELISA

In order to screen antibodies raised against the C-terminus peptide, 50 µg of the antigen was dissolved in 50 mM $NaHCO_3$ buffer (pH 9.5) and applied to 96-well plates at 100 µl/well. This was placed in a moist polystyrene box and left overnight at 4° C. Wells were then washed five times with wash buffer (1.2% NaCl + 0.05% Tween 80 in distilled H_2O) and blocked with 200 µl/ well of ELISA Blocking Reagent (Boehringer) for 1 hr at room temperature. The wells were washed five times with wash buffer and then incubated with 75 µl of tissue culture supernatant and 75 µl of conjugate buffer (50 mM Na_2HPO_4 + 1.2% NaCl + 0.5% Tween 80, pH 7). This was left on a shaker for 2 hrs at room temperature. Wells were then washed five times with wash buffer and then coated with 100 µl of a 1:1000 dilution of horseradish peroxidase-conjugated anti-mouse rabbit Ig (P260; Dako) in conjugate buffer. This was left incubating on a shaker at room temperature for 1 hr. Wells

were washed seven times with wash buffer and then developed with 100 µl of developing solution [70 mgs o-phenylenediamine di-HCl dissolved in 50 mls citrate buffer (525 mgs citric acid + 890 mgs Na_2HPO_4 titrated to pH 5) with a final addition of 20 µl hydrogen peroxide]. This was left for about 5-10 mins, or until the solution turns yellow. To stop this reaction, 50 µl/well of 2.5 M H_2SO_4 was applied. Absorbance readings were done at 492 nm. Positive wells were grown and subcultured by limiting dilution, following the procedure mentioned in an earlier section.

Isotyping

Determination of the isotype of the four mAbs was carried out using a mouse monoclonal antibody (mAb) isotyping kit (Serotec). In brief, a 1:50 dilution of the hybridoma supernatant to be tested was prepared. This dilution presupposes that the supernatant contains approximately 5-20 µg/ml mAb concentration. If the concentration is suspected to be lower than this value, then a 1:10 dilution is prepared instead. Based on empirical knowledge, neat tissue culture supernatants usually contain approximately 1 µg/ml of mAb. To a total of eight wells across a "U"-bottomed microtitre plate, 30 µl of this supernatant dilution was placed. A further 30 µl of each specific isotyping reagent, previously reconstituted and shaked was added. The plate was covered with a lid and placed on a plate shaker for six seconds to

allow optimal mixture. After about 1 hr, results could already be read. A part or full carpet agglutination meant a positive response to the isotype, whilst a small red circle or button was interpreted as a negative result.

Gel Filtration Chromatography for the Purification of IgM

Frozen spent tissue culture supernatants were thawed and clarified by centrifugation at 10,000 g for 5 min at 4° C. A saturated solution of ammonium sulphate (titrated to pH 7.4 with ammonium hydroxide at 4° C) was prepared and gently added to the supernatant with stirring to achieve a 45% solution. This was allowed to mix in a rotator for 1 hr at 4° C. Later, the mixture was centrifuged at 10,000 g for 15 min at 4° C and then, the supernatant was discarded and the pellet was resuspended with a volume equal to the original of 100 mM Tris, 150 mM NaCl (pH 8.0). This was dialyzed overnight against the same buffer at 4° C. The next day, the dialyzed material was centrifuged at 10,000 g for 15 min. at 4° C to get rid of precipitated material. The supernatant was then passed through a 0.2 μm filter and subjected to high performance liquid chromatography (Model 2248; LKB Bromma) on a size exclusion column (Hydropore-HIC; Rainin) with 100 mM Tris, 150 mM NaCl (pH 8.0) at a flow rate of 125 μl/min at 4-6° C. Fractions of 125 μl were collected on a microfraction collector (Model 203; Gilson).

Western immunoblotting

Immediately following electrophoresis, gels were transferred to nitrocellulose paper (BA 83; Schleicher & Schuell) and blotted using a semi-dry electroblotter (Ancos). The electrophoresis buffer used was: 25 mM Tris + 40 mM 6-amino-n-hexanoic acid + 20% methanol (cathodic side); 25 mM Tris + 20% methanol (anodic II side); and 300 mM Tris + 20% methanol (anodic I side). For IEF gels, pre-incubation (3 x 5 min) in 1% SDS + 50% methanol was deemed to improve protein transfer. Protein transfer was accomplished using a constant current of 2.5 mA/cm^2 of gel with a maximum voltage set at 100 V for 1.5 hrs. After transfer, the nitrocellulose was blocked with blocking reagent (Boehringer Mannheim) for 1 hr before overnight incubation with the respective mAbs at 4^{o} C. This was then incubated for 2 hrs at R.T. with a 1:1000 dilution of rabbit anti-mouse Ig (Z259; Dako), washed, and then incubated with a 1:2000 dilution of alkaline phosphatase (AP)-conjugated swine anti-rabbit Ig (D306; Dako) for 1 hr at R.T. Bound AP was detected using 5-Brom-4-chlor-3-indoyl-phosphate (Boehringer Mannheim) and 4-Nitro blue tetrazolium chloride (Boehringer Mannheim) as substrate and chromagen, respectively. The substrate stock solution is prepared as 50 mg 5-Br-4-Cl-3-indoyl phosphate in 1 ml N,N-dimethylformamide (DMF; Sigma); whilst that of the chromagen as 100 mg nitro blue tetrazolium in 910 µl DMF and 390 µl H_2O. The nitrocellulose blots were incubated in a buffer of 100 mM Tris-HCl, 100 mM NaCl, 50

mM $MgCl_2$ (pH 9.5) containing 37.5 µl of the substrate stock solution and 50 µl of the chromagen stock solution. Incubation was carried out in the dark and stopped once bands or dots are detected.

Sucrose density gradient centrifugation

The sedimentation of AChE and AChE + immunoglobulin complexes was examined on linear sucrose gradients (5-20% w/v) prepared in 10 mM Tris-HCl, 100 mM NaCl and 0.5% Triton X-100 (pH 7.4). Before sedimentation experiments were conducted, AChE was incubated with neat concentrations of antibody or incubated per se with buffer, for 4 hrs at 30° C. Samples were centrifuged at 28,000 rpm in an SW-28 rotor for 18 hrs at 4° C. Fractions of about 100 µl were collected from the bottom with a syringe needle by means of a peristaltic pump, and AChE activity was measured by the Ellman assay mentioned above.

Glycosidase incubation

Caudate nucleus AChE was incubated with either: neuraminidase- 100 mU/100 µl 50 mM sodium acetate buffer, pH 5 for 15 hrs at 37° C, or endoglycosidase F- 1 U/100 µl 50 mM sodium acetate buffer, pH 5 for 15 hrs at 37° C.

Immunocytochemistry

In brief, immunocytochemistry was performed on 4:6 acetone/alcohol-fixed frozen 6 µm sections of human caudate nucleus and temporal cortex. Complementary experiments were

also carried out with 10% methanol/3% H_2O_2 in PBS-fixed frozen 10 μm sections of rat striatum as well as rat striatum sections from animals perfused with periodate-lysine-paraformaldehyde according to the method of Mclean and Nakane, 1974. The sections were washed three times for 3 min in PBS containing 0.05% Triton X-100. This buffer was used throughout the washing procedure. Endogenous peroxidase was blocked by placing sections in 1.5% aqueous hydrogen peroxide for 10 min. After a brief rinse in distilled water, sections were washed in PBS and incubated at room temperature for 20 min in blocking serum (1:20 goat serum). Sections were incubated overnight at 4° C with 200 μl of the mAbs raised in the present study. After washing as before and rinsing of excess liquid, the sections were incubated for 1.5 hrs with 200 μl of a 1:200 dilution of biotinylated goat anti-mouse IgM (Vector). After further washes, the sections were incubated with elite ABC (Vector) at 1:200 dilution for 60 min at room temperature. After final washes, the sections were visualized with 50 μg/ml diaminobenzidine tetrachloride (DAB) and 1% H_2O_2 at 37° C for about 5 min. This was then washed and counterstained lightly for 2 min by dunking in a 1:1 solution of Mayer's haemalum in warm tap water. This was then differentiated by light washes in warm tap water, stabilized in PBS (minus Triton), and then mounted with glycerol gelatin (Sigma).

Chapter 3.

ACETYLCHOLINESTERASE PURIFICATION

INTRODUCTION

Most reports on the purification of AChE have focused on the use of affinity chromotography, which is a fast and simple procedure allowing selective adsorption of AChE from crude preparations. Using this method, AChE from the following have been purified:

bovine caudate nucleus	(Chan *et al.*, 1972),
pig cerebral cortex	(Reavill and Plummer, 1978),
rat brain	(Rakonczay and Brimijoin, 1986),
human erythrocyte	(Ott *et al.*, 1975),
human caudate nucleus	(Sorensen *et al.*, 1982),
Torpedo electric organ	(Stieger and Brodbeck, 1985),
mouse neuroblastoma NB2A cells	(Stieger *et al.*, 1989).

From these studies, it can be gathered that the choice of ligands for affinity columns differs from one source of AChE to another.

Employing a two-step procedure, it has been possible to purify substantial quantities of foetal bovine AChE: an initial preparative batch adsorption onto a procainamide Sepharose 4B gel, was followed by an analytical procainamide affinity chromatography of the step-1 product (de la Hoz *et al.*, 1986). Similarly, AChE from the larval trematode *Schistosoma mansoni,* was affinity purified on a Sepharose 4B gel of the inhibitor [N-(6-aminocaproyl-6-aminocaproyl)-m-aminophenyl] trimethyl ammonium. In this case, purified AChE was eluted from the column by decamethonium bromide (Goldlust *et al.*, 1986). Another group has synthesized a novel affinity matrix by coupling edrophonium chloride (a reversible cholinesterase

inhibitor) to epoxy-activated Sepharose. This matrix possessed a very stable property and had the unique advantage of selectively binding to the soluble molecular form of AChE from mammalian cerebrospinal fluid (Hodgson and Chubb, 1983). It was suggested that the chemistry of edrophonium coupled to Sepharose allows binding of sites on AChE which distinguish it from other molecular forms.

Affinity chromatography may also be used to investigate the interactions between AChE and other molecules. It was noted that sepharose-immobilized wheat-germ lectin bound AChE more markedly than concanavalin-A (Ravazzolo *et al.*, 1983) due to the presence of n-acetyl-glucosamine in the glycoprotein. In another scheme, the efficacy of N-methylacridinium as a ligand for affinity purification of AChE from chicken, rat, calf, and human brain, as well as *Torpedo* electric organ was investigated. Adsorption of AChE on the N-methylacridinium column was favourable for all mentioned, except for chicken AChE (Vallette *et al.*, 1983). In a similar study, the quaternization reactions of various 9-aminoalkylacridines and 9-aminoacridine and their relative binding affinities to AChE were compared. It was suggested that the unquaternized 9-aminoalkylacridine compounds were the preferred affinity ligand for *Electrophorus* electric organ AChE (Taylor *et al.*, 1983). This unquaternized acridinium resin was also said to be efficient in purifying AChE from the heads of the fruit fly *Drosophila* (Gnagey *et al.*, 1986) and the housefly *Musca domestica* (Fournier *et al.*, 1987).

Finally, the relative binding of human and horse serum AChE to procainamide-, methylacridinium-, m-trimethylammoniophenyl-and p-trimethylammoniophenyl-Sepharose 4B have been quantified . For example, it was noted that methylacridinium had a better binding capacity with human AChE, whilst procainamide was more favourable for horse AChE (Ralston *et al.*, 1984).

Other schemes for purification relied on mAbs raised against AChE using immunoaffinity chromatography. The asymmetric forms of AChE from the electric organs of the electric rays *Narke japonica* and *Torpedo californica* were purified on a column of Nj-601-Sepharose, using a low pH eluent (Sakai *et al.*, 1985). Nj-601 is a mAb raised against *Narke* electric organs AChE (Abe *et al.*, 1983). Likewise, A2-45-16, a mAb raised against human erythrocyte AChE, was used in the initial purification step for rabbit brain AChE, since it cross-reacts with the latter (Mintz and Brimijoin, 1985a).

The use of gel-filtration or ion-exchange chromatography as a single-step procedure in AChE purification is not widely used. Nonetheless, partial purification of AChE by gel-filtration chromatography has been attempted on the membrane-bound form of AChE from chicken brain (Rotundo, 1984b), the soluble form of rabbit erythrocyte AChE (Sine and Colas, 1987), and AChE from the venom of the shore pit viper *Trimeresurus* (Tan and Tan, 1988). As for the latter, the collected fraction was further purified on an ion-exchange chromatography.

In some cases, there is a need to combine two or even three procedures to achieve optimal purification of the enzyme. Foetal bovine serum AChE, for example, was purified in a procedure involving procainamide affinity chromatography, followed by gel-filtration (Sepharose 6B), then ion-exchange chromatography (DEAE-Sephadex) (Ralston *et al.*, 1983). A similar scheme was reported for bovine erythrocyte AChE, albeit a slight modification in the order of steps (i.e. DEAE cellulose, followed by affinity gel and Sepharose 6B) was made (Taguchi *et al.*, 1984). In another report, rabbit brain AChE was purified 2000-fold by immunoaffinity chromatography with a mAb raised against human erythrocyte AChE. This preparation was further purified by affinity on phenyltrimethylammonium-Sepharose 4B with decamethonium elution (Mintz and Brimijoin, 1985b). Finally, human membrane-bound erythrocyte AChE was purified first by a trimethyl(m-aminophenyl)-ammonium chloride affinity column, and then by gel-filtration on Sepharose 4-B (Ott *et al.*, 1975).

In toto, several procedures are currently available for the purification of AChE from a variety of sources. Unfortunately, most of these are lengthy, expensive, laborious, require the synthesis of ligands, or produce low yields. In this chapter, we shall attempt to explore optimal procedures and conditions for the purification of human CSF and brain AChE, which so far, has not been extensively undertaken by other researchers.

RESULTS

Chromatofocussing of AChE

Charge polymorphism of AChE can be quantitatively elucidated by the procedure of chromatofocussing on an anion-exchange column. Chromatofocussing of cerebrospinal fluid AChE was carried out on a pI range of 7.2 to 4.0. Optical density (at 280 nm) traces (chart recordings not shown) showed two major peaks eluting at fractions 55-68 and 85-95. (Total protein was assayed with a Biorad protein assay reagent, using BSA as standard.) AChE activity assay of aliquots from all the fractions collected revealed two major activity peaks, corresponding to fractions no. 55-61 and 62-68. The fractions containing the highest AChE activity were at 58/59 and 63/64, having a pI of 5.57-5.46 and 5.30-5.24, respectively (Figure 1). The other major protein peak (i.e. 85-95) corresponds to albumin, based on elution profiles done in a preliminary experiment using protein markers. The elution of albumin was slightly towards the acidic end of the gradient, between pI 4.75 - 4.38. Quantitative examination of aliquots from the two major activity peaks was undertaken by isoelectric focussing using Ampholine with a pI of 5-7 (Figure 2). Activity stain of a focussed sample of crude CSF revealed several bands spread across a pI range of approximately 4.5 - 6.0. These bands correspond to the different isoforms of AChE. The band patterns from different fractions obtained by chromatofocussing did not differ from each other, and

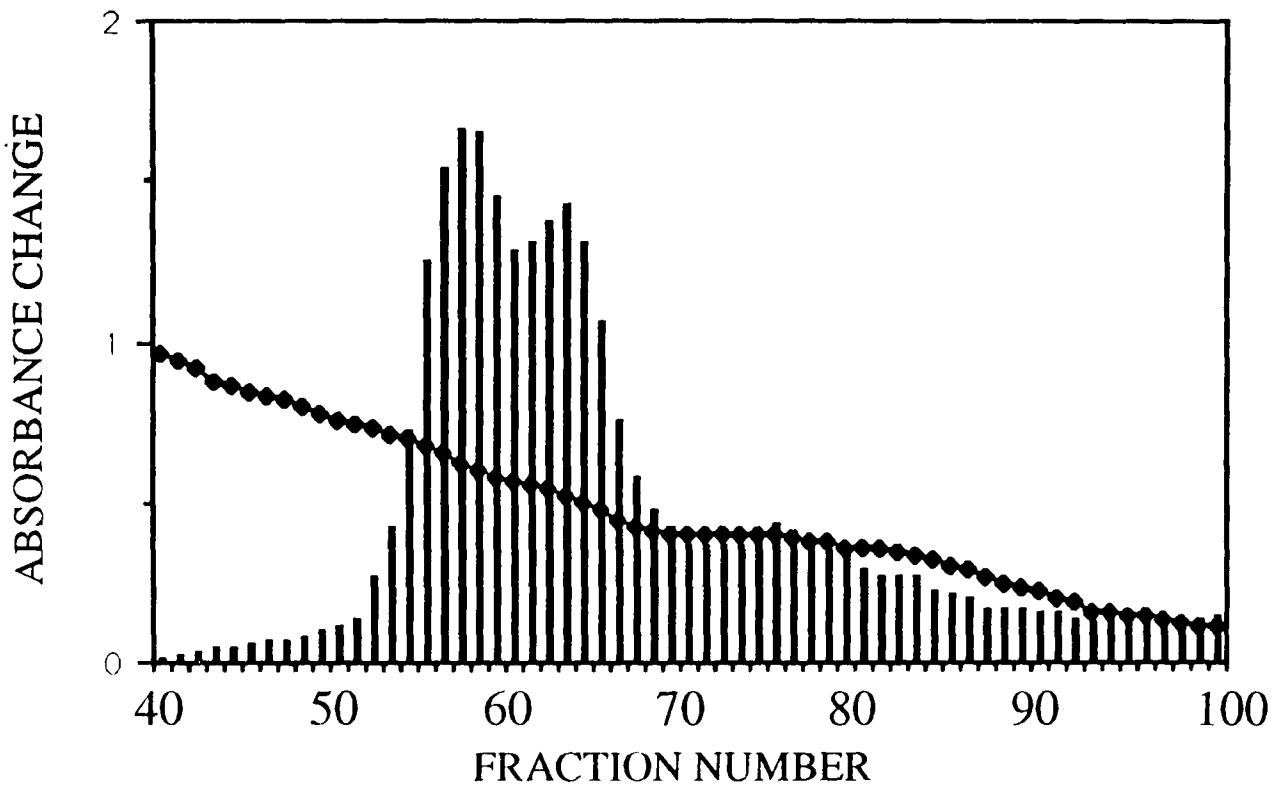


FIGURE 1. Chromatofocussing of human cerebrospinal fluid (CSF) AChE. 19 mls of pooled CSF (total activity= 258.4 mU) which had been previously desalted on a QA-Trisacryl Sephadex column and filtered through a 0.2 µm Whatman filter was injected onto a Mono-P 5/20 anion-exchange column at a flow rate of 350 µl/min.
Start buffer: 25 mM Bis-Tris pH 7.2
Elution buffer: polybuffer 74 pH 4.0
Solid bars indicate values of absorbance change (405 nm) in arbitrary units, taken over a 30-min period for each fraction collected. Diagonal line represents the pH value (ranging from pH 6.4 to 4.16) of the respective fractions.

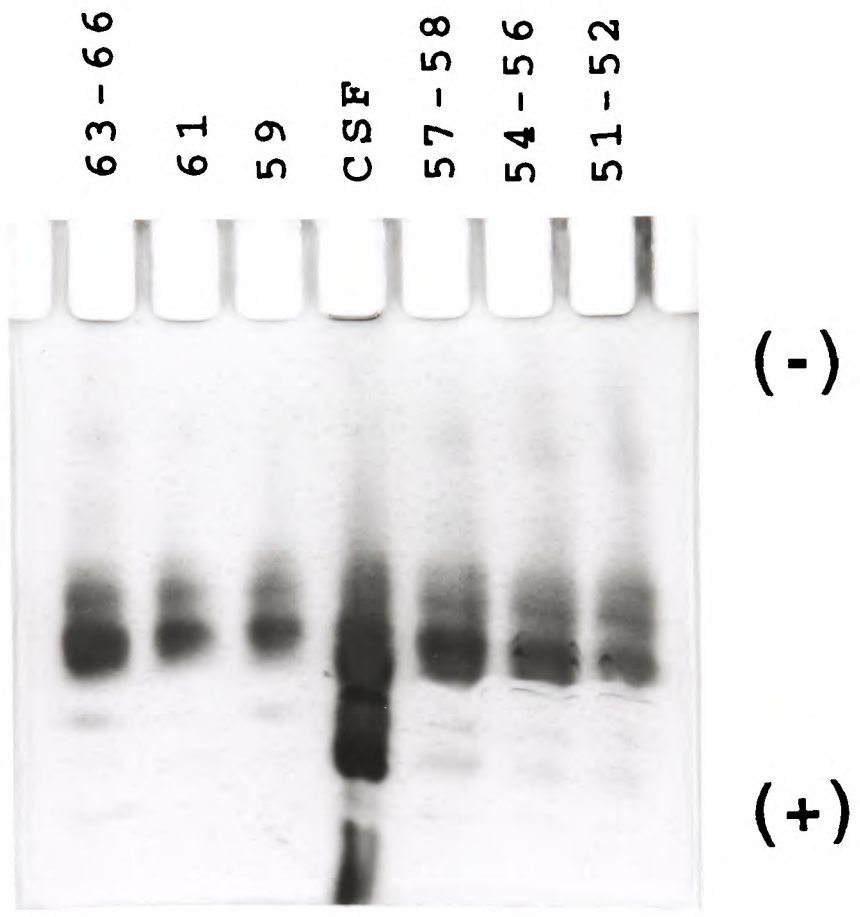
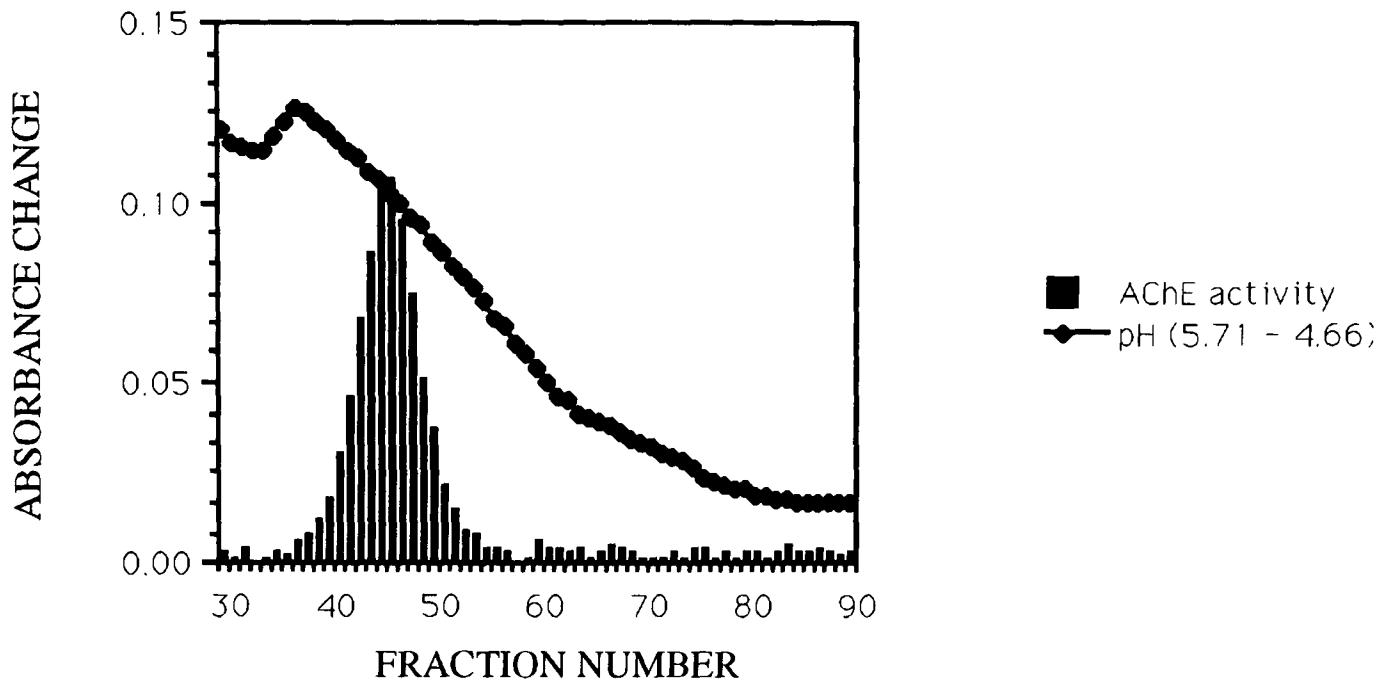


FIGURE 2. Aliquots of fractions from figure 1 were run on an isoelectric focussing (IEF) gel. Electrophoresis was performed on a pI 5-7 gel. Top panel shows respective fraction numbers and a tract with 4 mU crude CSF for comparison. The gel was stained in AChE activity incubation medium.

were also similar to the control CSF banding pattern, with the exception of some isoforms on the cathodic end which either could not be detected by the activity stain or are absent.

With the aim of trying to purify the different isoforms of AChE into separate fractions each, peak activity fractions 58/59 and 63/64 were re-injected into an HPLC and subjected to chromatofocussing (Figure 3). Upon assaying the collected fractions for AChE activity, major peaks were determined to be at fractions 40-50 (for sample 58/59) and 51-59 (for sample 63/64). The fractions containing the highest activity were at 45/46 and 54/55, corresponding to a pI of 5.57-5.53 and 5.26-5.22, respectively. Upon qualitative analysis by IEF of the collected fractions, it was noted that most of the isoforms still banded together in groups, similar to the pattern seen in the earlier IEF runs (Figure 4). After several other attempts, most of these under narrower pI ranges, it was still impossible to separate AChE into its individual isoforms.

Human caudate nucleus supernatant was tested with the view of comparing the elution pattern and chromatofocussing behaviour of AChE from another source. Elution conditions were done in a narrow pH gradient of 6.3 - 4.5. Based on optical density recordings, caudate nucleus protein eluted as a major peak in fraction nos. 50-70. Assay of individual fractions for AChE activity indicated



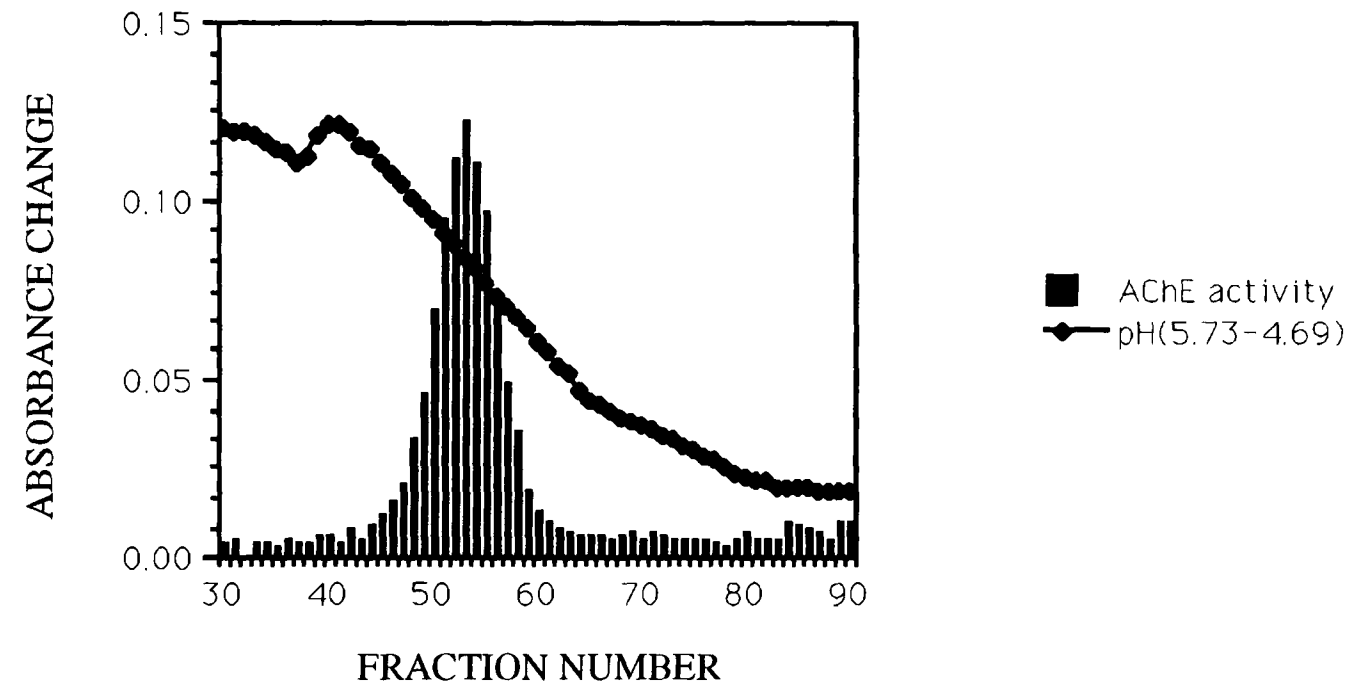


FIGURE 3. Top box: Chromatofocussing of fractions 58 & 59 from the run shown in figure 1. Bottom box: Chromatofocussing of peak fractions 63 & 64 from figure 1. All chromatographic conditions similar to figure 1. Solid bars represent absorbance change in AChE activity (presented in arbitrary units) taken within a 30 min period. Diagonal line shows pH values of respective fractions, ranging from pH 5.71 to 4.69.

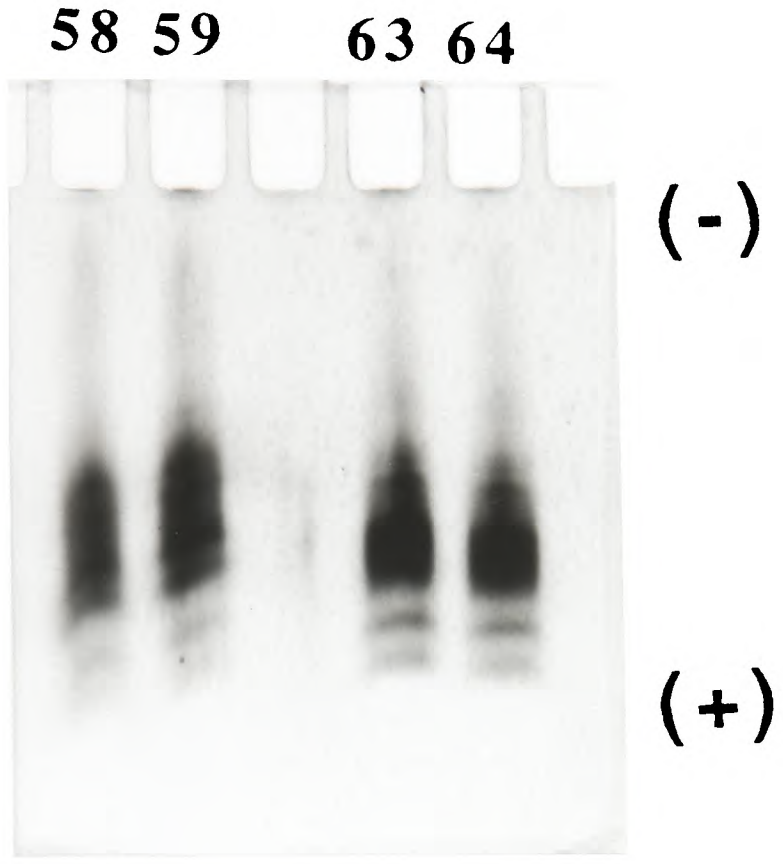


FIGURE 4. Top gel: Isoelectric focussing of peak fractions 58/59 and 63/64 from the purification of figure 1. Bottom gel: Peak fractions 54/55 and 45/46 from the purification of figure 3.

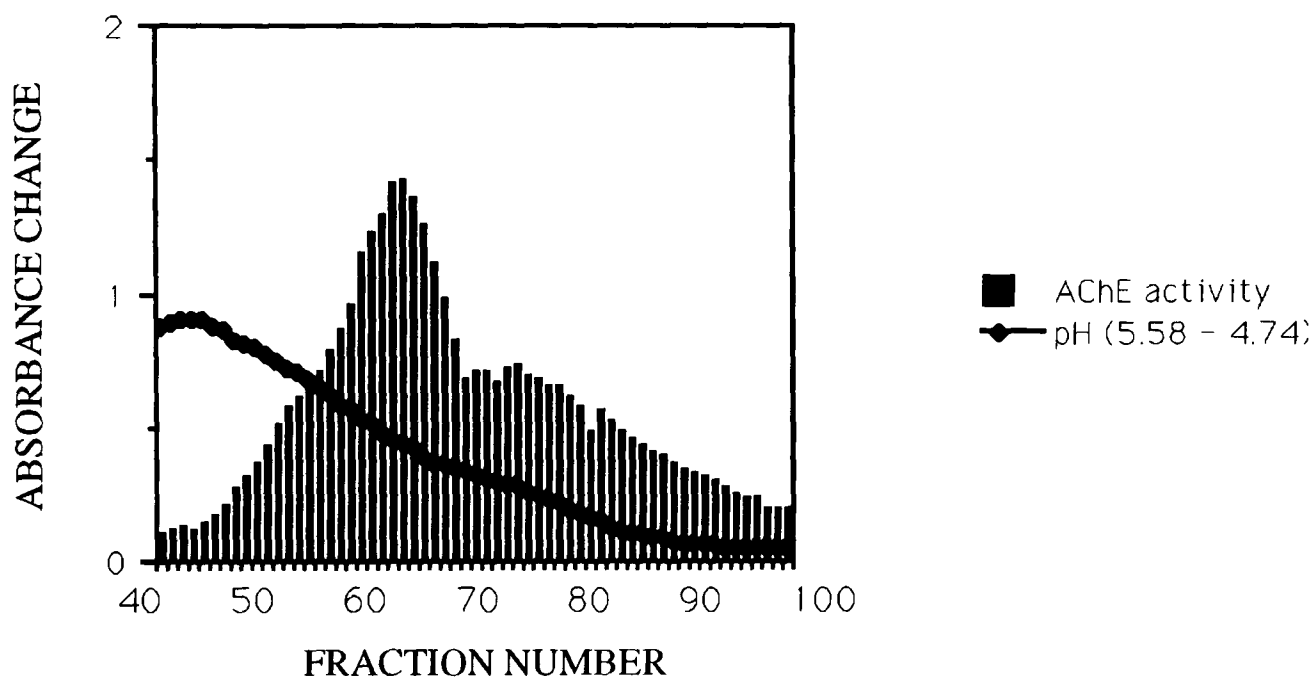


FIGURE 5. Chromatofocussing of brain homogenate. A total of 14 mls of caudate nucleus supernatant (total activity= 1673 mU), which had been previously desalted, filtered, and injected under the same conditions as in figure 1.
Start buffer: 25 mM Piperazine pH 6.3
Elution buffer: Polybuffer 74 pH 4.5
Solid columns indicate values for AChE activity (presented as arbitrary units) taken over a 30 min period for each fraction collected. Diagonal line represents the pH values (ranging from 5.58 to 4.74) of respective fractions.

one major activity peak, comprising fractions 58-69. The pI range of this peak corresponds to 5.45-5.20. A trailing pattern was also observed from fractions 70-90 (Figure 5). IEF of caudate nucleus AChE gave rise to a heterogeneous diffuse banding pattern, similar to that of CSF AChE, with isoforms in the region between pI of about 4.5 and 6.0. IEF runs of selected fractions from Figure 5 also showed no marked difference in banding patterns (Figure 6). The inability to separate each individual isoform may be due to AChE aggregating whilst in the column matrix.

From the above-mentioned findings, it can be assumed that the elution behaviour of CSF and caudate nucleus AChE are more or less similar, both eluting at pI of roughly between 5.5 - 5.2. The only marked difference is that CSF AChE has two major activity peaks whereas caudate nucleus AChE has only one. It is tempting to assume that the two major activity peaks seen with CSF AChE may actually reflect G_4 and G_1 AChE forms.

Affinity chromatography of AChE

Human brain AChE was extracted from either pons or caudate nucleus samples. In the case of pons samples, an average activity of 57 mU/mg wet weight was obtained. Purification of the soluble form of AChE was carried out by affinity chromatography on an acridinium column. The results of this purification are presented in Table I. When these samples were applied to the column, roughly 97.5% of AChE activity was bound. To ensure that the matrix

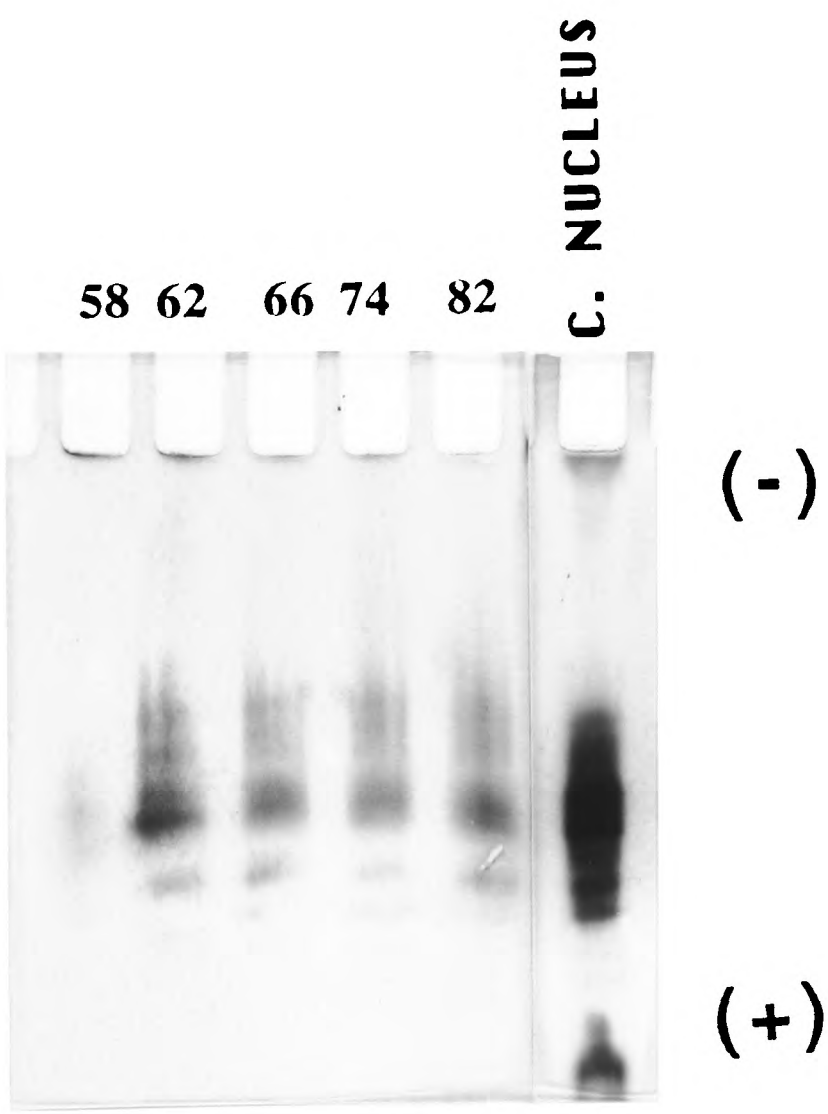


FIGURE 6. Aliquots of fractions from figure 5 were run on an isoelectric focussing gel. Electrophoresis was performed in a pI 5-7 gel. Top panel shows corresponding fraction numbers and a tract containing 4 mU of crude caudate nucleus.

TABLE I. Preparative isolation of AChE by affinity chromatography on acridinium-Sepharose.

	PONS AChE Activity (mU/mg wet weight)	S.E.M. (n=11)	Yield (%)
Starting Material	57.5	3.3	100%
Unbound fraction	1.4	0.6	2.5%
Low-salt wash	1.2	0.7	2.2%
High-salt wash	21.9	5.0	38.2%
TEA/ Decamethonium eluate	5.8	1.5	10.1%

did not adsorb proteins nonspecifically, bovine serum albumin was applied to the column. Nonspecific adsorption was almost nil, since >99% of the BSA applied was unretarded (data not shown). A further consideration was the flow rate of application. The optimal flow rate for maximum AChE retention was established to be 200 µl/min. When flow rates were increased, the degree of AChE retention decreases substantially (data not shown). It was also necessary to wash the column with low- and high-salt buffers in order to remove any proteins that were nonspecifically bound to the matrix. I usually applied a total of ten column volumes for each wash. When washes were made with less than ten column volumes, residual proteins were usually seen under SDS-PAGE. Only a minimal percentage (2.2%) of AChE is washed away under the low-salt wash. On the contrary, quite a large proportion (38.2%) is washed with the high-salt buffer. Bound AChE was eluted with a solution of tetraethylammonium (TEA; 400 mM) and decamethonium (10 mM), and the collected fractions were passed through a Sephadex column to remove the inhibitors. Roughly 10.1% of the bound fraction is eluted.

The possibility that some other affinity ligand might be more effective was investigated. Results obtained with the use of procainamide are presented in Table II. The first purification step involved adsorption of AChE from caudate nucleus supernatants to procainamide affinity gel. Up to 25 mls of supernatant was usually processed on a single 1 ml column. Approximately 92.3% of the AChE

TABLE II. Preparative isolation of AChE by affinity chromatography on procainamide-Sepharose.

	Caudate Nucleus Activity (mU/mg wet weight)	S.E.M. (n=11)	Yield (%)
Starting material	194.8	28.2	100%
Unbound fraction	15.1	4.2	7.7%
Low-salt wash	15.9	5.7	8.2%
High-salt wash	79.8	16.3	40.9%
TEA/ Decamethonium wash	83.4	12.2	42.8%

activity bound to the gel. The percentage of AChE washed away under the low- and high-salt buffers is more or less similar to that of the acridinium column. Selective elution with TEA and decamethonium resulted in recovery of roughly 42.8% of the applied AChE.

CSF AChE was also purified on a procainamide column. Percentage of elution for CSF AChE was slightly lower at about 25 - 30% (personal communication: R. Hale). To determine whether the eluted material was purified to electrophoretic homogeneity, aliquots were subjected to SDS-PAGE. Four prominent bands were visualized by Coomassie staining, and no traces of contaminating proteins were noted (gel not shown). Upon immunoblotting of this gel with the polyclonal antibody R431, the four bands were identified, indicating that these contain epitopes peculiar to AChE (Figure 7).

Comparison of affinity-purified brain and erythrocyte AChE by gel filtration

As a method of comparing the molecular size of AChE, I subjected acridinium affinity-purified brain and erythrocyte AChE to gel filtration in a Zorbax Bio-series GF-450 column. A good linear relationship was obtained with the protein markers used. Upon plotting the elution profile of the purified samples against this linear graph, a putative molecular mass of 310 and 235 kDa was calculated for brain and erythrocyte AChE, respectively (Figure 8).

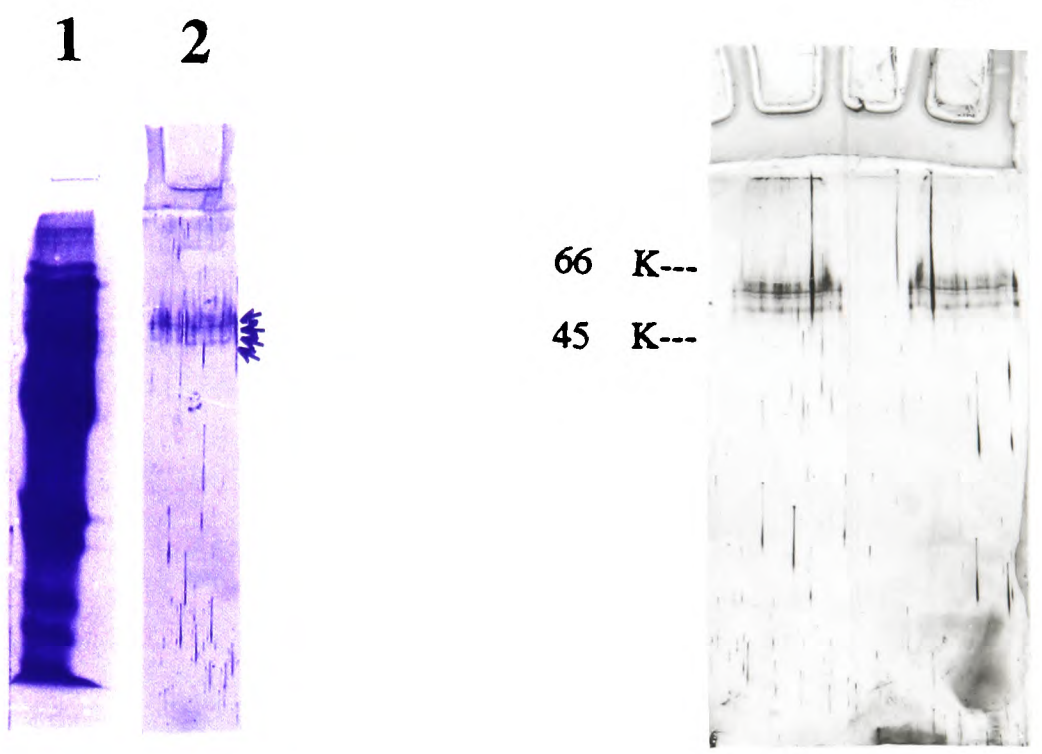


FIGURE 7. Procainamide affinity purified (A) cerebrospinal fluid and (B) caudate nucleus AChE at 25 mU per track were subjected to SDS-PAGE (12% Laemlli) and then Western blotted. Left figure: Western blot of SDS-denatured (1) CSF starting material and (2) affinity-purified CSF. Total protein bands were revealed by Coomassie staining. Arrows point to position of four AChE sub-units. Right figure: Immunodetection was carried out using the rabbit antiserum R431. Molecular weight markers used are indicated on the left.

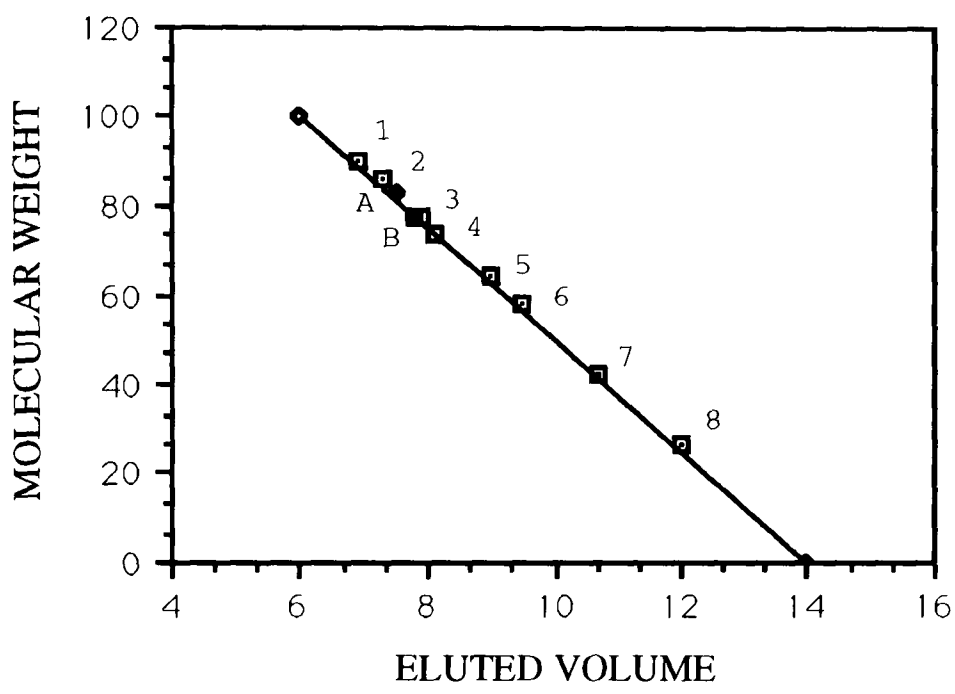


FIGURE 8. Gel filtration chromatography of acridinium purified human AChE. Open squares indicate point of elution of the following protein markers with their respective molecular weights: (1) thyroglobulin, 660 kDa; (2) ferritin, 445 kDa; (3) beta-amylase, 200 kDa; (4) alcohol dehydrogenase, 150 kDa; (5) beta-lactoglobulin, 54 kDa; (6) carbonic anhydrase, 29 kDa; (7) trasylol, 6.5 kDa; and (8) cobalamin, 1.35 kDa. The point of elution of (A) brain AChE is indicated by a filled diamond, whilst that of (B) erythrocyte AChE by a filled square. Elution volumes are expressed in mls. The values for the vertical axis are expressed in kDa.

Purification on a Pharmacia SMART System

SMART System is state-of-the-art technology which allows efficient micropurification of ng and μg quantities of biomolecules. This allows high recovery and preserved biological activity of biomolecules. Affinity-purified caudate nucleus AChE was applied into a Mono S cation exchange column. The latter has $CH_2SO_3^-$ charged groups in its narrow particle size MonoBeads, providing high resolution and stability. The activity profile of the eluted fractions is given in Figure 9. This shows one major peak with trailing from fractions 12-20. Qualitative examination by IEF of the eluted fractions revealed that under a salt gradient, no marked separation of the individual isoforms was achieved (Figure 10).

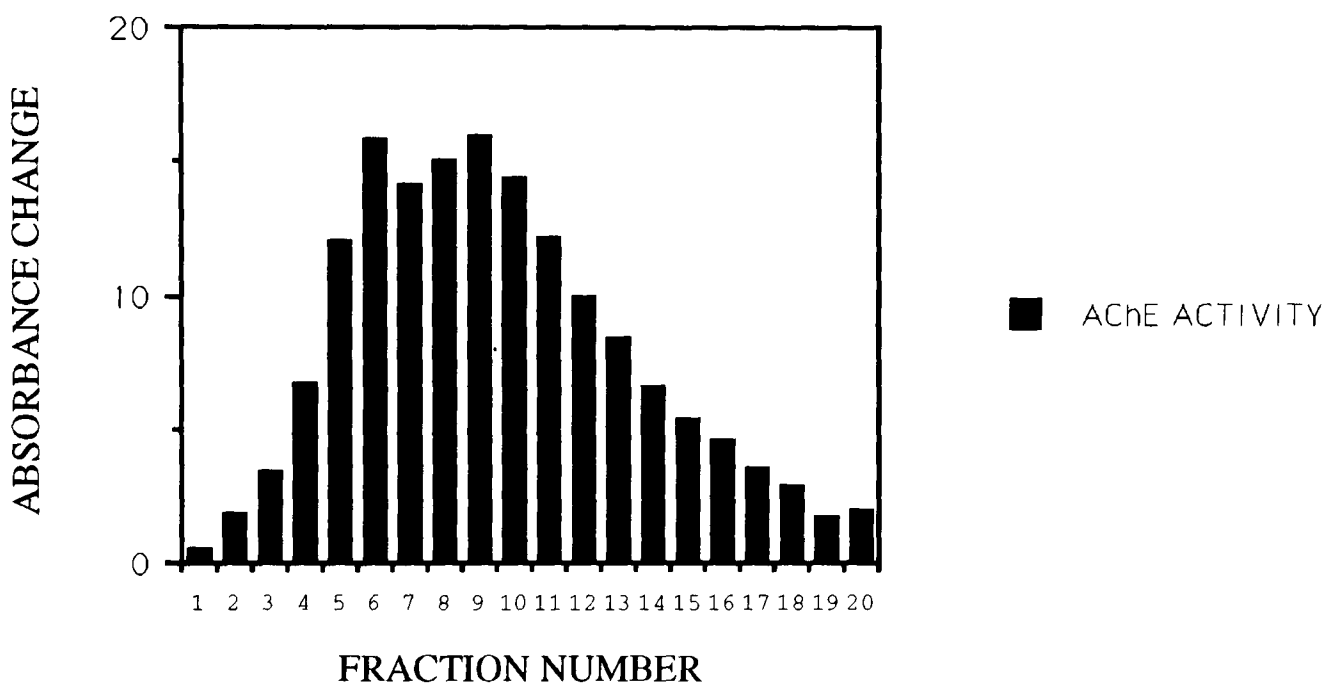


FIGURE 9. Ion exchange chromatography of affinity purified brain material on SMART System (Pharmacia). A 350 µl sample of procainamide affinity purified caudate nucleus AChE (total activity= 105 mU) was injected onto a Mono S-PC 1.6/5 ion-exchange column equilibrated with solvent A (50 mM succinate pH 5.35) at a flow rate of 100 µl/min. Elution with a gradient of solvent B (50 mM succinate + 300 mM NaCl pH 5.35) was started at fraction number 1. Solid columns indicate AChE activity (in arbitrary units) for each fraction collected.

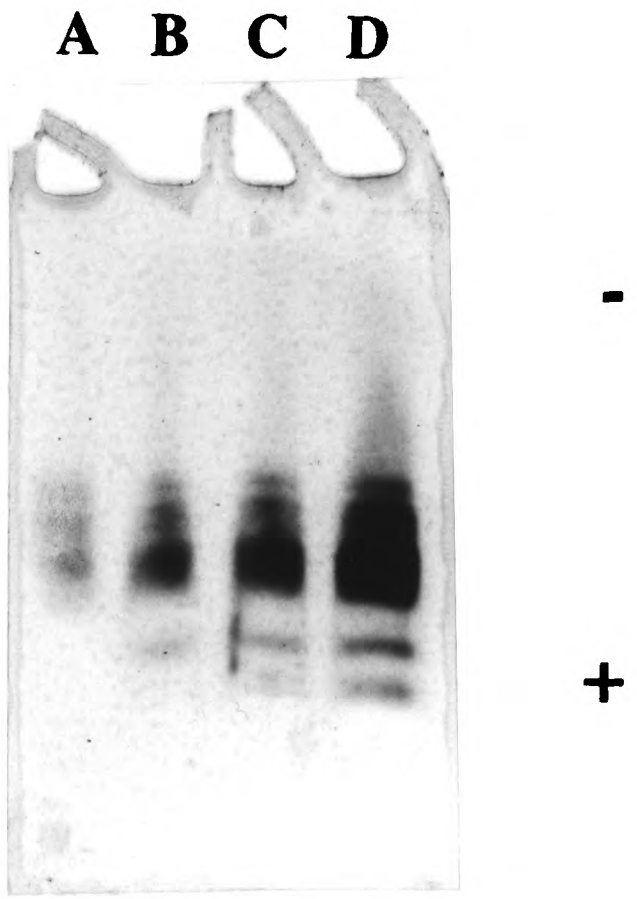


FIGURE 10. Aliquots of fractions from figure 9 were run on an isoelectric focussing gel. Electrophoresis was performed on a pI 5-7 gel. Top panel refers to fraction numbers (A) 12 (B) 10 (C) 8 (D) 6. The gel was stained in AChE activity incubation medium.

DISCUSSION

It is always the case that whenever one is faced with the onus of working on a material with biological significance (e.g. proteins, carbohydrates, lipids), the first important and indispensable step in this task would be to explore methods for its optimal purification. In my case, the need to produce highly purified samples of brain acetylcholinesterase (AChE) was essential, since I envisaged to utilize these for both immunization work and detailed characterization of the enzyme. Reports on the purification of human "membrane-bound" brain AChE have been previously documented (Rakonczay *et al.*, 1981; Sorensen *et al.*, 1982). To the best of my knowledge, however, the findings earlier shown in this chapter are the first to deal with purification methods for "soluble" brain AChE.

Current methods for separation usually rely on procedures which cause minimal damage to the physicochemical properties and biological activity of the material. The three main approaches to purifying a biological molecule rely on differences in their size, charge, and binding affinity. For example, gel-filtration chromatography allows separations on the basis of size: a sample is applied to a column of hydrated polymer beads (e.g. dextran, agarose, or polyacrylamide) which allows larger molecules to pass through more readily than smaller ones. In contrast, ion-exchange chromatography allows separations on the basis of net charge: viz, anionic (negatively-charged) molecules can be chromatographed on positively-charged DEAE- (diethylaminoethyl) cellulose

columns, whilst cationic (positively-charged) molecules can be separated on negatively-charged CM (carboxymethyl)-cellulose columns. Finally, affinity chromatography relies on the high binding affinity of a molecule for a specific chemical group. This is a type of adsorption chromatography in which a biomolecule is specifically and reversibly adsorbed by a complementary ligand which is immobilized on an insoluble matrix. Any unbound material is washed away and the bound biomolecule is then eluted with a high concentration of antagonist.

Chromatofocussing separates proteins on the basis of their isoelectric points, making it the liquid chromatographic equivalent of isoelectric focussing (IEF). Separation is based on the formation of linear pH gradients which cause elution of proteins bound to the ion exchanger in the order of their isoelectric points (pI) (Sluyterman *et al.*, 1978). My initial interest in the use of chromatofocussing was as a preparative means of purifying AChE isoforms for further studies on their structure and biological function. This was viewed in line with a recent finding by a colleague (Navaratnam *et al.*, 1991) who discovered an anomalous molecular form of AChE present at an apparent pI in the region of 5.3 - 5.5 in IEF runs of CSF from histologically-diagnosed Alzheimer's patients. Age-matched controls did not have this anomalous isoform. Unfortunately, my attempts at purifying the heterogeneous set of isoforms were unsuccessful, even at narrow pI ranges. What factors may have contributed to the

difficulty in separating these isoforms? Three possible reasons are suggested: Firstly, AChE exhibit a high degree of precipitation at its pI as the gradient migrates down the column. Although precipitation can be prevented by increasing the concentration of salt or Polybuffer, some loss in resolution is also unavoidable (personal communication: Pharmacia Technical Staff). Secondly, AChE may interact with other proteins of high avidity present in the sample. This is also coupled with the fact that the different AChE isoforms have very close pIs. Thirdly, irregularities in column packing may lead to poor resolutions. The latter is however unlikely because in preliminary experiments where test markers were used, sharp resolving peaks were obtained. It is proposed that chromatofocussing may be more suitable as a last-step procedure, in combination with other techniques such as gel filtration or affinity chromatography.

At any rate, chromatofocussing has shed some information on the differences in charge polymorphism between CSF and caudate nucleus AChE. In terms of major isoforms, IEF revealed similar band patterns for both CSF and caudate nucleus AChE. Ocular inspection suggests the presence of eight isoforms, within the pI range of 4.8 - 6.0. In contrast, cobra venom AChE has about 14-16 isoforms, obtained in the pI range of 5.2 - 6.2 (Raba *et al.*, 1979) whilst human erythrocyte AChE has five within the pI of 4.55 - 5.18 (Ott *et al.*, 1975). The existence of these isoforms may be partly due to glycosylation or

heterogeneity at the level of the polypeptide chain (Martinat *et al.*, 1990). The latter explanation is of course highly unlikely because CSF and caudate nucleus AChE are both of the G_4 form. It is more likely that AChE charge polymorphism arises from additional microheterogeneity in the glycosidic part of the molecule. Other possible sources of molecular charge differences include degrees of phosphorylation of seryl or threonyl residues, and/or partial deamidation of glutaminyl and asparaginyl residues (Oetting *et al.*, 1986).

The use of acridinium as an affinity ligand for mammalian AChE purification was first tested on pig cerebral cortex. A 900-fold purification was achieved and a recovery of 44% of the applied enzyme was eluted from the column (Reavill and Plummer, 1978). This ligand was also used for purifying plant cholinesterase (from bush bean roots), yielding a 78-fold purification (Mansfield *et al.*, 1978). With the development of a procedure for optimum purification of mammalian AChE based on acridinium affinity chromatography, this ligand became widely used for purifying human erythrocyte AChE because mg quantities of electrophoretically homogenous samples could be obtained from this tissue (Rosenberry and Scoggin, 1984). The efficacy of acridinium was also tested on other sources such as chicken, rat, calf and human brain; and from *Torpedo* electric organ. Retention of AChE on the acridinium column exceeded 90% for all sources, with the exception of chicken AChE where only 40-80% retention was

observed (Vallette *et al.*, 1983). This finding is consistent with the retention values of >97% which I obtained for pons and erythrocyte AChE. The major difference lies on yield values, of which I obtained very low figures (i.e. 10.1% for brain and 18-25% for erythrocyte AChE). In comparison for example, a recovery of 44% was reported for pig cerebral cortex AChE on an acridinium gel (Reavill and Plummer, 1978). It is possible that the low yield which I obtained with the brain enzyme was due to a loss in most of the eluted enzyme's activity. Indeed, granting that a yield of 10.1% was obtained and a total of 40.2% is released during the low- and high-salt washes, there still is about 50% of the bound enzyme worth accounting for. Technical factors may have brought about loss of enzyme activity during the purification process. However, this seems highly unlikely because all procedures were carried out in the cold room and assays were done right after "desalting" of the eluted material. A more likely explanation for this loss in activity is that G_4 AChE binds strongly to acridinium in both its active and esteratic sites. As such, this tight binding may cause steric hindrance which in turn may lead to structural disconfiguration and eventual decimation in AChE activity. Of course, there is the obvious explanation that the "unaccounted AChE" may still be bound to the column. Since high-salt buffers were used to wash away any material bound ionically, it is then possible that the non-eluted AChE is bound hydrophobically. Finally, in an earlier study, it

was reported that unquaternized acridine ligands were comparable in efficacy to the routinely employed quaternized acridine ligand. The ease of synthesis coupled with the column performance of these unquaternized compounds make them a preferred affinity ligand for AChE (Taylor *et al.*, 1983). As a result, unquaternized acridinium was used in the foregoing experiments.

The use of procainamide as a ligand was first described for the purification of horse and human serum BuChE, with the former showing a better yield than the latter. However, when *Electrophorus* AChE was applied, it was discovered that this bound 10-100 times more tightly to procainamide than what was seen with BuChE (Ralston *et al.*, 1983). Procainamide affinity ligand has been successfully employed for purification of foetal bovine serum (FBS) AChE (de la Hoz *et al.*, 1986). In an earlier quantitative study, FBS AChE was purified to approximately 44,000 fold, corresponding to an overall yield of 45% (Ralston *et al.*, 1985). This value is almost comparable to the yield of 42.8% which I obtained on purifying caudate nucleus AChE.

When employing affinity chromatography, recovery was contingent on several factors such as the presence of Triton X-100, the ionic strength, the inhibitor used for elution, and the nature of the affinity ligand. In the absence of a detergent, AChE assumes different states of aggregation. It was also reported that losses of enzyme activity up to 90% and more were encountered during

prolonged incubation of AChE in the absence of Triton X-100 (Ott *et al.*, 1975). I have also noted that a 10% (approx) decrease in AChE binding usually occurs after reuse of both affinity gels. Such behaviour is not uncommon (de la Hoz *et al.*, 1986) and can be solved by regeneration of the gel with 1 M NaOH washes.

Based on the present findings, brain AChE appears to have a better purification yield when using procainamide as a ligand as opposed to acridinium. It can be postulated that the weaker binding with procainamide allows maximum elution under less harsh conditions. However, in the absence of inhibition constants data, a putative mode of action cannot be suggested. I have also found that slight differences in AChE retention are seen in both ligands. The slightly lower AChE retention in the procainamide gel may be due to the ligands lying spatially too close to the gel matrix, thereby sterically shielding any interaction with the active site of AChE (Berman and Young, 1971). This of course can be remedied by further extending the ligand from the gel.

I have tried using edrophonium affinity ligand, which has been shown to successfully purify secretory soluble AChE (Hodgson and Chubb, 1983). Percentage elution is 86% for bovine CSF, 53% for foetal bovine serum, and 28% for rabbit brain. In preliminary experiments, however, I was unable to demonstrate any significant retention for human brain AChE. It is reasonable to assume that edrophonium binds only to soluble AChE which has the electrophoretic

and size characteristics of secretory AChE (Chubb *et al.*, 1976).

Under SDS-PAGE, four bands migrating between 50 - 62 kDa were seen for both brain and erythrocyte AChE. This varies significantly from other studies that reported values given in the table below:

Detergent-soluble		
Human brain	**78 kDa**	**Sorensen *et al.*, 1982**
***Torpedo* electroplax**	**80 kDa**	**Viratelle & Bernhard, 1980**
Ascidian larva	**65-70 kDa**	**Meedel, 1980**
***Bungarus* venom**	**70 kDa**	**Grossmann & Lieflander, 1979**
Naja venom	**69 kDa**	**Grossmann & Lieflander, 1979**

The values that I obtained for both brain and erythrocyte AChE were lower than the average molecular weight of the smallest catalytically active molecular form from the various animal tissues presented above. On the other hand, one paper presented values of 64 - 68 kDa for mammalian brain, erythrocyte and neuroblastomas AChE (Raba *et al.*, 1979). The highest band which I obtained (62 kDa) is almost compatible with the values just mentioned. The presence of the three other bands may be due to an artifact of proteolysis caused by an endogenous protease contaminant (Gnagey *et al.*, 1986) or of differential processing of a single precursor polypeptide chain (Rotundo, 1984a).

When affinity-purified samples were subjected to gel filtration, the putative molecular masses of brain and erythrocyte AChE were determined to be 310 and 235 kDa,

respectively. The former is more or less in agreement with a previously calculated value of 320 kDa for the "membrane-bound" form of human brain AChE, representing a tetramer of roughly 78 kDa per subunit (Sorensen *et al.*, 1982). In comparison, the molecular masses of rabbit brain (Mintz and Brimijoin, 1985b), foetal bovine serum (Ralston *et al.*, 1985), and chicken brain AChE (Rotundo, 1984) were estimated to be 280, 340, and 420 kDa, respectively. These are also close to the value of 328 kDa found for the rat brain enzyme (Rakonczay *et al.*, 1981). However, the molecular mass which I calculated for erythrocyte AChE is not in consonance with an earlier reported value of 159 kDa (Ott *et al.*, 1975). This disparity may be due in part to differences in hydrodynamic properties between G_2 and G_4 AChE. In the case of FBS AChE (a G_4 form), for example, Triton X-100 has no effect on its physical state. On the other hand, erythrocyte AChE forms polydisperse aggregates in the absence of Triton X-100, but disaggregates in its presence (Ralston *et al.*, 1984). It is likely that the value obtained for erythrocyte AChE represented aggregated forms of this enzyme. Indeed, under gel filtration, the appearance of intermediate AChE forms along with very high molecular weight material may suggest the involvement of a complex process of disaggregation and reaggregation (Mintz and Brimijoin, 1985b). Triton X-100 was not included in the gel filtration buffer, since this caused column blockage and pressure increase in preliminary runs. Another explanation worth mentioning is the possibility that the

high apparent molecular mass for erythrocyte AChE is due to its high degree of glycosylation. It has previously been mentioned that erythrocyte AChE is more highly glycosylated than its brain counterpart (Liao *et al.*, 1991).

It must be emphasised that the values given by previous investigators are only rough estimates. The use of gel filtration for molecular mass determinations is somewhat contentious, depending on one's vantage point (a physical chemist vs a biochemist). In my case, estimation of the above molecular masses was straightforwardly made according to the enzyme's elution relative to protein markers, without taking into account Stokes radius and sedimentation coefficients. Inclusion of these two parameters is unnecessary and would be too pedantic (personal communication: Dr. J. D. Priddle).

With the advent of Pharmacia's SMART System, I decided to take advantage of this technology's unique features in trying to purify human brain AChE into its isoforms. However, IEF analysis showed that the isoforms still eluted as a heterogenous group. This is quite discouraging, granting that samples from crude preparations such as chick optic lobe extract and calcitonin peptide have been efficiently resolved, according to the manufacturer's fact sheet (SMART System Data file). Several reasons are suggested for this poor resolution: (1) Since the pI of AChE is roughly within 5.0 - 5.6, I should really be working with a buffer having a pH of 4.0 and not pH 5.35. This however poses a crucial problem on preserving the

activity of the enzyme. (2) The salt gradient used was too steep. Shallower gradients tend to give better resolutions. (3) The sample applied, with a total activity of 105 mU, may have been too much. Due to the highly sensitive nature of this system, a lower concentration of the sample should have been applied.

The activity profile of fractions collected from the SMART System showed some degree of trailing. This trailing has also been encountered in another study, pointing to it as an artefactual behaviour brought about by a complex equilibrium existing between the free enzyme and aggregates thereof (Ott *et al.*, 1975). I have not attempted to do further work on this subject. Nonetheless, different parameters for optimizing resolution of AChE isoforms are currently being investigated by another colleague.

To recapitulate, I initially hoped to purify human brain AChE into its isoforms using preparative techniques like chromatofocussing and cation exchange chromatography. Unfortunately, these were unsuccessful. I surmise that AChE aggregation is one major obstacle precluding good resolution. Indeed, clusters of two or three tetramers can form in vitro under appropriate conditions, although it is uncertain whether such associations occur in vivo (Rotundo, 1984b). Purification by affinity chromatography gives better preparative yields with the use of procainamide as a ligand. These affinity-purified samples were used as antigen for monoclonal antibody production, and shall be discussed in the proceeding chapters.

Chapter 4.

PRODUCTION OF MONOCLONAL ANTIBODIES TO HUMAN ACETYLCHOLINESTERASE BY IN VIVO IMMUNIZATION

INTRODUCTION

In order to gain an insight into the structure, biology, evolution, and biosynthesis of acetylcholinesterase (AChE; EC 3.1.1.7), the need to raise monoclonal antibodies (mAbs) with extreme specificity and homogeneity has become important. Possible applications of these mAbs are in the histochemical localization of the different forms of AChE in tissue sections, analysis of the anatomical and molecular physiological basis of AChE polymorphism, enzyme immunoassay of AChE concentration in tissues, and purification of AChE by immunoaffinity chromatography. In the past, polyclonal antiserums against rat (Marsh *et al.*, 1977; Zanetta *et al.*, 1981), mouse (Adamson, 1977), electric eel (Greenberg *et al.*, 1977), trematode (Espinoza *et al.*, 1991), and bovine (Holmes *et al.*, 1973) AChE were raised, although their usage was somewhat limited due to their heterogeneous and incompletely defined nature. Moreover, the long-term benefits of polyclonal antisera is precluded because of its low titers, limited supply, and lack of reproducibility. Notwithstanding these drawbacks, much useful information has been obtained with their application. Using polyclonal antiserum raised against electric eel AChE, Greenberg *et al.* (1977) demonstrated a high degree of antigenic cross-reactivity existing between globular and asymmetric forms of AChE. Similarly, previous studies involving anti-AChE polyclonal antisera also showed no differences in the immunodominant antigenic determinant between the globular

and asymmetric forms of AChE (Rieger *et al.*, 1973; Gurari *et al.*, 1974). In another study, polyclonal antisera raised against AChE from *Schistosoma mansoni* (which causes a parasitic disease in humans called schistosomiasis or Bilharzia) led to marked complement-killing of schistosomes in vitro. It is suggested that AChE is involved in the immune response towards the parasite during the initial stages of infection, thus giving the possibility of this antiserum to be used for vaccination purposes (Espinoza *et al.*, 1991). In clinical applications, polyclonal antiserum was used in an enzyme antigen immunoassay to measure AChE in amniotic fluid specimens. This approach allows possible diagnosis of open fetal abnormalities, such as neural tube defects (Rasmussen *et al.*, 1989). Finally, polyclonal antisera to AChE have allowed further analysis of the structural forms of this enzyme, such as differences between "membrane-bound" and "soluble" brain AChE (Sorensen *et al.*, 1982), elucidation of the collagen nature of the elongated tail unit of asymmetric AChE (Anglister *et al.*, 1978), and immunological similarities between AChE and butyrylcholinesterase (Marsh *et al.*, 1984).

With the advent of Kohler and Milstein's (1975) watershed discovery on the successful fusion of myeloma cells with lymphocytes of immunized animals, their method for producing mAbs has become widely used not only in immunology, but also in most fields of medical science. As such, mAbs against AChE from a variety of sources, such as chicken brain (Rotundo, 1984b), rabbit brain (Mintz and

Brimijoin, 1985a), rat brain (Rakonczay and Brimijoin, 1986), *Torpedo* electric organs (Musset *et al.*, 1987), human erythrocytes (Sorensen *et al.*, 1987a), and human brain (Rakonczay and Brimijoin, 1988) have been successfully raised.

Several of these anti-AChE mAbs displayed interesting characteristics. AE-2 was raised against human erythrocyte membrane-bound dimeric AChE (Fambrough *et al.*, 1982) and demonstrated cross-reactivity with human and monkey muscular junction AChE. Immunocytochemical staining patterns of this mAb corresponded to the distribution of junctional AChE. Hence it was speculated that a high level of homology exists between the AChE of erythrocytes and neuromuscular junctions. A further study on AE-2 demonstrated its non-competitive inhibition of AChE activity. In theory, the catalytic center of AChE consists of two active sub-sites, the esteratic (catalytic) sub-site and the adjacent anionic (substrate-binding) one, with the latter mediating binding of the cationic substrate (Wilson and Bergman, 1950). AE-2 inhibition was fully reversible in a time-dependent manner, and inhibited human adult and foetal brain AChE, but only to a lesser extent bovine brain AChE (Sorensen *et al.*, 1987a). In comparison, C1B7 demonstrated slightly greater AChE inhibition vis-à-vis AE-2. It is suggested that AE-2 and C1B7 both decrease the catalytic rate of enzyme activity by inducing an allosteric long-distance interaction between the antibody-binding site and the anionic sub-site (Olson *et al.*,1990). In contrast,

F3-43 inhibits rabbit brain AChE activity by inducing conformational changes that are unfavorable for catalysis. This mechanism might involve direct antibody binding to the catalytic site of the enzyme (Brimijoin *et al.*, 1985). In a similar manner, Nj-501, raised against *Narke japonica* electric organs, inhibited the activities of the asymmetric, detergent-soluble, and low salt-soluble electric organ AChE. The strong inhibition of AChE by Nj-501 suggests that this antibody was directed to the active site of catalytic subunits of AChE (Abe *et al.*, 1983). Finally, mAb 25B1 demonstrated markedly different features from the above-mentioned anti-AChE inhibitory mAbs, in terms of its degree of inhibition (greater than 98%), high degree of specificity, and stability of antigen-antibody complex. It is suggested that 25B1 is directed against a conformational epitope located in close proximity to the catalytic center of this enzyme (Ashani *et al.*, 1990).

One set of anti-AChE mAbs demonstrated varied functional effects. Intrastriatal injection of anti-AChE mAbs in rats produced disappearance of AChE-positive nerve terminals within the striatum and cortex. It is suggested that this mAb produces selective effects on cholinergic terminals possessing functional deficits (Bean *et al.*, 1991). Another mAb, systemically injected in adult rats, caused a syndrome with complement-mediated destruction of presynaptic fibers in sympathetic ganglia and adrenal medulla. This mAb may serve as a tool for selective cholinergic autoimmunity, which is relevant to the

pathogenesis of human dysautonomias (Brimijoin and Lennon, 1990).

It is likely that immunologic differences among the AChE forms may be due to variations in the degree of protein glycosylation. One group of anti-AChE mAbs was found to recognize carbohydrate moieties glycosylated to AChE. These antibodies could possibly shed light on the glycosylation of AChE, in general, and about structural similarities of N-linked carbohydrates between vertebrate AChE, in particular. One such mAb, Elec-39, raised against *Electrophorus* electric organ AChE, has been shown to react with globular AChE (amphiphilic G2) from *Torpedo* electric organs, but does not react with asymmetric AChE from *Torpedo* electric organs or with AChE from other tissues of *Electrophorus* and *Torpedo*. Elec-39 has a specificity resembling that of other anti-carbohydrate IgMs such as HNK-1, NC-1, L2, and NSP-4, which belong to a family of mAbs that bind membrane-bound and matrix proteins appearing to be involved in cell to cell and cell to substrate interaction (Kuchler *et al.*, 1991). It is therefore speculated that AChE may play the role of a cell adhesion protein, specific to electric organs because of its high concentration in this tissue (Bon *et al.*, 1987). Another carbohydrate-specific mAb, 2G8, was raised against *Torpedo nacline timilei* electric organ AChE, and displayed a wide range of cross-reactivity with AChE from different vertebrates. This mAb recognizes a continuous and not a conformational N-linked oligosaccharide epitope

which appears to be highly conserved during evolution (Liao *et al.*, 1991).

At the present, efforts are being made to raise mAbs against human AChE. The major application of these mAbs would be in diagnostic assays, since AChE has been recently implicated in several pathologies, such as Alzheimer's disease, Parkinson's disease, Gerstmann-Straussler syndrome, Down's syndrome (Alzheimer-type pathology derived in middle age), and Korsakoff's psychosis (Perry, 1986). One such mAb with possible clinical usefulness is 4F19 which showed preferential binding to AChE of neuronal origin and was used to determine increased levels of AChE in amniotic fluid of foetal neural tube defects. Indeed, 4F19 has a higher affinity for the tetrameric AChE of neuronal origin than for the dimeric erythrocyte membrane enzyme. Further, 4F19 is adequate for discrimination between normal and pathological samples as long as these are not blood contaminated (Rasmussen *et al.*, 1987). Presently, 4F19 is being used in determining amniotic fluid AChE as a procedure for antenatal diagnosis of fetal neural tube defects (Loft, 1990). It was suggested that the use of 4F19 in immunoassay is more reliable than the gel electrophoretic or spectrophotometric procedures used for neural tube defects diagnosis (Smith *et al.*, 1979). Further, 4F19 is also being used for determining AChE levels in cerebrospinal fluid of patients with various neurological disorders (Mader *et al.*, 1991). MAbs HR2, HR3, and HR5 were raised against human cerebellum AChE, but

cross-reacts with erythrocyte AChE with nearly equal affinity (Rakonczay and Brimijoin, 1988). A major application of these mAbs, however, would be in the quantitative immunoassay of AChE, where extreme specificity and the ability to measure catalytically inactive AChE are called for. The clinical application of these mAbs may help in resolving conflicting information on the effects of neuromuscular disease and dementia on plasma AChE (Festoff and Fernandez, 1981; Atack *et al.*, 1988). Recently, another mAb, C1B7, was raised against human erythrocyte AChE, but its cross-reactivity with brain AChE has yet to be tested as such (Olson *et al.*, 1990).

Obviously, any attempts at raising anti-AChE mAbs as diagnostic tools in neuropathology must aim at distinguishing brain from erythrocyte AChE. In the present study, I have attempted to raise mAbs directed against the soluble brain form of this enzyme. My raison d'être for carrying out this work is to eventually develop, in the long-term, a diagnostic test for Alzheimer's disease, based on a recent finding in our laboratory whereby isoelectric focussing gels of CSF AChE from histologically-diagnosed Alzheimer's disease patients has been shown to possess an anomalous AChE isoform which was absent in age-matched controls (Navaratnam *et al.*, 1991). Here I describe the immunological properties of four IgMs raised against human brain and erythrocyte AChE using a dual-antigen priming protocol.

RESULTS

Antigen preparation

Soluble caudate nucleus and membrane-bound erythrocyte AChE were affinity purified on an acridinium column, as described in an earlier chapter. A good recovery yield for erythrocyte AChE was obtained (data not given), although this was not the case for caudate nucleus AChE, where only a final yield of 10.1% was achieved. Nonetheless, 3.5 μg of brain AChE was pooled and used as antigen.

Monoclonal antibody production

Two mice were primed with affinity-purified erythrocyte or caudate nucleus AChE, according to the protocol described in Materials and Methods. One spleen cell-myeloma fusion was performed and from among 960 wells seeded, a total of 75.1% (721/960) showed primary hybridoma culture growth. From this initial screening, a total of sixty-six (66) anti-AChE mAb-secreting clones were identified, based on a modified ELISA capture assay which regarded any absorbance readings >0.050 against background values as "positive cultures", i.e. secreting mAbs to AChE. A single stable mAb-secreting cell line was established from each "positive culture" by three subcultures at limiting dilution. To rule out the possibility of false positives, more specifically the influence of Ig from the serum used, control wells coated with rabbit anti-mouse Ig were incubated with neat foetal calf serum or tissue

culture medium. The absorbance values of these wells did not vary significantly from those of the blank control.

In the end, I pencil-focussed on four clones, which somehow managed to survive the vagaries of mAb production, such as contamination, poor viability, and loss of antibody production capacity. The four cell lines which were grown to monoclonality and expanded are: BMS(Bristol-Myers Squibb)-3E4, BMS-6D6, BMS-7G10, and BMS-9F4. Isotyping of these mAbs showed that these were IgM heavy chain-subtypes (Figure 1). The isotyping method was based on the principle of red cell agglutination (reverse passive). A positive, agglutinated result is produced when highly specific antibody (coupled to sheep RBC) recognizes and binds to the isotype to which it is directed. This binding forms a lattice on the bottom of the microtitre plate- termed "agglutination". A negative result is produced when the reagent cells are put into supernatant containing a class of antibody which they do not recognise. The reagent erythrocytes fall to the bottom of the U-shaped microtitre plate wells forming a small circle or button in the centre.

Binding affinity for AChE

To initially screen the antigen preference of the four mAbs, a straightforward dot immunobinding assay was made, using several dilutions (expressed in mU of activity) of brain and erythrocyte AChE. As shown in Table I, one group of mAbs (BMS-3E4, BMS-7G10, BMS-9F4) seemed to bind with

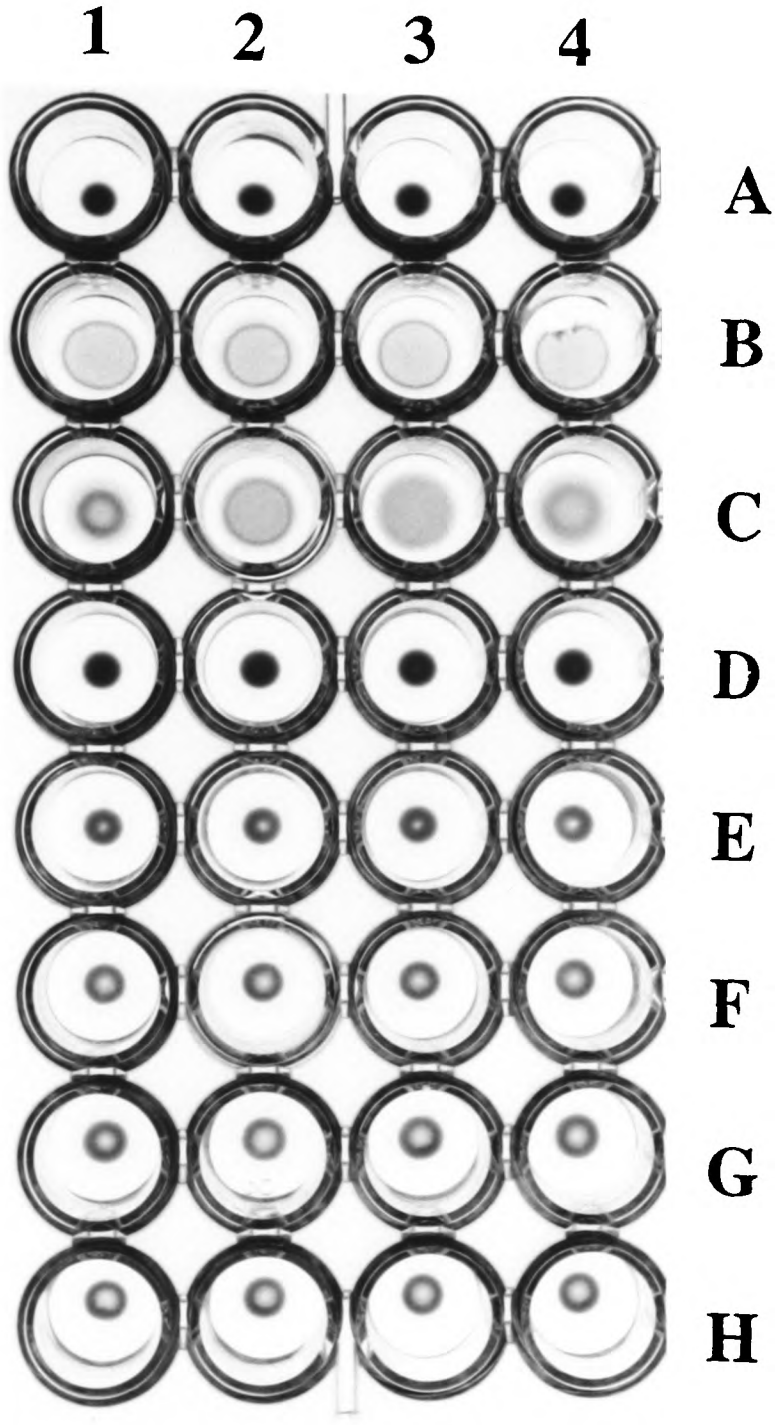


FIGURE 1. Isotyping of the class and subclass of the four mAbs. (1) BMS-3E4 (2) BMS-6D6 (3) BMS-7G10 (4) BMS-9F4. (A) Negative Control (B) Positive Control (C) IgM (D) IgA (E) IgG3 (F) IgG2b (G) IgG2a (H) IgG1.

TABLE I. Dot immunobinding assays using the four mAbs and immobilized caudate nucleus and erythrocyte AChE, human serum BuChE, and two control proteins.

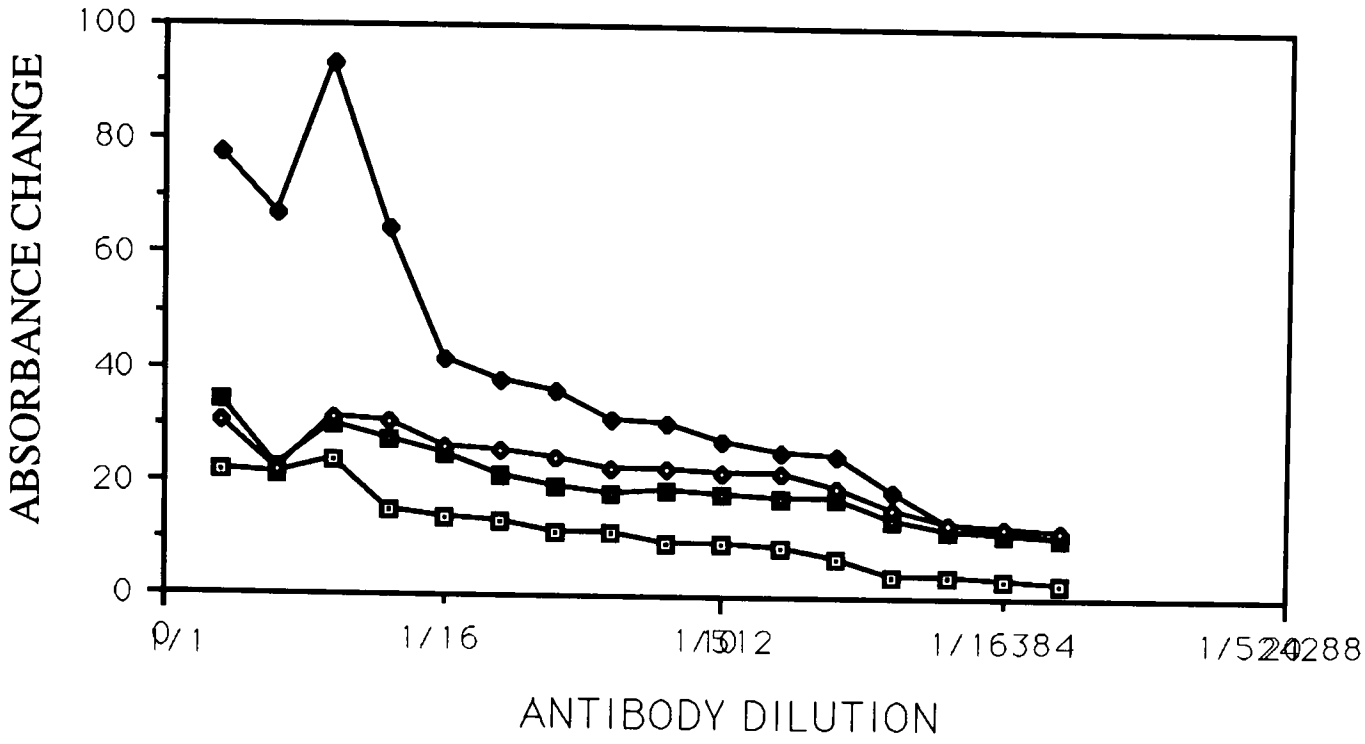
	Caudate nucleus AChE			Erythrocyte AChE					
MAbs	5mU	3mU	1mU	5mU	3mU	1mU	BSA	OA	BuChE
BMS-3E4	+	-	-	+	+	+	-	-	-
BMS-6D6	+	+	+	+	-	-	-	-	-
BMS-7G10	+	-	-	+	+	-	-	-	-
BMS-9F4	+	-	-	+	+	+	-	-	-

(+) positive reaction
(-) negative reaction
BSA, bovine serum albumin
OA, ovalbumin

erythrocyte AChE, whereas BMS-6D6 displayed preferential binding to caudate nucleus AChE. To rule out the possibility of non-specific binding, bovine serum albumin and ovalbumin were used as controls. No positive reaction to the latter was noted.

In view of the structural and functional similarities between AChE and BuChE, the four mAbs were also tested for their possible cross-reactivity with BuChE. Crude human serum was blotted onto nitrocellulose paper and incubated with the mAbs. None of the present four mAbs bound to BuChE, even when dilutions of this enzyme were tested.

To further evaluate antibody binding affinity, various dilutions of the present mAbs were incubated with erythrocyte or caudate nucleus AChE. The titration curves obtained are shown in Figure 2. In agreement with the immunodot assays, BMS-6D6 displayed a stronger dose-response curve with caudate nucleus AChE. In contrast, BMS-3E4 and BMS-9F4 showed a stronger titre with erythrocyte AChE. On the other hand, BMS-7G10, which recognized erythrocyte AChE in immunodot assay, showed very weak or in some cases failed to show a similar reaction in the ELISA system. In the titration curves, one can observe an initial drop and thereafter an increase in percentage response. This is referred to as a "prozone phenomenon" and is a result of an excess of mAbs relative to the antigen, thereby creating an initial displacement effect. In spite of its very little documentation, this effect is quite well established and encountered by many



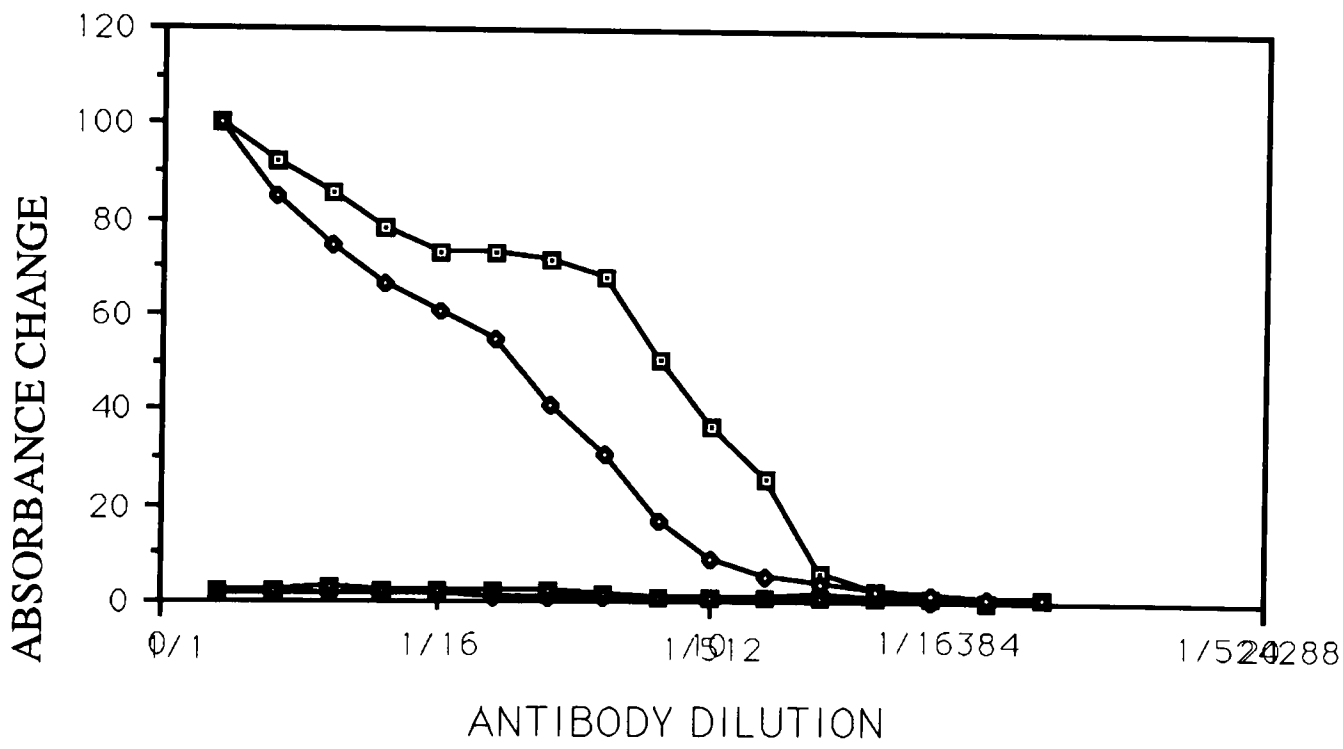


FIGURE 2. Titration (dose-response) curves obtained after incubating wells with decreasing dilutions of mAbs BMS-3E4 (open square), BMS-6D6 (filled diamond), BMS-7G10 (filled square), and BMS-9F4 (open diamond), to which caudate nucleus (top diagram) and erythrocyte (bottom diagram) extracts containing 50 mU AChE per well were added. The vertical axis shows AChE activity on an arbitrary scale.

investigators in the field. The exact mechanism of this phenomenon is unknown, although it is suggested that steric hindrance between binding antibodies may be responsible (personal communication: Dr. C. Popham). The present mAbs yielded different titres to the two sources of antigen tested. The titre values at which one-half maximum AChE activity is achieved are given in Table II.

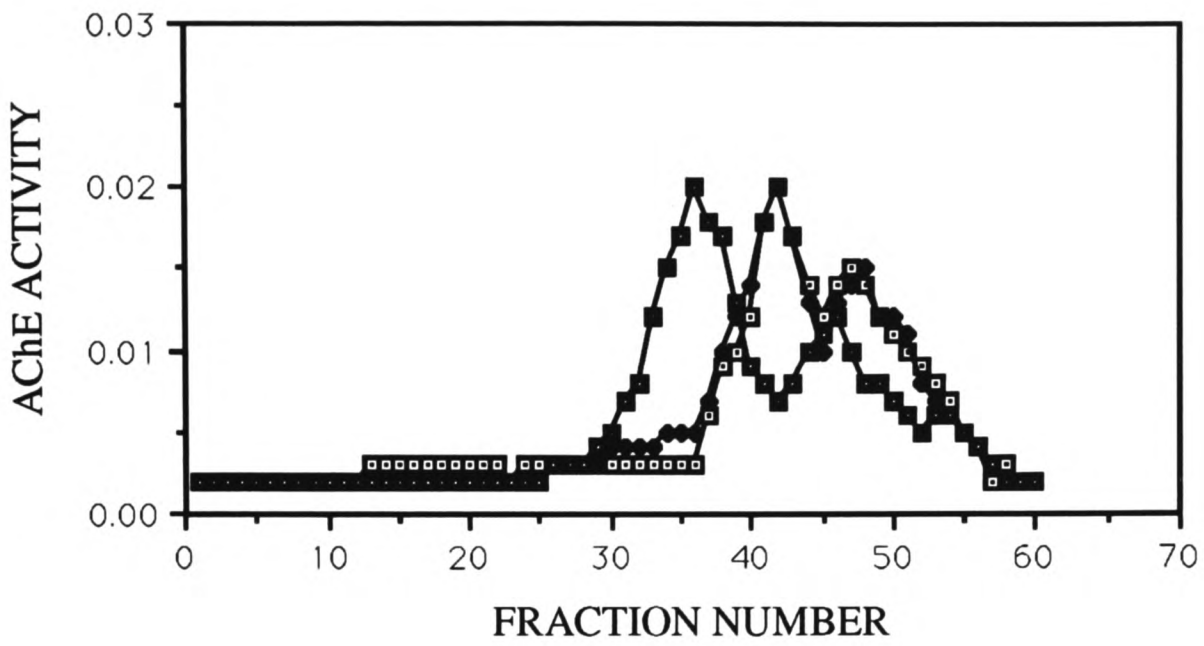
Sedimentation analyses

In the above-mentioned ELISA experiments, the antigen used for capture assays were tissue supernatants (i.e. not affinity purified). It was possible that the present mAbs may have reacted to proteins in the supernatant, other than AChE. To preclude this possibility, sucrose density gradients were made under the rationale that if a mAb bound specifically to AChE, the rate at which the antigen-antibody complex sediments when centrifuged on a sucrose gradient would be uniformly increased. The sedimentation profile obtained following preincubation of the mAbs with the respective antigens is shown in Figure 3. In a control gradient, the activity of AChE from caudate nucleus and erythrocyte preparations yielded symmetrical peaks with the former eluting earlier than the latter. This behaviour is characteristic of the fact that brain AChE is tetrameric, whereas erythrocyte AChE is dimeric. It should also be noted that brain AChE sedimentation displays a second minor peak, which may represent dimeric forms of the enzyme brought about by dissociation of the tetrameric form due to

TABLE II. Elisa titres of the four mAbs.

MAbs	Caudate nucleus AChE	Erythrocyte AChE
	ED_{50} +/- SEM	ED_{50} +/- SEM
BMS-3E4	-ve	8.8×10^{-4} +/- 0.302
BMS-6D6	4.5×10^{-4} +/- 0.167	-ve
BMS-7G10	neat	-ve
BMS-9F4	-ve	5.5×10^{-3} +/- 0.171

-ve, negative response
ED_{50}, dilution of neat tissue culture supernatant containing mAbs which yields one-half of maximum AChE activity



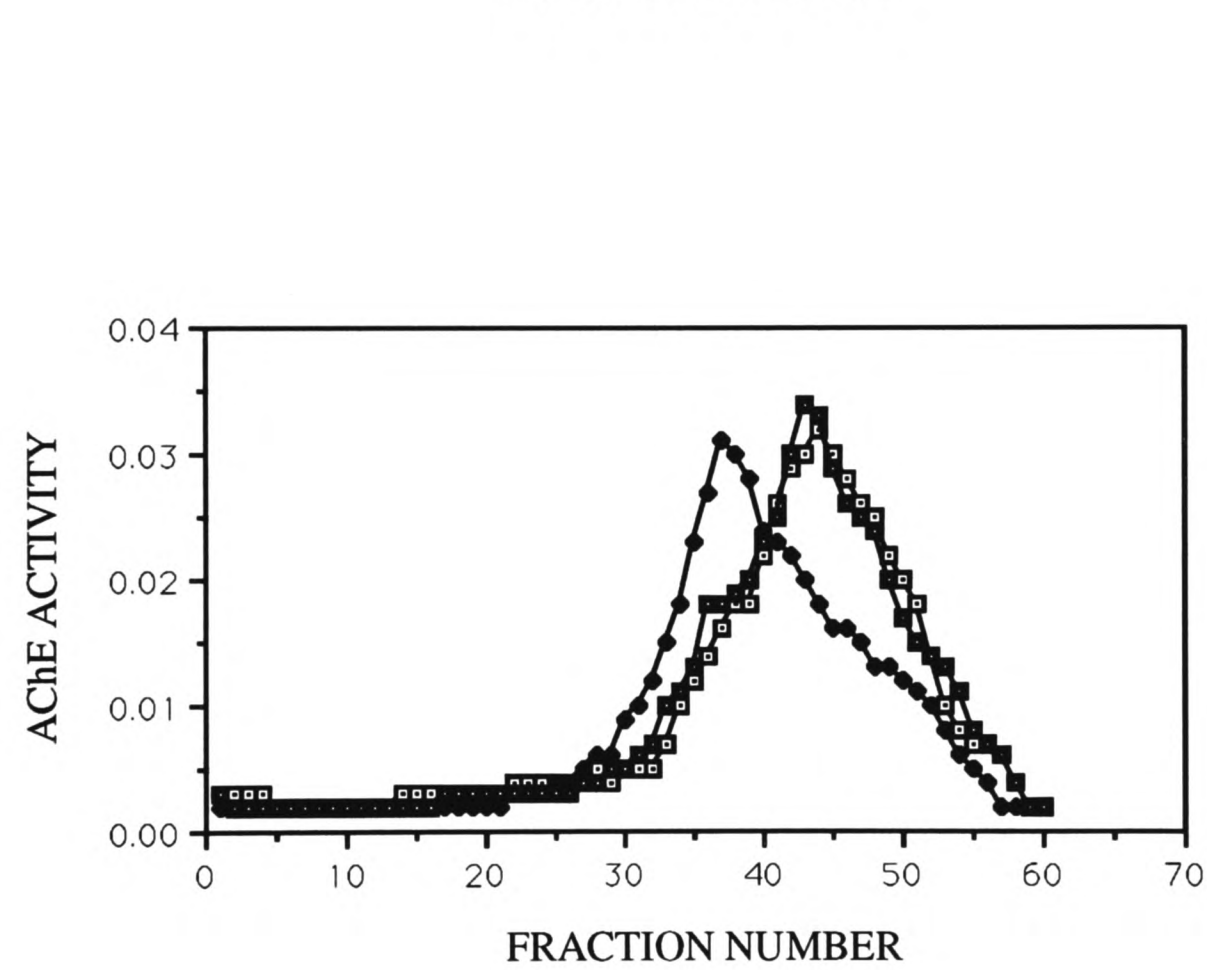


FIGURE 3. Effect of preincubation of caudate nucleus (top diagram) and erythrocyte (bottom diagram) AChE with tissue culture medium (open square), BMS-3E4 (filled diamond), and BMS-6D6 (filled square) on the subsequent sedimentation of the antigen-antibody complex through a sucrose density gradient. AChE activity was assayed under similar conditions for control and mAb samples, and is presented on an arbitrary scale.

the harsh conditions of tissue preparation, centrifugation, etc. Incubation of brain AChE with mAb BMS-6D6 markedly shifted the sedimentation profile to the left, although BMS-3E4 remained unaltered. Similarly, the sedimentation profile of erythrocyte AChE preincubated with BMS-3E4 was shifted to the left, although the one of BMS-6D6 remained unaltered. From these findings, it can be deduced that BMS-6D6 formed a specific antigen-antibody complex with brain AChE, while BMS-3E4 formed a similar one with erythrocyte AChE. The binding of AChE in the culture medium with these mAbs can be ruled out, because in dot blot experiments, no cross-reactivity to FBS AChE was shown.

Gel electrophoresis and immunoblotting

In examining the immunoreactivity of the present mAbs to AChE loaded on a non-denaturing Clarke gel and Western blotted onto nitrocellulose, it was observed that all four mAbs recognized two bands of brain AChE but did not recognise erythrocyte AChE (Figure 4). That these two bands correspond to AChE and not BuChE were verified by incubating gels with an AChE inhibitor, BW284C51, which abolished the staining of these two bands. Similarly, when these mAbs were tested on immunoblotted isoelectric focussing (IEF) gels (loaded with purified AChE), all the four mAbs recognized the different brain AChE isoforms, with no reactivity to the erythrocyte AChE isoforms. These mAbs seemed to recognize most of the AChE isoforms which

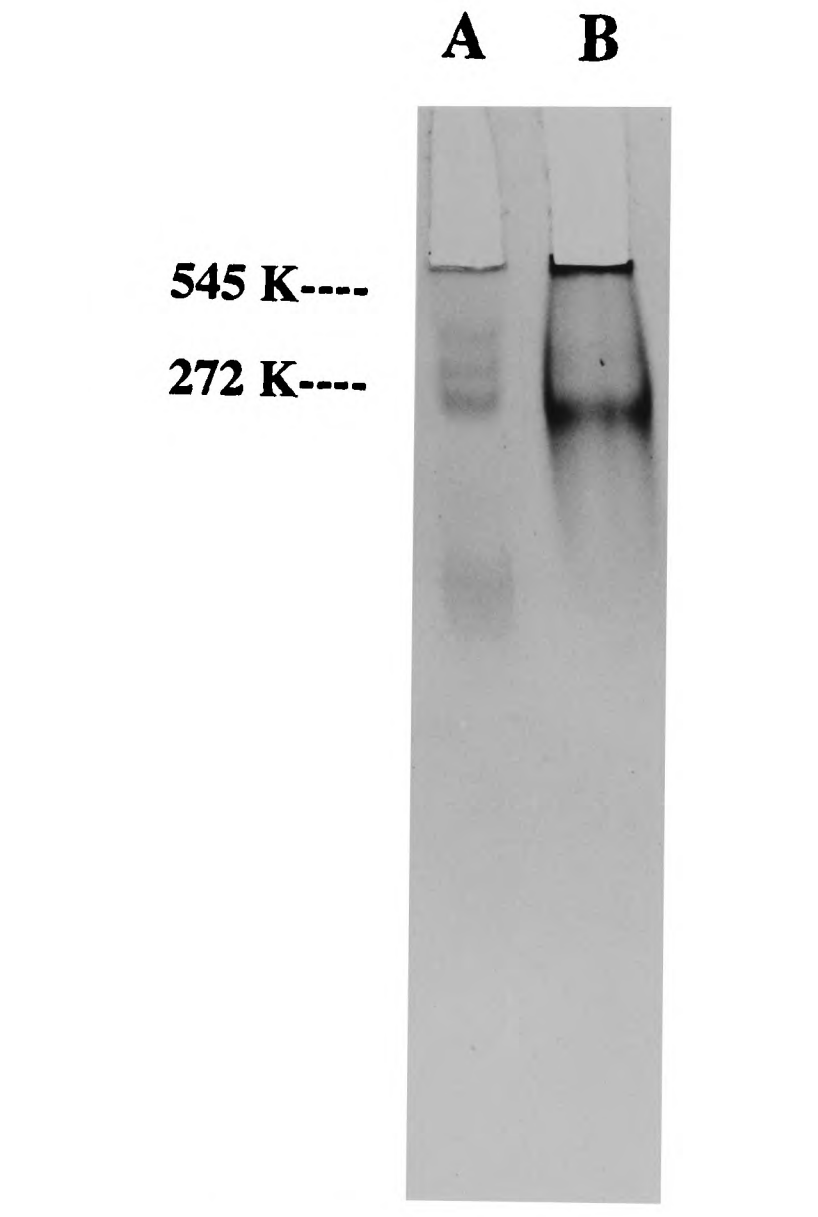


FIGURE 4. Detection of non-denatured AChE by the four mAbs. Top figure: Affinity purified (A) caudate nucleus and (B) erythrocyte AChE at 1 mU for each tract were run on a 7% non-denaturing Clarke gel, and stained to reveal cholinesterase activity. Bottom figure: Immunoblot of A and B ran on a 7% Clarke gel. Blots were incubated with (1) BMS-3E4 (2) BMS-6D6 (3) BMS 7G10 (4) BMS 9F4 (5) HAH 1-1. The migration of jack bean urease hexamer (545 KDa) and dimer (272 KDa) is indicated to the left of the gel for purposes of comparison.

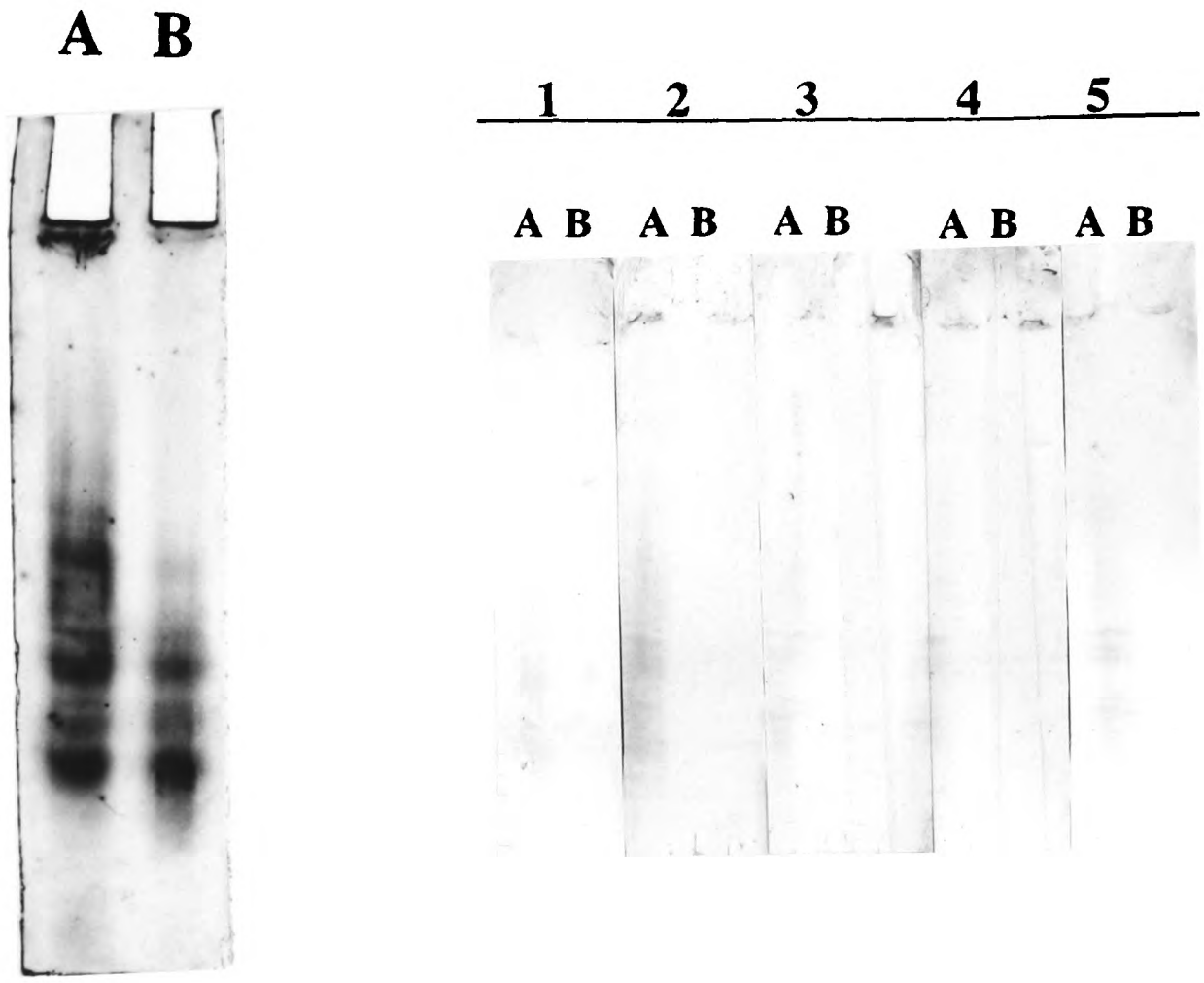


FIGURE 5. Detection by the four mAbs of different isoforms of AChE following isoelectric focussing (IEF). Affinity purified (A) caudate nucleus and (B) erythrocyte AChE at 4 mU each were run on an IEF gel. Electrophoresis was performed in a pH 5-7 polyacrylamide gel, and then the gel was incubated to reveal cholinesterase activity (left figure). A similar gel was Western blotted and blots (right figure) were incubated with mAbs (1) BMS-3E4 (2) BMS-6D6 (3) BMS-7G10 (4) BMS-9F4 (5) HAH 1-1.

are revealed in activity stains of the IEF gel (Figure 5). The reactivity of these mAbs was also tested on SDS gels under denaturing conditions. It was found that none of the four mAbs recognized the denatured forms of the enzyme (data not shown). The effect of other available mAbs to AChE were tested on SDS-denatured AChE (Figure 6). A032 is a mAb raised against human serum cholinesterase, whilst AE-2, 4F19, and HAH 1-1 are mAbs raised against human erythrocyte AChE. It was found that A032 and HAH 1-1 recognized bands of both brain and erythrocyte AChE, displaying some degree of cross reactivity. On the other hand, AE-2 and 4F19 did not recognize any of the denatured AChE bands. From the above findings, it is apparent that in the case of three mAbs (BMS-3E4, BMS-7G10, BMS-9F4), a puzzling behaviour in binding patterns was observed between the liquid (i.e. capture assay) and solid immobilized (i.e. immunoblotting) phases. Furthermore, the seeming inability to recognize denatured forms of the enzyme may suggest that the said mAbs may be binding to conformational epitopes, rather than linear peptide epitopes.

Binding to "membrane-bound" AChE

The binding of the four mAbs to Triton-solubilized "membrane-bound" caudate nucleus AChE was tested on IEF immunoblots (Figure 7). None of the present mAbs was able to recognize this "detergent-soluble" form. Basically, the IEF banding pattern of "soluble" and "membrane-bound" brain is very similar, except for the presence of minor bands in

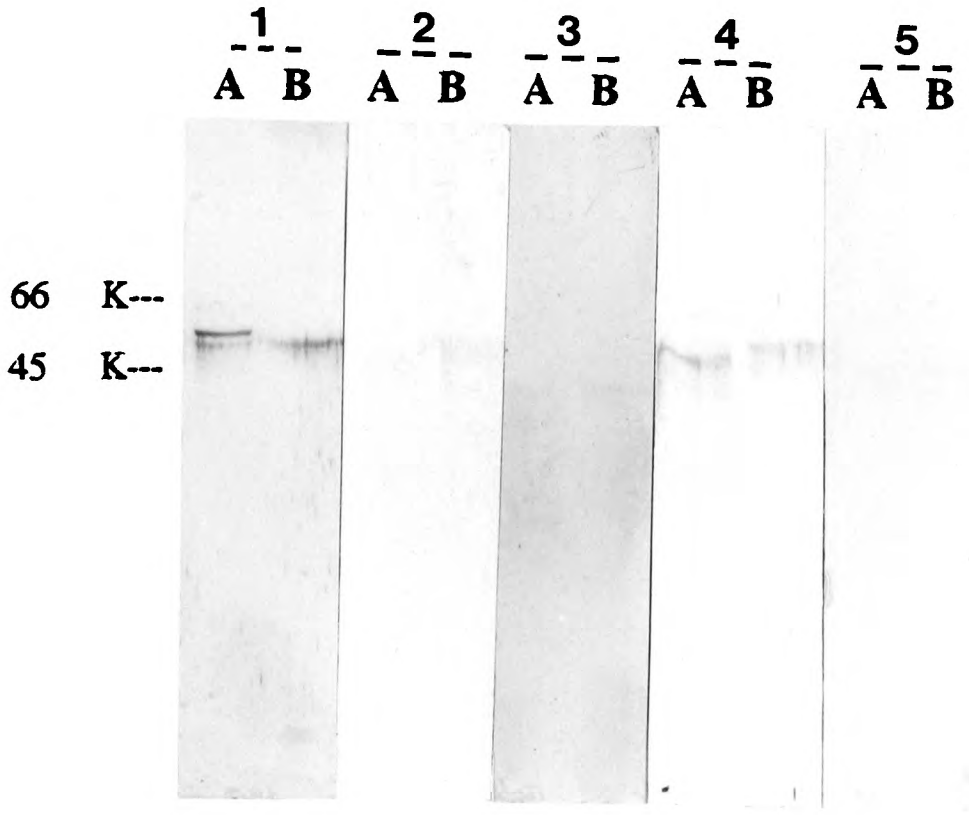


FIGURE 6. Detection by other mAbs of affinity purified (A) caudate nucleus and (B) erythrocyte AChE ran on an SDS-PAGE (12% Laemlli). The migration and mass of bovine serum albumin (66 kDa) and ovalbumin (45 kDa) is indicated on the left of the figure. Gels were Western blotted and blots were incubated with (1) A032 (2) AE-2 (3) 4F19 (4) HAH 1-1 mabs, and (5) tissue culture medium. The latter was used as a control.

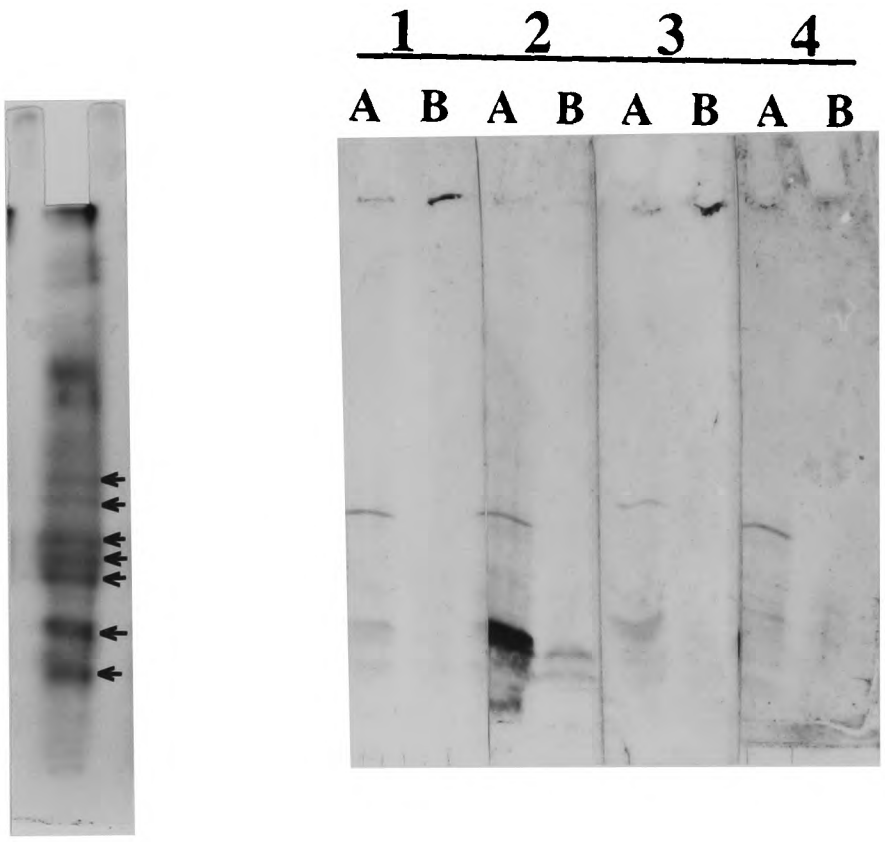


FIGURE 7. Interaction of the four mAbs with (A) Affinity purified cerebrospinal AChE and (B) Triton-solubilized caudate nucleus AChE. A and B were ran at 4 mU each on an IEF gel. The gel was Western blotted and incubated with mAbs (1) BMS-3E4 (2) BMS-6D6 (3) BMS-7G10 (4) BMS-9F4. The figure at the left is an activity stain of tract A, with arrows pointing to the different isoforms of AChE.

the anodic region of the latter.

Effect of anti-ubiquitin and anti-phosphotyrosine mAbs

The possibility that AChE is either ubiquitinylated or phosphotyrosinylated was tested using mAbs against ubiquitin (Z458; Dako) and phosphotyrosine (Figure 8). In the presence of an anti-ubiquitin mAb, four major bands and two major bands were recognized for SDS-denatured brain and erythrocyte AChE, respectively. On the other hand, anti-phosphotyrosine faintly recognized one band for both affinity-purified brain and erythrocyte AChE. These bands are within the region where SDS-reduced AChE has been reported. This finding may shed light onto the possible novel functionary role of AChE.

Deglycosylation studies

To determine whether the present mAbs recognized carbohydrate residues attached to AChE, deglycosylation experiments have been carried out. Endoglycosidase F cleaves N-linked oligosaccharides, whereas neuraminidase cleaves sialic acids. When purified caudate nucleus AChE was separately preincubated with the two enzymes, Western blot analysis of IEF gels showed that none of the mAbs was able to recognize the different isoforms of AChE (Figure 9). Similar findings have been observed on dot blot assays (figure not shown). It can be speculated from this result that an essential part of the epitope recognized by the four mAbs is carbohydrate residues linked to AChE.

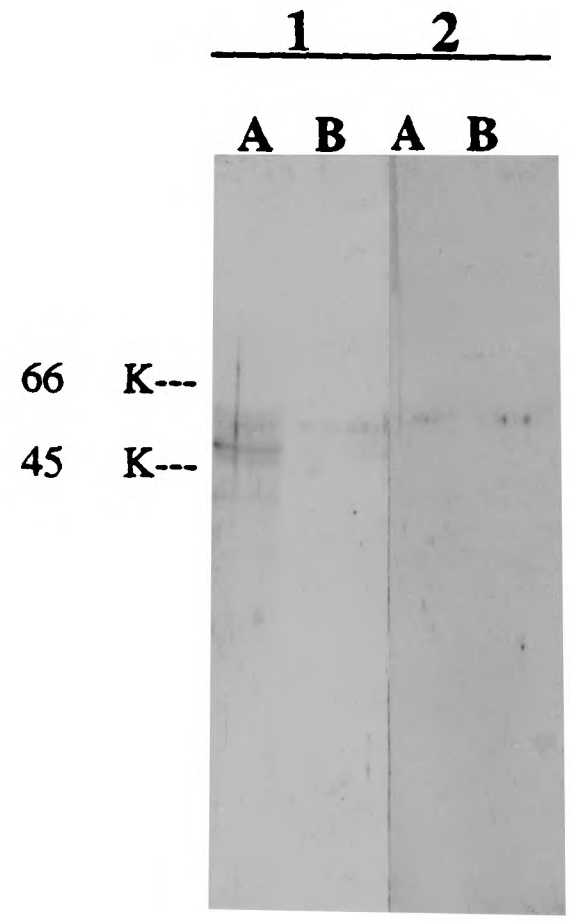


FIGURE 8. Effect of (1) anti-ubiquitin and (2) anti-phosphotyrosine mAbs on affinity-purified (A) caudate nucleus and (B) erythrocyte AChE. A and B were ran at 25 mU each on a 12% Laemlli gel.

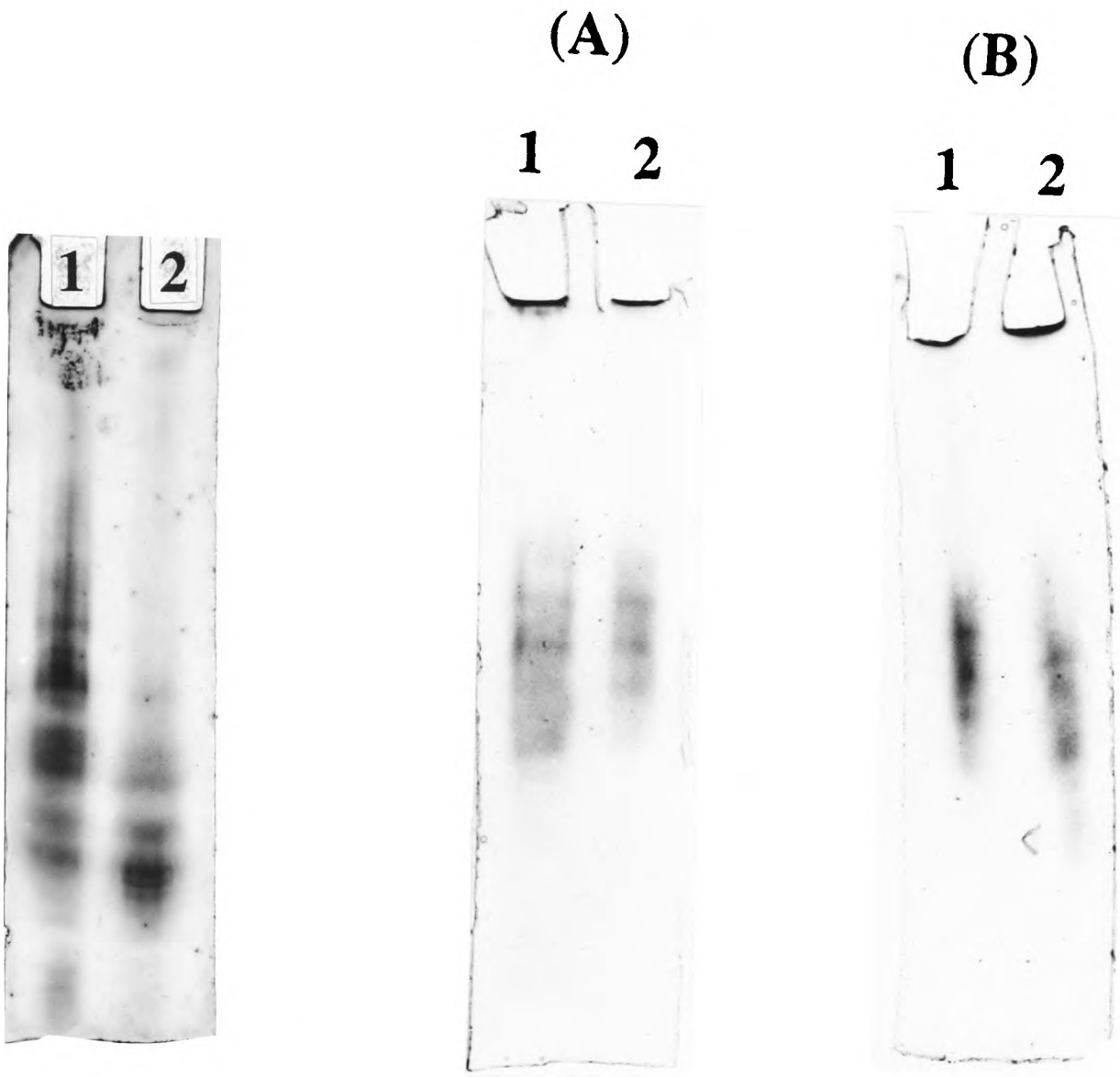


FIGURE 9. Affinity purified caudate nucleus AChE was incubated with (A) endoglycosidase-F and (B) neuraminidase, run on an IEF gel, Western blotted, and detected with the designated mAbs. Center and right-hand figure: Activity stain of (1) caudate nucleus and (2) erythrocyte AChE after deglycosylation with (A) and (B). Note that majority of isoforms are shifted towards to basic end, compared with non-glycosylated (1) brain and (2) erythrocyte AChE [gel shown on the left]. No immunodetection by the four mAbs could be detected in blots, hence Western blot data is not presented.

DISCUSSION

In carrying out the present work, my initial aim was to develop mAbs with high selectivity against human brain AChE. The prospects of achieving this would be beneficial in the clinical diagnosis of certain neuropathologies like Alzheimer's (Marquis *et al.*, 1985), Huntington's (Hammond & Brimijoin, 1988) and motor neurone diseases (Rasool *et al.*, 1983), where changes in the level of AChE activity have been reported. Indeed, in the case of 4F19, this mAb has been used in a procedure for antenatal diagnosis of neural tube defects by determination of AChE in amniotic fluid (Loft, 1990). Although mAbs against AChE from a plethora of sources, such as *Torpedo* and *Electrophorus* electroplax, chicken and rabbit brain, and human erythrocyte tissues have been raised (see review: Brimijoin *et al.*, 1986), the present collection is still inadequate to further elucidate the cytochemical biology of the different AChE molecular forms. Moreover, based on my literature search, only one group has so far attempted to raise mAbs against human brain AChE (Rakonczay and Brimijoin, 1988), although their mAbs, HR3 and HR5 also cross-reacted with erythrocyte AChE. Apparently, one major obstacle is that mAbs which have so far been raised against human AChE do not display any clear specificity for either the G_2 or G_4 forms of this enzyme. The apparent cross-reactivity may suggest that minor structural differences exist between the two molecular forms and these are not sufficient to trigger an antigenic response (Marsh *et al.*, 1984). It may also be possible that the immunological

cross-reactivity between G_2 and G_4 may be due to the presence of common antigenic sites (Rieger *et al.*, 1976). In relation to this, it has been previously suggested that erythrocyte AChE is immunochemically similar to human brain AChE, although markedly different from human serum BuChE. Perhaps the highly glycosylated nature of BuChE masks the antigenic determinants that it shares with AChE, thereby making immunologic differentiation of BuChE easier (Brimijoin *et al.*, 1983). In view of the lack of specificity to human brain AChE, it became apparent to me that additional mAbs were needed, and it was this vacuum that I hoped to fill in pursuing this work.

In order to target the production of mAbs against particular antigens (in this case, against brain AChE), a method using an immunosuppressant drug cyclophosphamide to block the immune response of a mouse to immunodominant or common antigens was proposed (personal communication with my supervisor; reference can also be made to Ou *et al.*, 1991). In this scheme, cyclophosphamide suppresses the immunogenic response of a mouse to common antigens in a first extract (erythrocyte AChE) before the injection of a second extract (brain AChE) containing the target antigen of interest.

In the early stages of this project, much of the mouse priming was carried out by a technical support team (from the Oxford Brookes University). I had access to two mice which were previously primed with erythrocyte AChE for a project to be undertaken by a colleague (Dr. D.

Navaratnam). This project was later abandoned, hence the mice were passed on to me. They were then injected with brain antigen. Later on, however, after fusions and capture assays were made, I had discovered that cyclophosphamide was not given to these mice (due to laboratory miscommunication). Nonetheless, because capture assays revealed some mAbs already raised against brain AChE and since we did not want to use more mice than was deemed necessary, the use of the cyclophosphamide method was abandoned.

I have obtained four hybridoma clones which were grown to monoclonality by three passages of limiting dilution. I was convinced of the monoclonality of these cell lines based on the following criteria, as suggested by Brimijoin *et al.* (1983): (a) a clear single heavy chain and a light chain on reduced SDS-PAGE; (b) smooth symmetrical shifts in antigen-antibody sedimentation complexes; and (3) stability in antigen binding affinity of mAbs grown under successive cultures. Three of these mAbs were directed against the erythrocyte form, whilst one mAb was against the brain form of AChE, as shown by enzyme antibody immunoassay. All of the four mAbs were of the IgM type, which was quite unusual under an in vivo immunization schedule where IgGs are more commonly obtained. It is speculated that the final boosting elicited a primary response, because by the time I performed this, eight weeks had already lapsed since the initial priming. This protracted interim, brought about by technical vagaries, was certainly longer than the commonly

recommended period of 3-4 weeks (Brown and Ling, 1988). It is established that IgMs are the first antibody class to be produced after exposure to an antigen. Upon a second delayed exposure to the same antigen, IgGs are then synthesized as a result of class switching (Hayzer and Jaton, 1985). As such, the high percentage of IgM clones produced most likely represents an increased frequency of fusion of immature plasma cells with myeloma cells to form the hybridoma (Pardue *et al.*, 1983). It is also possible that AChE possesses a relatively weak immunogenicity. Such weakly-immunogenic antigens are known to be biased towards IgM production, as in the case of mAbs raised against an autoantigen, myelin basic protein (Sheng *et al.*, 1987). This explanation however is highly unlikely because previous investigators have successfully raised anti-AChE IgGs, such as mAbs HR2, HR3 and HR5, raised against human cerebellar AChE, which all bound to human brain and erythrocyte AChE with nearly equal affinity (Rakonczay and Brimijoin, 1987), and mAbs AE-2 (Fambrough *et al.*, 1982), 4F19 (Rasmussen *et al.*, 1987), and C1B7 (Olson *et al.*, 1990) which were all raised against human erythrocyte AChE.

One could speculate further that factors such as recognition (antigenic) sites and hydrophobicity may account for the higher antigenicity of the final antigen priming (i.e. brain AChE), which then led to a primary immunogenic response. Indeed, antigenic determinants are said to differ significantly between the different AChE molecular forms (Rakonczay and Brimijoin, 1986). However,

antigenic sites per se do not dictate the imunogenicity of a molecule, especially when we consider that AChE was injected in its non-denatured form, thereby raising the possibility of the antigenic site being obscured by the molecule's three-dimensional conformation. Hydrophobicity may also account for an antigen's high immunogenicity (personal communication: Dr. C. Popham). Cholinesterases are glycoproteins which have been shown to present a number of electrophoretic variants, which probably differ in their sialic acid content (Massoulie and Bon, 1982). It is possible that the more glycosylated a molecule is, a priori, the more immunogenic it becomes. Indeed, when an antigen is a carbohydrate, IgM and IgG3 are commonly elicited (Perlmutter *et al.*, 1978). Following this argument, it may be said that the tetrameric form is more glycosylated than the dimeric form. However, evidence for this is to the contrary, since dimeric erythrocyte AChE is said to contain more carbohydrate residues than tetrameric brain enzyme (Liao *et al.*, 1991). Finally, it is also plausible that molecular mass, coupled with another biological variable, renders a molecule more immunogenic. It follows then that brain AChE (a tetramer) would exert greater immunogenic response than its dimer counterpart. Differences in immunogenicity between G_1 and A_{12} forms of AChE have been shown in the production of polyclonal antibodies. A_{12} AChE showed greater immunoreactivity, possibly due to its aggregative state which gives it an increased antigenic valence (Gurari *et al.*, 1974). Hence,

in the case of G_4 AChE, its higher valency might result from a larger number of catalytic subunits vis-à-vis G_2 AChE, and not due to any additional determinants.

Another likely explanation is that the primary and booster immunizations behaved in a classical way to produce IgGs. However, the novel epitopes of brain AChE presented in the final boost would have produced a primary (IgM) response. The protacted time period up to the final boost is unlikely to have had much effect. Further, there are cases with some antigens where a single immunization gives a better response than a series of booster injections. This is the case when the mouse immune system will eliminate any antibody secreting cells as having the potential to cause an autoimmune condition, i.e. negative clonal selection (personal communication: Dr. C. Popham).

Binding affinity is an important feature in assessing the potentials of a mAb for immunohistochemistry and antigen purification. I noted that depending on the mode of assay used (i.e. liquid vs. solid immobilized phases), the binding of one mAb to a particular antigen may be altered. Such was the phenomenon which I observed with BMS-3E4, BMS-7G10, BMS-9F4 which showed preferential binding to erythrocyte AChE under ELISA and sucrose gradient systems. However, under immunoblotting systems, these three mAbs recognized brain and not erythrocyte AChE. This problem is not unusual and has been similarly encountered by other investigators (Ellis *et al.*, 1989). For example, mAbs Tor-1A5 and Tor-3E5 recognized *Torpedo*

AChE under ELISA, but failed to cross-react with the immunoblotted enzyme. Similarly, Elec-106 only faintly recognized the immunoblotted catalytic subunit of *Electrophorus* AChE, although it had a strong affinity for it under ELISA (Musset *et al.*, 1987). Furthermore, 2G8 gave a stronger response to immunoblotted flounder body muscle AChE than that observed in ELISA (Liao *et al.*, 1991). I proffer the following explanations: the binding orientation of the antigen varies in nitrocellulose and in polystyrene, or the conditions of electrophoresis destroy the antigenicity of recognition sites. The observation whereby the present findings from immunoblot assays were compatible with those obtained under ELISA and sucrose gradient systems may give credence to the latter explanation.

The sedimentation velocities in sucrose gradients of immunocomplexes were examined to shed information on antigen-antibody interactions. My findings here support those obtained in ELISA studies, i.e. BMS-3E4 had preferential binding to erythrocyte AChE, whilst BMS-6D6 bound to brain AChE. The formation of immunocomplexes was mutually compatible, indicating that the epitopes recognized by these two mAbs are possibly distinct between both enzymes. No attempts were made to quantify the degree of shift of the respective peaks because I was only interested in further assessing the selective binding affinity of mAbs BMS-3E4 and BMS-6D6. The peaks observed were sharp and symmetrical, indicating that single

immunocomplexes predominated, formed between a single divalent antibody molecule and a single antigenic site in one catalytic subunit (Fambrough *et al.*, 1982). Interpretation of this peak is not simple because sedimentation values are usually affected by the isotype of the mAb and in some cases, the antigen-antibody complex formed may actually turn out to be associating-dissociating systems which lead to peak broadening and slowing (Conti-Tronconi *et al.*, 1981). It may be reasonable to speculate, however, that the induced marked shifts may be due to the formation of larger immunocomplexes by IgM, possibly because of its pentameric structure. In the case of brain AChE, a minor peak was present which may represent monomers as by-products of tetramers brought about by the harsh conditions of centrifugation.

It is likely that conformational determinants are implicated, since the four mAbs failed to bind AChE denatured by treatment with SDS, mercaptoethanol, and heat. The finding that the present mAbs do not recognize the "membrane-bound" form of brain AChE may mean that the existence of a 20 kDa hydrophobic anchor peptide in this molecule could create a steric hindrance in the binding of the mAb to its conformational epitope (Inestrosa *et al.*, 1987). It is difficult to elucidate the nature of the epitopes recognized by my mAbs, although the possibility that the present mAbs may be directed against carbohydrate moieties cannot be excluded. This notion was supported by results obtained from treatments with endoglycosidase F, an

enzyme which cleaves N-linked oligosaccharides; and neuraminidase, which cleaves sialic acids. Activity stain of IEF gels loaded with deglycosylated brain AChE revealed bands which had shifted to the basic end compared with intact AChE. This shift is caused by the removal of negative charges which cause a corresponding increase in the isoform's pI. These bands were not detected by the four mAbs on Western blots, thereby possibly demonstrating that the epitope(s) to which the present mAbs are directed to consist of carbohydrate moieties (or one spanning both an antigen site and the glycosylated portion). The finding that my four IgMs had a common antigenic motif may suggest that this carbohydrate possesses a highly immunogenic property. As postulated by Schumacher *et al.* (1986) and Sikorav *et al.* (1987), each catalytic subunit of *Torpedo* AChE contains four potential N-glycosylation sites. Hence, each catalytic subunit may have four distinct carbohydrate attachments. Currently, very little is known about AChE glycosylation, and I have not come across any study revealing the definite number of oligosaccharide chains nor their sites of attachment in human G_2 or G_4 AChE. Similar anti-AChE mAbs which are directed towards carbohydrate epitopes have been previously reported. Elec-39 is an IgM raised against *Electrophorus* A_{12} AChE which recognizes a carbohydrate antigenic motif, comparable to that recognized by other anti-carbohydrate IgMs such as HNK-1, NC-1, and NSP-4 (Bon *et al.*, 1987). Carbohydrates are thought to subserve functions in cellular interactions (Keilhauer *et*

al., 1985), and as a result, it was speculated that AChE may function as a cell-cell or cell-substrate adhesion protein. Another of these mAbs, 2G8, raised against *Torpedo* electroplax AChE, also bound to an N-linked carbohydrate epitope which seems to be highly conserved during evolution (Liao *et al.*, 1991). At this point, it is tempting to speculate that the IgM nature of Elec-39 and my four mAbs, coupled with their binding towards carbohydrate moieties, may suggest that these were triggered against thymus-independent antigens. This is a scenario whereby humoral antibody formation takes place independent of thymus cell action (Anderson and Blomgren, 1971).

Other previously raised anti-AChE mAbs were found to inhibit AChE enzyme activity, such as Nj-501 against *Narke japonica* AChE (Abe *et al.*, 1983), F3-43 against rabbit brain AChE (Brimijoin *et al.*, 1985), and AE-2 (Sorensen *et al.*, 1987b) and C1B7 (Olson *et al.*, 1990) against human erythrocyte AChE. Their relative degree of inhibition is F3-43 (90%) > C1B7 (84%) > Nj-501 (75-80%) > AE-2 (72%). The mechanism of inhibition may be due to a restriction of the active site by the mAb, resulting in direct steric hindrance which blocks access of macromolecular substrates (Gurari *et al.*, 1974). Alternatively, indirect restrictions may be involved such as that causing conformational changes in the active site or by a process which prevents substrate-induced conformational change (Holmes *et al.*, 1973). It is claimed that mAbs which can bind to the active site of AChE may be useful, as in the

case of an inhibitory anti-BuChe mAb which provides an alternative diagnostic test for BuChE variants to predict succinylcholine-induced apnea (Brimijoin *et al.*, 1983). The finding that no direct inhibitory action was detected by my four IgMs may indicate that these mAbs do not directly bind to AChE's active site, further strengthening an earlier view that the present mAbs are directed against a conformational rather than a continuous epitope. Unfortunately, in the absence of data on binding stoichiometry and competition between these mAbs, determination of the site and number of epitopes recognized is precluded.

The finding that AChE may be ubiquitinylated deserves some mentioning. Ubiquitin is a highly conserved protein, about 8.5 kDa, which is essential in tagging proteins for destruction. The C-terminus glycine of ubiquitin is covalently attached to the lysine residues of proteins destined to be degraded. A protein destined for degradation usually acquires several molecules of ubiquitin. Further, ubiquitin on a protein may also induce proteolysis, although the mechanism is still unknown (Ciechanover, 1987). Could it be possible that the reported non-classical carboxypeptidase or trypsin-like activity of AChE (Small, 1990) may be due to the presence of ubiquitin in this molecule? Further investigation into this area may shed insights into the non-cholinergic functions of AChE.

In the final analysis, I believe that additional studies are warranted in order to further elucidate the

immunological features of the present mAbs. Immunohistochemistry on brain tissues, which will be dealt with under a separate chapter, should provide straightforward answers on the fine specificities of these mAbs. Suffice it to say, I am convinced that the present mAbs possess interesting properties which may be useful for immunologic differentiation of brain and erythrocyte AChE.

Chapter 5.

PURIFICATION OF IMMUNOGLOBULIN M

INTRODUCTION

In medical and basic research applications where monoclonal antibodies (mAb) are employed, it is sometimes necessary to use highly purified immunoglobulin samples, devoid of proteins such as albumins and other immunoglobulins which are the major constituents of ascites fluids or tissue culture media. In choosing a favourable purification method, several parameters must be taken into consideration, such as the concentration of mAb in the starting material, extent of purity required, scale of the experiment, the need for minimal processing time, cost effectiveness of purification, mAb isotype, and above all the ability to achieve a high yield and to retain antibody immunoreactivity.

The usual methods employed for mAb purification are Protein-A sepharose affinity chromatography (Ey *et al.*, 1978), ion-exchange and gel filtration chromatography (Goding, 1980), DEAE-Affinity gel blue (Bruck *et al.*, 1982), and caprylic acid precipitation (Reik *et al.*, 1987). Purification by Protein-A affinity chromatography is a convenient one-step procedure which is especially appropriate only for IgG2a, IgG2b, and IgG3. Unfortunately, the various commercially-available Protein-A samples in the market vary in their binding capacity for IgG2 and IgG3. Further, the IgG2 subclasses have specific elution requirements which necessitates harsh conditions (pH 5 or lower) that may alter their immunological properties (Seppala *et al.*, 1981). A combination of ion-exchange and

gel filtration chromatography offers a suitable way for purifying IgG1, although it is a labyrinthine procedure which requires precipitation of murine ascites or culture supernatant with ammonium sulfate followed by extensive dialysis. Further, the eluted material is usually contaminated with ascites proteins and its specific immunoreactivity is reduced (Dissanayake and Hay, 1975). In a similar manner, purification by DEAE-Affinity gel blue is a time consuming process which also requires ammonium sulfate precipitation and is only suitable for IgG1 purification (Bruck *et al.*, 1982). Recently, caprylic acid precipitation has been shown to be effective in separating IgG1, IgG2a and IgG2b from murine ascites and culture supernatant. This is a rapid and inexpensive procedure which combines precipitation of contaminating proteins with caprylic acid and then precipitation of immunoglobulin with ammonium sulfate (Reik *et al.*, 1987). Finally, immunoaffinity chromatography methods are highly specific and straightforward, although harsh conditions are required for elution which lead to reduced or even loss of immunoreactivity.

From the above-mentioned examples, it is evident that various conventional biochemical methods are available for Ig purification. In the case of mAbs of the IgM class, however, purification has been somewhat more difficult due to their high avidity and lower affinity, vis-à-vis other Ig classes. Nonetheless, several procedures have been proposed, although these often include many time-consuming

steps and their uses are not at all exhaustive. In most of these procedures, an initial step of ammonium sulfate precipitation is required to eliminate protein contaminants. Further, some IgMs can reputedly be precipitated in the cold, especially if these IgMs possess cryoglobulin properties (Kalpaktsolou *et al.*, 1973).

In one scheme, ascites fluid was ammonium sulfate precipitated, redissolved, dialyzed, subjected to gel filtration chromatography on Ultrogel AcA22 and finally ran on ion exchange chromatography on DEAE-Sepharose (Knutson *et al.*, 1991). A two-step rapid procedure, similar to that mentioned, has also been described for the isolation of human IgM paraproteins from plasma samples. In this case, the overall yield of IgM was greater than 70% and the purified material was judged to be free from contaminants (Jehanli and Hough, 1981).

Mouse IgM and IgG3, human IgM, and, rat IgG2c and IgM all share two common properties: they are induced by the same types of T-independent antigens (Perlmutter *et al.*, 1978; Der Balian *et al.*, 1980), and they are all euglobulins. A purification method based on euglobulin precipitation has been previously examined on human IgM (Weir, 1978) and rat IgG2c (Bazin *et al.*, 1986). Recently, purification of murine IgM based on euglobulin precipitation in demineralized water was reported (Garcia-Gonzalez *et al.*, 1988). Similar results were obtained when a low ionic strength buffer (10 mM phosphate pH 8) was used instead of demineralized water. Although IgMs could be

purified this way, the degree of purity was more reproducible in the IgG3 group than in the IgM group.

Purification schemes based on affinity chromatography include the use of human secretory component bound covalently to Sepharose 4B (Jones *et al.*, 1987). Secretory component is a 75 kDa protein produced by mucosal epithelia. This binds covalently and non-covalently to polymeric immunoglobulins (Radl *et al.*, 1971; Brandtzaeg, 1985) but does not bind to monomeric or aggregated monomeric immunoglobulin (Newkirk *et al.*, 1983). Further, human secretory component has also been demonstrated to bind to polymeric immunoglobulins from other species (Socken and Underdown, 1978). A second rapid and simple affinity chromatography method for purifying IgM from enriched sources such as myeloma serum or ascites fluid employs a complement protein C1q coupled to Sepharose (Nethery *et al.*, 1990). C1q is a subunit of the first component of human complement which binds specifically to the Fc portions of IgG and IgM, having an affinity of 18-folds greater for IgM than for monomeric IgG (Sledge and Bing, 1973). It was noted that IgM has two identical and independent binding sites for C1q, and that C1q was attached to the IgM through its C1q heads. This implied that biologically significant binding sites were involved in this interaction (Poon *et al.*, 1985). One major drawback, however, is that IgG aggregates bind C1q with high affinity. Hence, samples containing IgG aggregates as contaminants may not be suitable for IgM purification. A

similar approach utilizes a complement component C4b-binding protein (C4BP) bound to DNA-cellulose. This procedure however entails ammonium sulfate precipitation followed by QAE-Sephadex, DNA-cellulose, and Sephacryl S-300 chromatographic steps, which are very time-consuming (Abdullah *et al.*, 1985). Another method employs a new mannose-specific plant lectin (GNA) immobilized on Sepharose 4B. GNA is derived from the snowdrop (*Galanthus nivalis*) bulb and is a lectin which is highly specific for D-mannose (Van Damme *et al.*, 1987). The unique binding specificity of GNA, i.e. a strict requirement for nonreducing terminal D-mannose residues, gives rise to a high selectivity for murine IgM which carries high-mannose-type glycan chains (Shibuya *et al.*, 1988). A further method is based on salt-promoted (salting-out) adsorption chromatography on a "thiophilic " adsorbent ("T-gel"), which was synthesized by covalently coupling mercaptoethanol to Sepharose 6B that had been activated and cross-linked by divinylsulfone (Porath *et al.*, 1985). The "thiophilic gel" has preferential affinity for immunoglobulins and macroglobulins from human serum proteins. The adsorption is salt promoted and its unique properties may be attributed to its sulfone group and thioether sulfur. Unfortunately, the recovery of purified IgM is considerably less than that for IgG, probably due to dissociation occurring during the adsorption-desorption process (Belew *et al.*, 1987).

Other protocols for rapid purification of IgMs employ single-step liquid chromatography techniques. The use of high-pressure liquid chromatography (HPLC) on an hydroxylapatite column allows the rapid processing of large volumes of ascites fluid containing either IgG or IgM antibodies, to yield highly purified mAb preparations. Further, this method seems to separate specific mAbs from contaminating antibodies regardless of the exact origin of these extraneous antibodies. Titration of the purified material yielded a recovery of 80-90%. It was suggested that the basis for this separation is brought about by variations in light chain compositions among the different mAb isotypes (Stanker *et al.*, 1985). In another approach, a fast protein liquid chromatography (FPLC) system, which incorporated the use of a strong anion exchanger, was evaluated as a method for rapid separation of serum IgM. A favourable feature of this method is that FPLC is capable of separating IgM from a sample more rapidly, in contrast to gel filtration chromatography (Sampson *et al.*, 1984). A similar procedure also corroborated the results obtained with FPLC, yielding efficient separation between IgG and IgM from human sera (Gallo *et al.*, 1987).

In a recent study, ion-exchange (Mono-Q), gel-filtration (Superose 6), and a combination of the latter two were compared to determine the ideal method and optimum conditions for the purification of IgM from ascites and culture supernatants. A comparison of these approaches revealed that mixed-mode chromatography was the most

efficient and yielded products of high purity (Chen *et al.*, 1988). The latter finding however, was refuted by another report (Coppola *et al.*, 1989) which claimed that the most efficient single-step purification method was based on gel-filtration chromatography on a high-resolution Superose 6 HR 10/30 FPLC column. The use of another size-exclusion column (Sephacryl S-300) gave lower yield and purity. Finally, this study also claimed that mixed-mode hydroxyapatite chromatography resulted in only a partially pure product.

In this chapter, we shall attempt to purify the mAbs raised to AChE by a straightforward size-exclusion column. We envisage to use these purified mAbs for future studies in immunocytochemistry.

RESULTS

In preliminary experiments (data not shown), I tested euglobulin precipitation for my IgMs, but was unable to obtain the reported recovery rates. I noticed that major contaminant proteins such as albumin and transferrin coprecipitated with the mAb. This was so the case even when I used a low ionic strength buffer (10 mM Na-phosphate, pH 7.5), in lieu of demineralized water. I surmise that the inability to apply this method on my IgMs may be due to the fact that I was working with tissue culture supernatants which contain lower amounts of mAbs vis-à-vis ascites fluids. Furthermore, euglobulin precipitation is said to be more efficient for IgG3, with recovery rates of >90% (Garcia-Gonzalez *et al.*, 1988).

Another procedure for preparative isolation of immunoglobulins involves the use of caprylic acid which precipitates albumin and other non-immunoglobulin proteins from the serum of a host of sources such as horse, rabbit, sheep, goat, rat, and mouse (McKinney and Parkinson, 1987). This is then followed by the addition of ammonium sulphate to 45% saturation. The functional basis for such an effect has not yet been well established. Nonetheless, this method was successfully applied on isolating a pool of immunoglobulins from guinea pig serum (Karol *et al.*, 1991). However, when applying this method on preliminary experiments with my IgMs (data not given), the samples obtained were found to coprecipitate with albumin. It is reasonable to speculate that the relatively lower amounts

of albumin present in tissue culture supernatants as compared with sera may have brought about coprecipitation of the IgMs with albumin. Finally, another method which I tried was precipitation with polyethylene glycol (PEG) 6000 at dilutions ranging from 3-6% (w/v). PEG precipitation of immune complexes in serum is an established way of isolating compounds circulating as soluble complexes in the blood. Unfortunately, this method failed to precipitate my mAbs, again possibly because of the low amounts of immunoglobulins present in culture supernatants.

Profile of IgM eluted by gel filtration chromatography

Injection of ammonium sulphate precipitated tissue culture medium on a gel filtration column yielded an elution profile of one minor peak and one major peak (Figure 1-A). The major peak corresponds to albumin, based on the elution profile of preliminary runs carried out with protein markers. Albumin is a major component of foetal calf serum, usually present at concentrations of 1.80 - 3.50 g/100 ml. The minor peak corresponds to globulin, which is also present in commercially-available foetal calf serum. These globulins are present as alpha-, beta-, and gamma-globulins at concentrations of 0.7 - 2.0 µg/ml, 0.3 - 0.9 µg/ml, and 10 - 200 µg/ml, respectively. The single peak which was noted may actually reflect these three globulins eluting as a macroglobulin.

In contrast, application of ammonium sulphate precipitated medium containing the mAb BMS-6D6 showed an

elution profile similar to that of the control medium, except for the presence of a clearly marked shoulder in the minor peak (Figure 1-B). This shoulder corresponds to the IgM antibody. During preliminary runs using thyroglobulin (molecular mass: 660 kDa) as a marker protein, this was seen to elute at fraction 37. It was then expected that IgM should elute earlier than this. However, this shoulder begins to appear at fraction 38. It is possible that the IgM molecule eluted together with the macroglobulin. This is not unexpected since IgM is prone to aggregate with itself and with other proteins.

SDS-PAGE Analysis of Eluted Fractions

When fractions from Figure 1-B were run on an SDS-PAGE, two prominent bands at 80 and 25 kDa were seen, corresponding to the molecular masses of the heavy and light chains of IgM, respectively (Figure 2). The purity of the different fractions differed from one another, but fraction 41 appeared to be the most pure (i.e. containing only the heavy and light chains). The other fractions also contained the heavy and light chains at greater density, although contaminant proteins were visible. For fractions 42-45, a band migrating close to the wells was visible. This may represent a subunit of globulin, which is reported to migrate in the region of 180 kDa. Albumin begins to appear at fraction 49, and peaks at fraction 62. In SDS-PAGE, the band corresponding to albumin is seen migrating

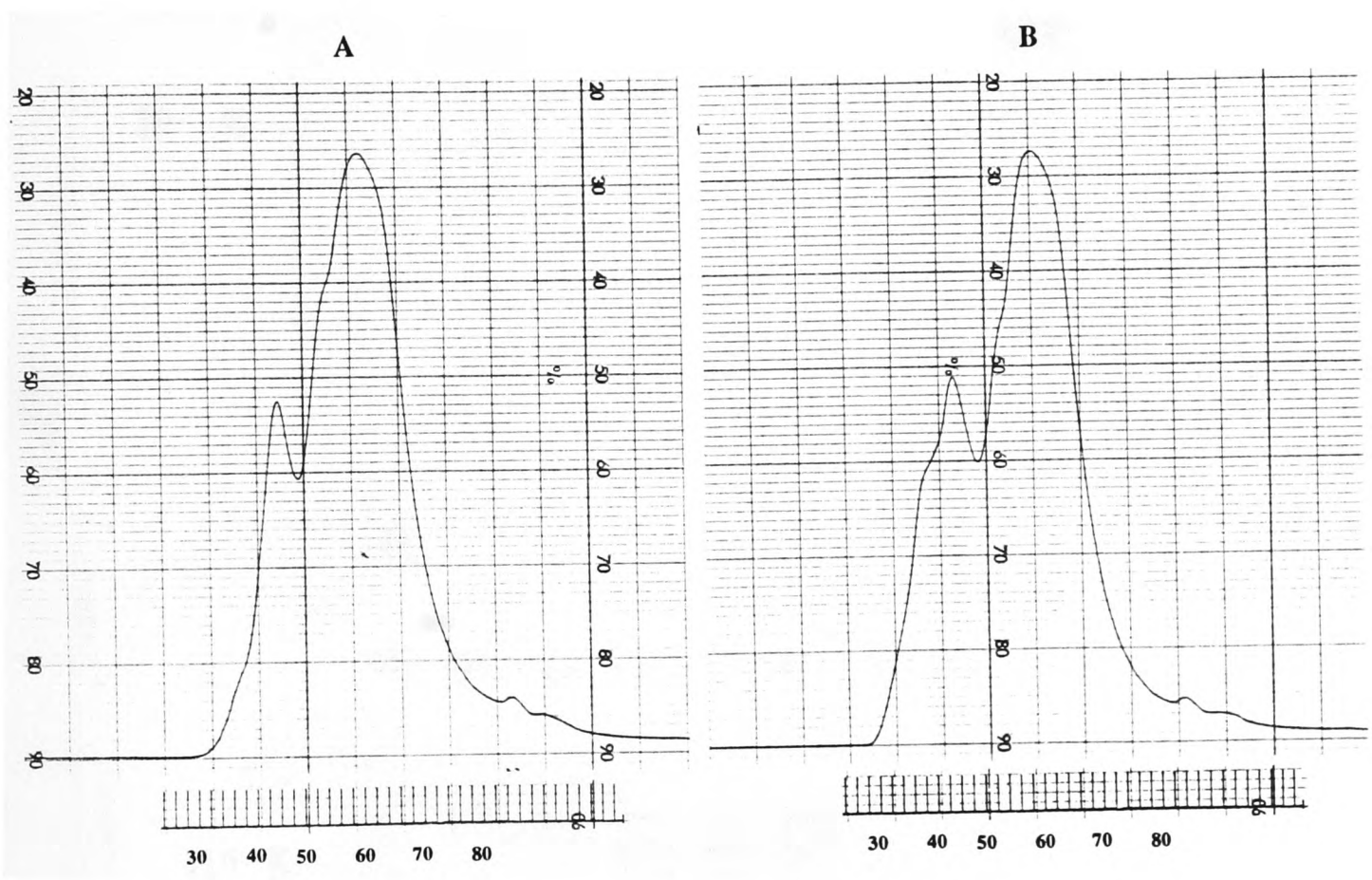
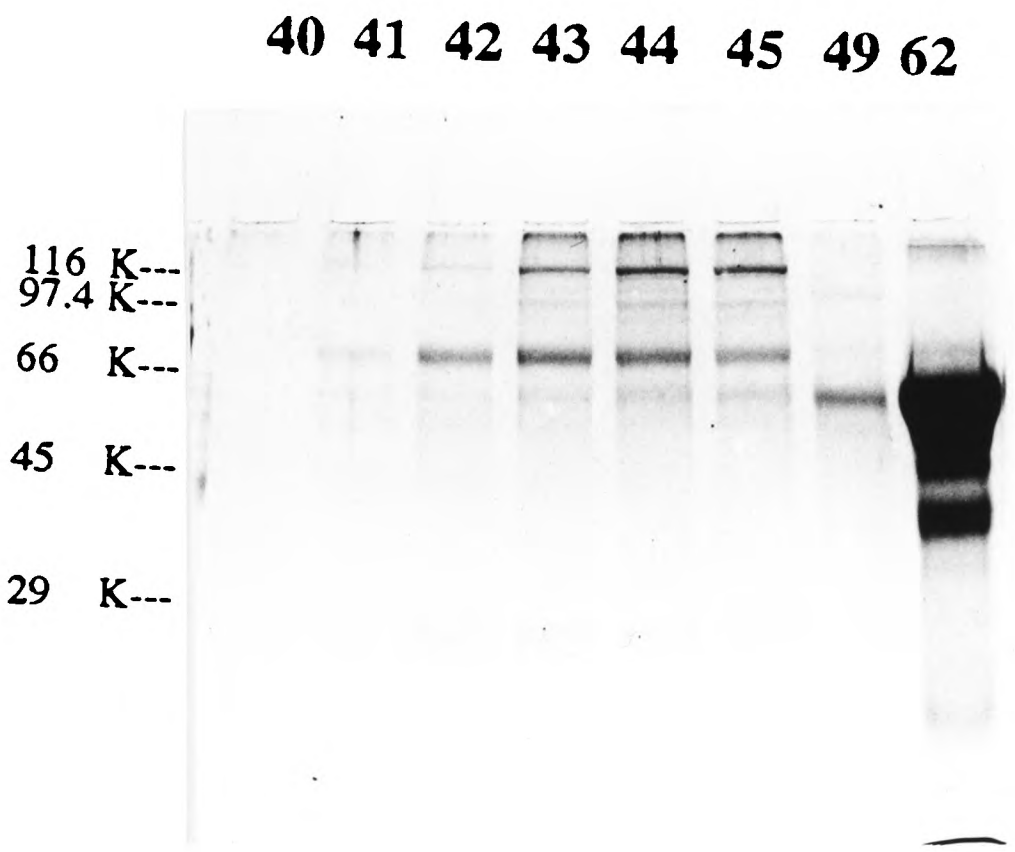
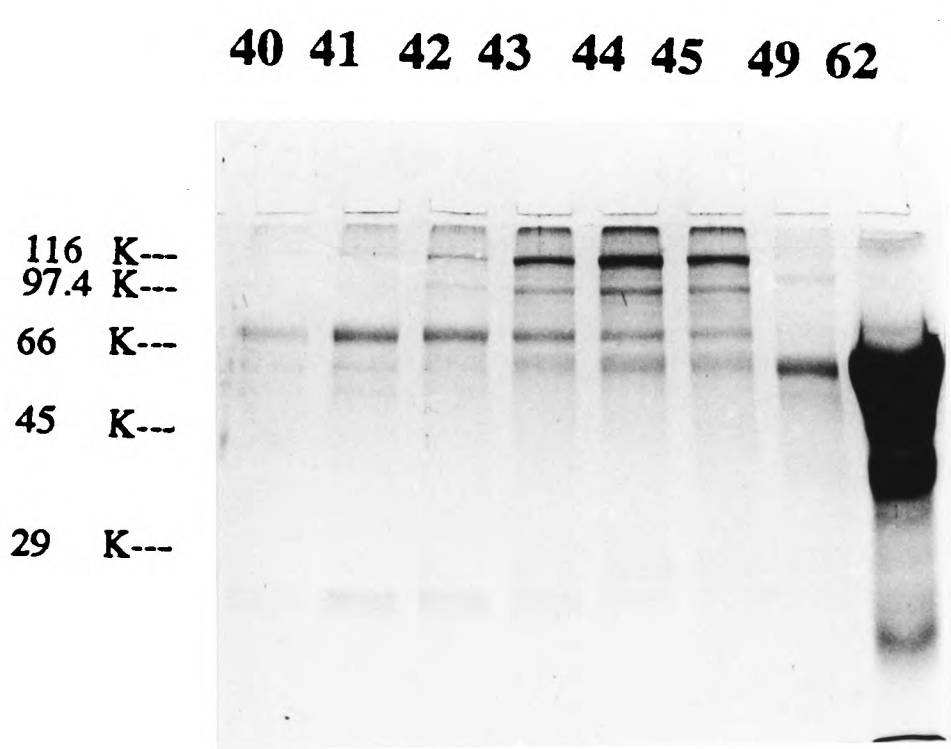


FIGURE 1. IgM purification by gel filtration chromatography. Elution profile of protein monitored at an absorbance of 280 nm. Elution profiles for (A) control supernatant and (B) supernatant containing mAb BMS-6D6. After ammonium sulphate precipitation (see Methods), the clarified sample was subjected to gel filtration chromatography on a Rainin size exclusion column with 100 mM Tris, 150 mM NaCl (pH 8.0) at a flow rate of 125 µl/min. Vertical bars indicate fraction numbers.

A


40 41 42 43 44 45 49 62
116 K---
97.4 K---
66 K---
45 K---
29 K---

B


40 41 42 43 44 45 49 62
116 K---
97.4 K---
66 K---
45 K---
29 K---

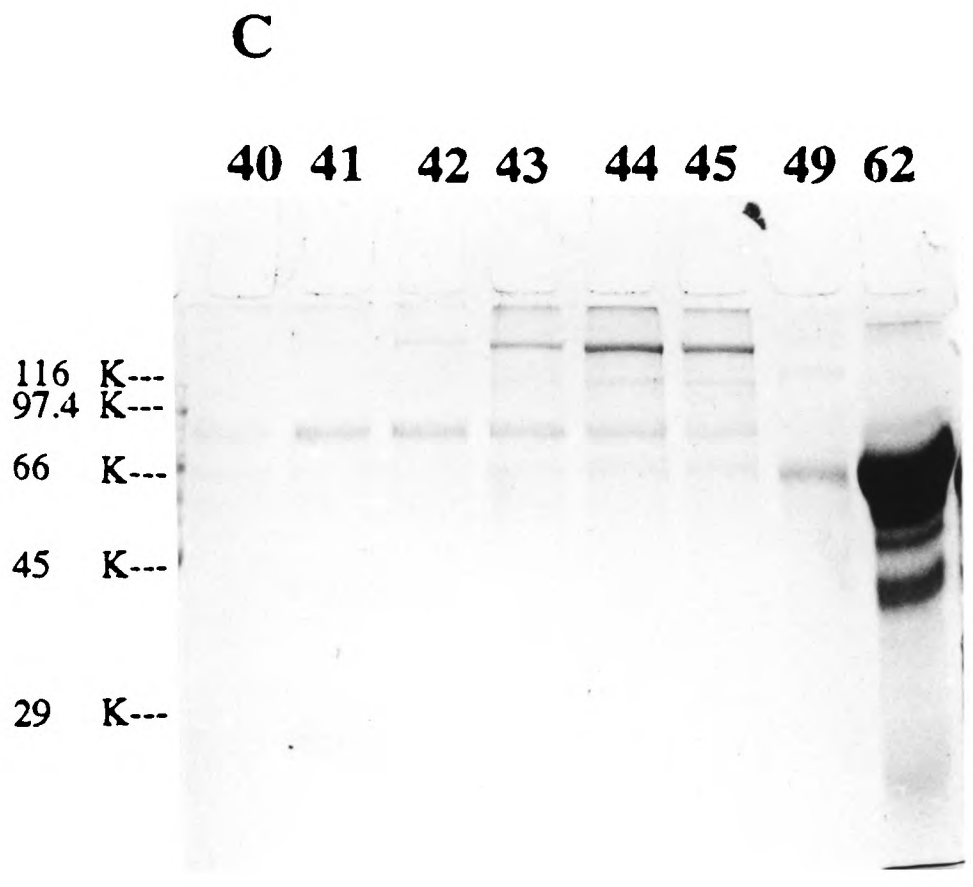


FIGURE 2. Reducing SDS-PAGE (12% Laemlli) of selected fractions eluted from the gel filtration step of mAbs (A) BMS-3E4 (B) BMS-6D6 (C) BMS-7G10 (D) BMS-9F4. The numbers in the upper panel refer to corresponding fraction numbers in Figure 1-B. The migration and mass of B-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) are indicated on the left of each gel.

at about 66 kDa, which corresponds to the known molecular weight of the bovine albumin monomer.

In contrast, SDS-PAGE of the control fractions from Figure 1-A shows that no bands migrated at 80 and 25 kDa (Figure 3). Hence, the two bands seen in Figure 2 clearly suggest that they correspond to IgM's heavy and light chains.

Western Blot of IgM Heavy and Light Chains

To further support my claim that the two bands seen correspond to the IgM heavy and light chains, the gels from Figures 2 and 3 were Western blotted and incubated with alkaline phosphatase-conjugated immunoglobulins to mouse Ig. Upon developing, two bands were detected at positions corresponding to where these were seen in SDS-PAGE (Figure 4). In contrast, Western blotting of the SDS-gel of control fractions (Figure 3) revealed no visible detection of these two bands.

Capture Assay of IgM Fractions

The collected fractions were subjected to enzyme capture assay to assess their immunoreactivity. In one typical result, fractions from the purification of BMS-6D6 were tested (Figure 5). From these, it was shown that immunoreactivity was not lost and this was evident in fractions 41-46, in agreement with the results seen in Figure 4.

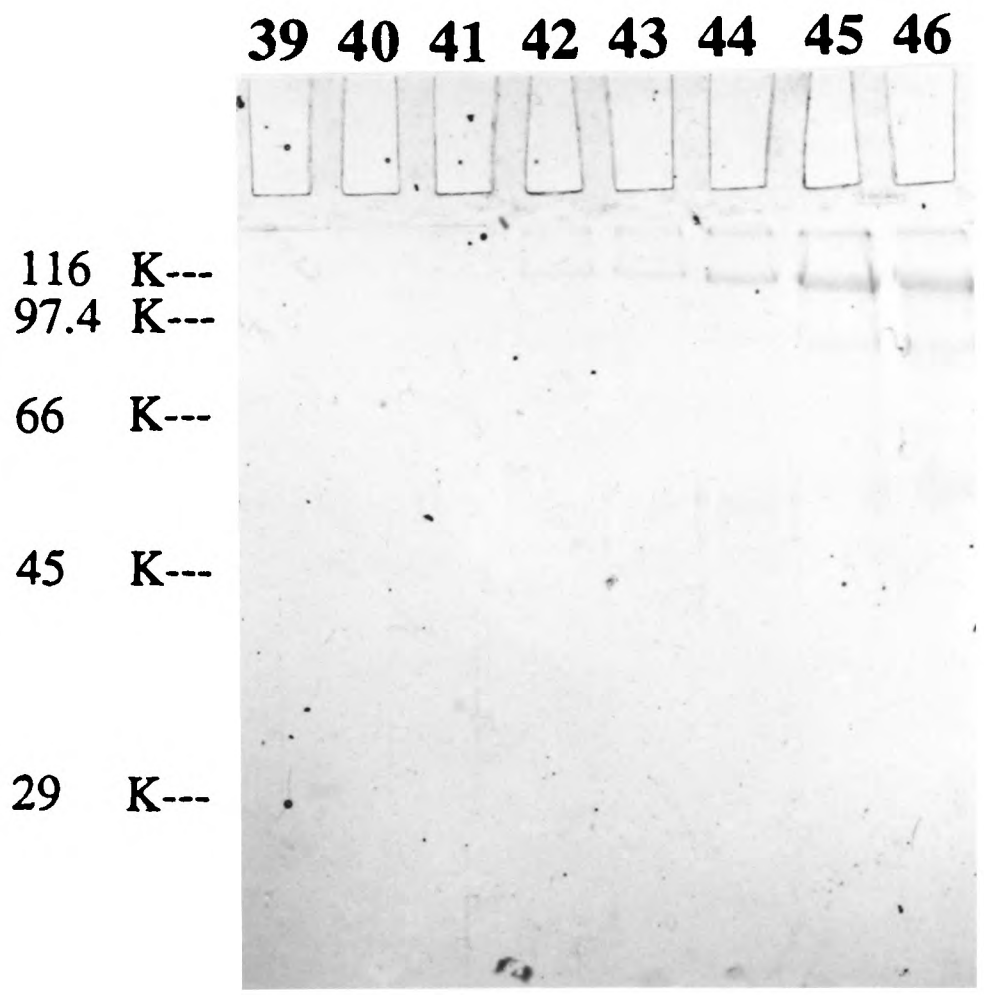


FIGURE 3. Reducing SDS-PAGE (12% Laemlli) of selected fractions eluted from the gel filtration step of control sample (i.e tissue culture medium), following ammonium sulphate precipitation. The numbers in the upper panel refer to corresponding fraction numbers in Figure 1-A. The migration and mass of molecular weight markers are indicated on the left of each gel.

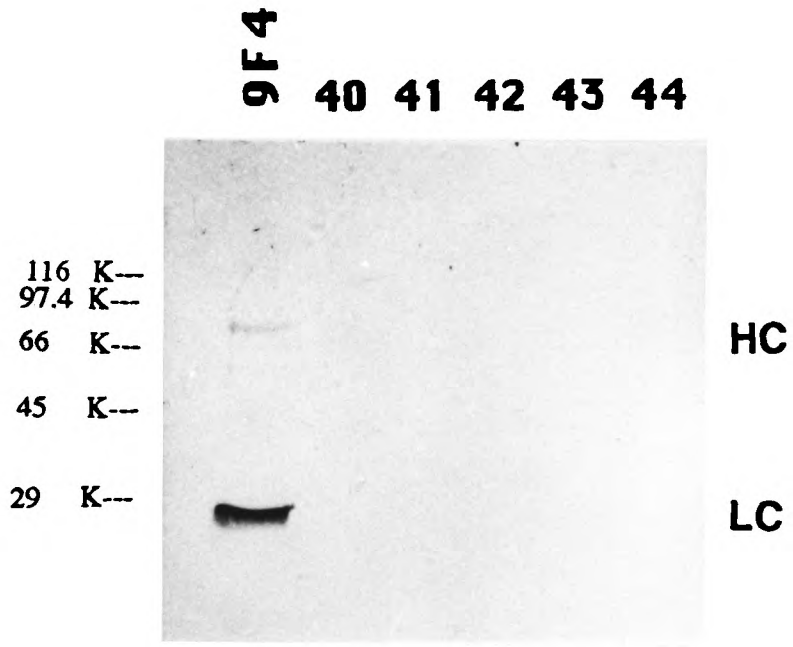


FIGURE 4. Western blot of SDS-PAGE runs of BMS-9F4 fraction no. 41 (from Figure 2-D) and control fractions 40-44 (from Figure 3). HC, heavy chain. LC, light chain. The migration and mass of molecular weight markers are indicated on the left. (Note that the fuzzy appearance of the light chain may have been brought about by aggregated dimers of J-chains.)

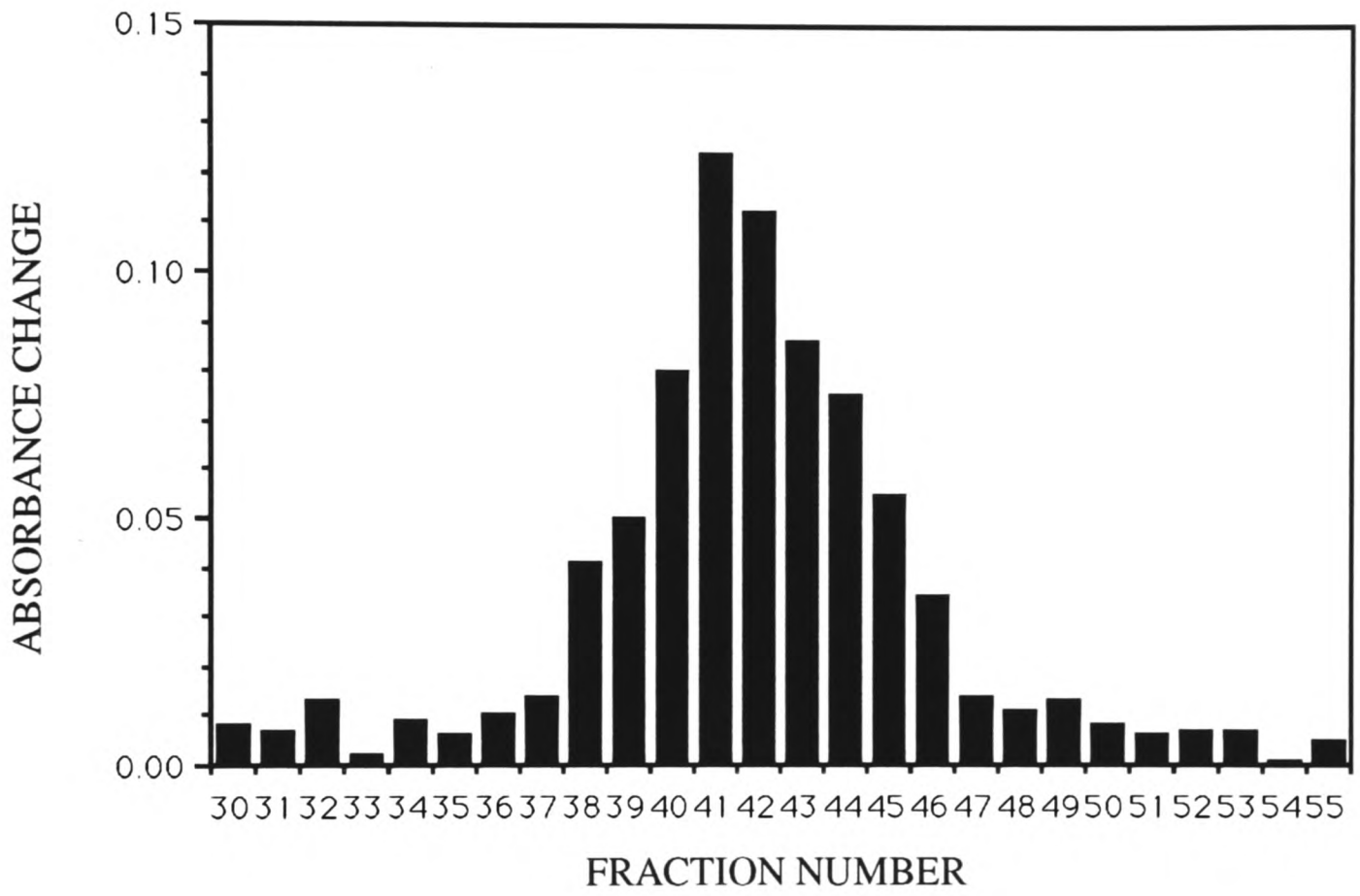


FIGURE 5. Immunoreactivity assay of fractions from the gel filtration run of BMS-6D6. Solid bars represent AChE activity based on a capture assay technique mentioned in the Materials and Methods. Absorbance change is presented in arbitrary units. Fractions correspond to the elution profile in Figure 1-B.

Rechromatography of Eluted Fractions

In an attempt to elucidate whether contaminant proteins in fractions 43-45 could still be removed, these fractions were pooled and subjected to a second round of gel filtration chromatography. In Figure 6-A, a typical elution profile of BMS-6D6 is shown, having a marked shoulder where the mAb is expected to elute. In Figure 6-B, elution profile of the rechromatographed fractions is shown. A major peak elutes at fraction 44, whilst a minor one elutes at fraction 60. Analysis of these fractions by SDS-PAGE (gel not shown) showed fractions 42-43 to be relatively free of contaminants.

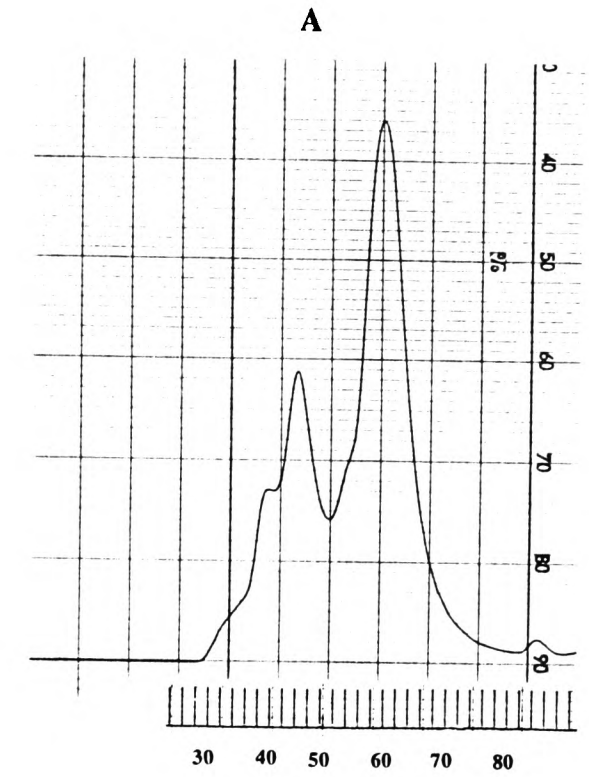


FIGURE 6. Rechromatography of fractions 41-46 from the run of figure 1-B. (A) Elution profile of BMS-6D6. (B) Elution profile of rechromatographed fractions 41-46. All chromatographic conditions are similar to that mentioned in figure 1.

DISCUSSION

With the view of testing the present mAbs in immunocytochemical studies on brain slices, it was necessary to obtain them in a purified form in order to preclude the effects of nonspecific binding of extraneous proteins. Isotyping of the four mAbs have revealed them to be of the IgM type. Under reducing conditions, two major bands at about 80 and 25 kDa were visible, corresponding to the masses of the heavy (mu) and light (kappa) chains, respectively. These values are consistent with those reported by other investigators, i.e. ranging from 76-80 kDa and 25-26 kDa for heavy and light chains, respectively (Karol *et al.*, 1991; Knutson *et al.*, 1991).

In relative terms, very few IgMs end up to be tested for their immunological properties or clinical applications. In most cases, investigators simply avoid working with IgMs, convinced that these mAbs are beset with problems in their technical applicability, due to their high avidity and relatively lower specificity (personal communication: Dr. N. Groom). In certain cases, however, success in the potential uses of IgMs has been documented. MARCH 1E11, a rat anti-lymphocyte IgM has been used to deplete donor bone marrow of lymphocytes because of its high binding and activating effect on human complement (Hale *et al.*, 1983). Other rat anti-lymphocyte IgMs which activate the human complement and may have potential uses in bone marrow transplantation have also been described.

Earlier in this chapter, I presented results on a simple procedure for carrying out purification of mAbs of the IgM class. This procedure entails the use of a coventional protein purification technique that is simple, inexpensive, and allows the retention of mAb immunoreactivity. As a preparative stage, I carried out precipitation of the mAbs with 45% ammonium sulphate. This was necessary in order to concentrate my mAbs and to remove major protein contaminants present in the tissue culture supernatant. The use of ammonium sulphate for mAb precipitation is quite commonly employed on murine ascitic fluid (Pavlu *et al.*, 1986). This method is applicable to immunoglobulins (Igs) of most species, but is regarded only as a partial purification step and way of concentrating Igs. Recovery of immunologic activity was reported to be greater than 52% in most cases (Knutson *et al.*, 1991).

Other methods are of course available, such as euglobulin precipitation, which was reportedly successful for human IgM (Weir, 1978) and rat IgG2c (Bazin *et al.*, 1986). This procedure is very simple and economical, only making use of dialysis against demineralized water to precipitate the IgM. For murine ascitic fluid, euglobulin precipitation yielded IgM recovery rates of 40-90%, in contrast to the very little or almost no precipitation for IgG1, IgG2a, and IgG2b (Garcia-Gonzalez *et al.*, 1988). In the end, the four mAbs were all precipitated with 45% saturated ammonium sulphate, which I empirically found to yield better optimal results.

Having obtained a partially-purified concentrated sample of IgM, it was then necessary to carry out further purification because in most immunological applications, it is ideal to work with highly purified preparations, devoid of extraneous proteins that may contribute to nonspecific binding and high "signal-to-noise" background levels. Such is the case when a cell line has not been grown to pure monoclonality. The mere presence of a minor Ig contaminant (e.g. IgG), which has the same specificity as the major Ig (e.g. IgM), may lead to a competitive inhibiting effect of IgM to the antigen (Filice *et al.*, 1980).

At this point, it should be emphasised that each class of mAbs has distinct physicochemical properties that may affect its purification, stability, and suitability for chemical modification. These factors include temperature solubility, ionic strength, protein aggregation, stability of the monomeric or polymeric structural domain, and isoelectric point (Smith-Gill *et al.*, 1985). Indeed, IgM is unique in its response to purification treatments, and one method which works for another isotype may not necessarily work well with IgM. In analysing what approach to take next, I also had to consider several factors such as overall simplicity, short processing time, yield, and preservation of specific binding affinity of the IgM to the antigen. Moreover, the choice of an ideal method also depended on the initial source of an antibody, for example, whether it is heterogeneous (i.e. normal serum), or relatively free of extraneous proteins (i.e. culture

supernatants), or containing higher levels of IgM (i.e. serum from Waldenstrom disease patients) (Hayzer and Jaton, 1985). Since IgM is a very large molecule (about 950 kDa), the only method that could take advantage of this feature as well as satisfy the earlier mentioned factors is gel filtration chromatography.

In my literature search, I have not come across any paper which uses gel filtration alone as a method for IgM purification. In most cases, gel filtration was used in combination with another chromatographic method like anion-exchange on DEAE-Sepharose (Jehanli and Hough, 1981; Knutson *et al.*, 1991), and on secretory component (Jones *et al.*, 1987). Although I could have religiously adhered to these documented protocols, I decided not to, due to reasons beyond my control, i.e. most of the ligands are not commercially available and the columns used are expensive. Indeed, it was candidly expressed by several investigators that HPLC ion-exchange chromatography coupled with gel filtration permits immunoglobulin separation to a high degree, but, without the use of expensive columns, this combined method is not suitable for purification purposes (Burchiel *et al.*, 1984; Boonekamp and Pomp, 1986). Over and above this factor however, I was mostly concerned with the labile nature of my IgMs and how this may be affected by immobilization on ion-exchange or affinity resin supports. In working out conditions for capture assay, I discovered that my mAbs were sensitive to extreme changes in pH (data not shown). This was something worth

considering especially since in most cases, harsh conditions of elution are involved (Sampson *et al.*, 1984). Earlier studies have reported high recovery rates for IgM using two-step chromatographic methods, or even with one-step affinity chromatography method using ligands such as *Galanthus* lectin (Shibuya *et al.*, 1988) and complement protein C1q (Nethery *et al.*, 1990). However, I question the potential uses of the highly-purified yield, since in all these reports, no assessment of IgM immunoreactivity was made. Only one report has delved into the immobilization lability of IgM (Knutson *et al.*, 1991). It must be emphasised that purity after ion-exchange chromatography was >95%, but only 10% of the initial activity could be recovered. It was concluded that if recovery of immunoreactive IgMs is expected, purification procedures should avoid ion-exchange or affinity chromatography. I postulate that in view of the pentameric structure of IgM, immobilization subjects it to potential steric forces. This factor, coupled with the fact that in affinity chromatography, IgMs have to be eluted in buffers with low pH (Boonekamp and Pomp, 1986), precludes recovery of IgM immunoreactivity. This decrease in activity of the purified IgM may have been brought about by dissociation of the pentameric IgM and subsequent loss of the J-chains during the adsorption-elution process (Belew *et al.*, 1987). Apart from this, immobilization reportedly leads to some changes in the property of IgM, such as its susceptibility to proteolysis (Plaut and Tomasi, 1970), its ability to bind

with *Staphylococcus aureus* protein A (Harboe and Folling, 1974), and binding with sugars (Jehanli and Hough, 1981). It was suggested that some of these properties are dependent on the structural conformation of IgM.

It is surprising that in spite of what has been reported (i.e. affinity chromatography subjects IgM to denaturing effects that may alter its conformation and henceforth its activity), methods for IgM purification involving immobilization are still commonly used. This may be due to its relatively satisfactory yield which ranges from 60% for Cibacron Blue F3GA affinity ligand (Johnson *et al.*, 1987) to 84% for human secretory component affinity ligand (Jones *et al.*, 1987). Unfortunately, none of these studies have delved into the immunoreactivity of the purified mAbs.

In the method that I used, I realized that in order to achieve a successful purification by gel filtration, factors such as pH and ionic strength were critical. In preliminary experiments, I was able to establish an optimal pH range of 7.5 - 8.0 for my IgMs. At extreme pH ranges, especially at pH <7.0, the IgMs co-eluted with the major protein contaminant (i.e. albumin). It is therefore essential that a carefully controlled pH was maintained. Ionic strength was a second critical parameter that I had to consider. I obtained best results in 150 mM NaCl. When lower concentrations of cations were used, I noted that precipitation of IgMs occurred during fractionation. In

other reports, an even higher salt concentration (500 mM NaCl) was recommended (Hayzer and Jaton, 1985).

The importance of establishing optimal values of these two parameters has also been noted in the use of cation-exchange chromatography for purifying IgMs (Boonekamp and Pomp, 1986). Under this method, optimal conditions were established at pH 5.5 and 100 mM NaCl. At a pH >5.0, IgMs were not retained on the column and were eluted with other ascites proteins. Under lower salt concentrations, elution was incomplete and at higher NaCl concentrations, shrinkage of the column occurred.

SDS-PAGE analysis of the fractions collected has shown that one fraction (no. 41) seems to be the least contaminated. Minor bands, close to the position of the heavy chain, may give the impression of contamination. However, their presence may just be due to overloading of the gels, which may lead to impartial reduction of the IgMs. Furthermore, for IgMs of most animal species, stronger dissociating agents, such as 8M urea or 6-7 M guanidinium chloride are recommended to yield highly pure H chains (Hayzer and Jaton, 1985). Under the present conditions which I used, interchain disulfide bond may have only been partially reduced by 2-mercaptoethanol. At any rate, the heavy and light chains of purified fraction no. 41 were clearly the dominant bands, and the putative impurities were of minor significance. In some cases, it was observed that the light chains were somewhat fuzzy in appearance. This observation has also been seen in other

SDS-PAGE analytical descriptions of the light chains (Belew *et al.*, 1987; Garcia-Gonzalez *et al.*, 1988). It should be noted that secreted IgMs contain the J-chain peptide which comprise 2% of the total weight of an IgM pentamer. This J-chain peptide has a molecular weight of about 15.6 kDa. It is possible that the fuzzy light chains may actually represent aggregated dimers of J-chains. Indeed, dimerized J-chain has previously been reported in fractions eluted from Sephadex G-100 chromatographic steps (Hayzer and Jaton, 1985).

To recapitulate, I have demonstrated in this chapter that the simple chromatographic method of gel filtration is capable of isolating almost-pure samples of IgM from tissue culture supernatants following ammonium sulphate precipitation. This procedure does not involve any step of immobilization which causes a marked loss in the mAb's immunoreactivity.

Chapter 6.

PRODUCTION OF MONOCLONAL ANTIBODIES TO THE C-TERMINUS PEPTIDE OF HUMAN BRAIN AChE BY IN VITRO IMMUNIZATION

INTRODUCTION

In vitro immunization is basically defined as a primary, antigen-specific response of splenic B lymphocytes in culture. The primed B lymphocytes can now be used in somatic cell hybridization for monoclonal antibody production. The first demonstration of in vitro immunization of dissociated lymphoid tissues was attempted by Carrel and Ingebrightsen (1912) using cultures of lymph node fragments and bone marrow. Early work in this field focussed on studies in cellular interactions for the initiation of immunity (reviewed by Dutton, 1967) and the development of better culture systems for in vitro immunization of murine splenocytes (Mishell and Dutton, 1966). With the advent of Kohler and Milstein's (1975) revolutionary technology for monoclonal antibody (mAb) production, the use of in vitro primed splenocytes for fusion with murine myeloma cells was successfully carried out (Hengartner *et al.*, 1978).

In retrospect, initial work on in vitro immunization focussed on attempts to understand the cellular basis of antibody production. Based on the two-signal model for the control of antibody production (Bretscher and Cohn, 1970), when B cells (bone marrow-derived cells) per se interact with the antigen, no antibody production occurs. However, when B cells interact with both the antigen and a signal from T cells (helper thymus-derived cells), antibody production ensues. The activation of murine splenocytes using thymus dependent antigens (i.e. antigens that require

helper T cells to initiate a B-cell activation) has been carried out during in vitro immunization attempts (Feldman, 1974). Examples of T-independent antigens in mice include lipopolysaccharide (LPS)-coated erythrocyte (Moller *et al.*, 1972), haptenated LPS (Coutinho *et al.*, 1974), and haptenated bacteria (Golding *et al.*, 1981).

Apart from T cells and B cells, macrophages are also required for inducing antibody production in vitro. Exposure of macrophages to antigen and then to T cells and B cells which were never exposed to antigen was sufficient to elicit an optimal response (Mosier, 1967). Most T-dependent and T-independent antigens require macrophages, both needed in antigen presentation and in stimulating B-cell proliferation (Unanue, 1981).

In addition to cellular interactions which are involved in inducing antibody production, T cells produce soluble factors, namely antigen specific factors (ASF) and antigen nonspecific factors (NSF), which are essential in stimulating B-cell immunization, proliferation, and maturation into antibody-secreting cells (Andersson *et al.*, 1977; Geha and Mudawwar, 1979). Both ASF and NSF act synergistically and may be important in in vitro immunization (Andersson *et al.*, 1980).

Recently, a T cell line has been established that continuously produces ASF (Apte *et al.*, 1981), and thus allowed its characterization. ASF are thought to represent secreted C57BL/6 T cell receptors which could specifically bind to the determinants encoded by the Ia portion of the

immune response (Ir) region of the BALB/c B-cell (Lonai *et al.*, 1981), and then stimulates the latter.

On the other hand, NSF are diffusible factors produced by T-cells, either upon antigen (Hartwell *et al.*, 1976) or mitogen (Kaattari and Rittenberg, 1982) presentation. NSF are characterized as 30-40 kDa factors which contain major histocompatibility complex and Ia component determinants (Naruo *et al.*, 1980). The properties of NSF are similar to those of interleukin-2 (IL-2), but its B-cell stimulating effects are quite different from IL-2 (Maizel *et al.*, 1982).

In addition to lymphokines, monokines (macrophage factors) and mitogens are also necessary to stimulate antibody production. On their own, macrophage factors have been demonstrated to promote the growth of unprimed B-cells (Kincade *et al.*, 1981) and myeloma cells (Cancro and Potter, 1976) in vitro. As a matter of fact, macrophages have been used to replace lymphokines in the thymocyte culture-conditioned medium (TCM) used for in vitro immunization (Jonak *et al.*, 1982). Finally, mitogens, such as polyclonal T-cell activators (e.g. Con A) and polyclonal B-cell activators (e.g. bacterial lipopolysaccharide, lipoprotein, poke weed mitogen, protein A, anti-immunoglobulin) can stimulate B-cell growth in long term in vitro cultures (Rosenberg, 1981; Andersson *et al.*, 1979; Radnay *et al.*, 1979; Kuritani *et al.*, 1980; Schribner and Moorhead, 1982; Sredni *et al.*, 1981). Other factors which are said to possess B-cell induction effects include

adjuvant peptides (Boss, 1984), a complex of nucleosides-inhibitors-silica beads (Van Ness *et al.*, 1984), and glass beads (Eddy *et al.*, 1985).

It is evident that in vitro immunization presents a feasible new approach to raising mAbs against most antigens, even if they are homologous or syngeneic in nature. In spite of its novelty, several studies on the production of mAbs against bacteria, cell membrane components, haptens, proteins, and virus have been successfully carried out. To name a few, mAbs by the in vitro approach have been raised against sheep red blood cell (Hengartner *et al.*, 1978), benzopyrene (Borrebaeck, 1983), mouse albumin and haemoglobin (Borrebaeck and Moller, 1986), bovine insulin (Rathjen and Underwood, 1985), and Rabies virus (Sethi and Brandis, 1981).

Major work is also being carried out in the application of in vitro immunization techniques for producing human mAbs. The therapeutic value of human mAbs may prove beneficial in certain areas such as passive immunization against viral and bacterial pathologies, drug and toxin elimination, diagnostic imaging of tumours, drug targeting to specific tumours, and modulation of autoimmune responses (James and Bell, 1987; Thompson, 1988). The advantages of human mAbs in clinical diagnosis and therapy are: (1) anti-immunoglobulin response is precluded when used in vivo; and (2) human mAbs recognize polymorphic major histocompatibility complex epitopes, tumour-associated antigens, and other determinants not commonly

detected by murine mAbs, which react mainly with immunodominant structures (reviewed by Borrebaeck, 1988). In spite of its clinical application potentials, technical procedures used for murine in vitro immunization cannot be used for its human counterpart. This is so because the peripheral blood lymphocytes which are used in human in vitro immunization are usually arrested in a phase which require further stimuli for activation (Lagace and Brodeur, 1985). Nonetheless, current work on antibody engineering, somatic cell hybridization, and Epstein-Barr virus transformation may provide the optimal conditions for the in vitro production of human mAbs.

In the present chapter, I report on the use of the relatively novel technology of in vitro immunization for the production of murine mAbs raised against the C-terminus portion of human brain acetylcholinesterase. The amino acid sequence of this C-terminus is also identical to the C-terminus of foetal bovine serum (FBS) AChE. Hence, any mAb raised against the human brain AChE C-terminus, should in theory also identify FBS AChE. Finally, the properties of the mAbs raised in this chapter were also characterized with respect to their binding affinities and specificities.

RESULTS

The antigen used for immunization was a synthetically-produced 16-amino acid peptide corresponding to the C-terminus of human brain AChE. Its sequence is as follows:

Lys-Asn-Gln-Phe-Asp-His-Tyr-Ser-Lys-Gln-Asp-Arg-Cys-Ser-Asp-Leu

Preparations containing two batches of antigen for in vitro immunization were rendered aseptic by irradiation procedures. Irradiation of this preparation was carried out at 50 Gy (5000 rads). A 15 μg preparation of this peptide fragment was used for the priming of a monolayer of support cells. After day 2, a fresh mixture of 15 μg peptide fragment and 15 μg peptide fragment coupled with succinylated BSA was incubated with the pre-primed support cells for another 4 days. After fusion of the immunized cells with myeloma cells, hybridoma colonies were visible in 33% of the 960 wells. The supernatant media from these microwells were shown by capture assay to contain mAbs reacting to immobilized succinylated peptide fragment, and not to BSA. Hybridomas from 10 positive wells with consistently strong assay signals were cloned, expanded, and prepared as spent tissue culture supernatant. In the end, I focussed on four mAbs, namely BMS-5, BMS-6, BMS-7, and BMS-8. These mAbs were all of the Ig M class (Figure 1). Isotyping was carried out by the method of red cell agglutination, as described in an earlier chapter.

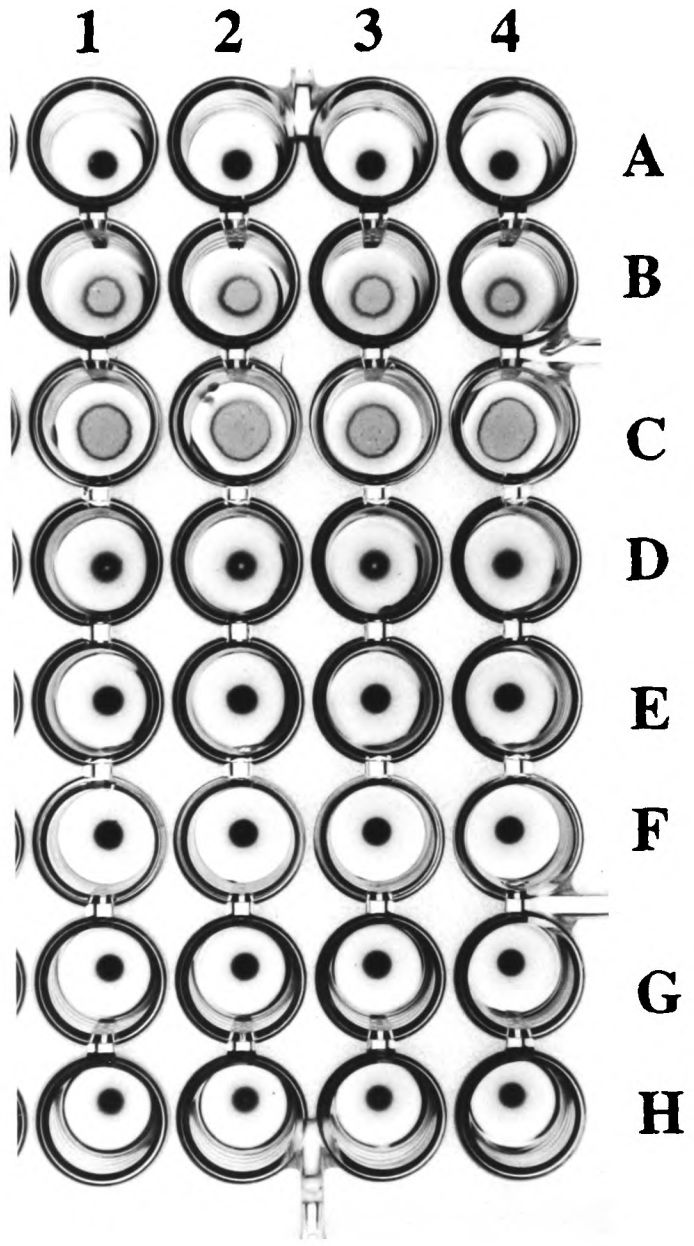


FIGURE 1. Isotyping of the class and subclass of the four mAbs raised by in vitro immunization. (1) BMS-5 (2) BMS-6 (3) BMS-7 (4) BMS-8. (A) Negative Control (B) Positive Control (C) IgM (D) IgA (E) IgG3 (F) IgG2b (G) IgG2a (H) IgG1.

Since the present mAbs were raised against a synthetic peptide fragment, and further screened using the same antigen, it was essential to determine whether or not it would recognize brain AChE in its native state. This was verified by immunoblot procedures on nitrocellulose paper (Figure 2). For mAbs BMS-5, -6, -7, and -8, a positive result was obtained with "soluble" caudate nucleus AChE at concentrations of 8, 6, 4 mU, whilst negative results were seen for erythrocyte and "membrane-bound" caudate nucleus AChE. Further, it was noted that the four mAbs did not show any cross-reactivity to BSA. The latter would suggest that the four mAbs were not raised against the succinylated BSA used to couple the peptide fragment to.

The present mAbs yielded different titres to caudate nucleus AChE (Table I). The four mAbs showed almost closely related positive titres for caudate nucleus AChE, in the order of BMS-7 > BMS-8 > BMS-6 > BMS-5. In comparison however, the titres of these mAbs are much lower than that of BMS-6D6, which was described in an earlier chapter. The four mAbs did not detect erythrocyte AChE (Figure 2).

It is a generally held view that if an antibody binds to an antigen, this phenomenon would increase the rate at which the antigen-antibody complex sediments when centrifuged on a sucrose gradient. Hence, experiments were performed to determine whether the four mAbs bound specifically to brain AChE. In the absence of mAb, caudate

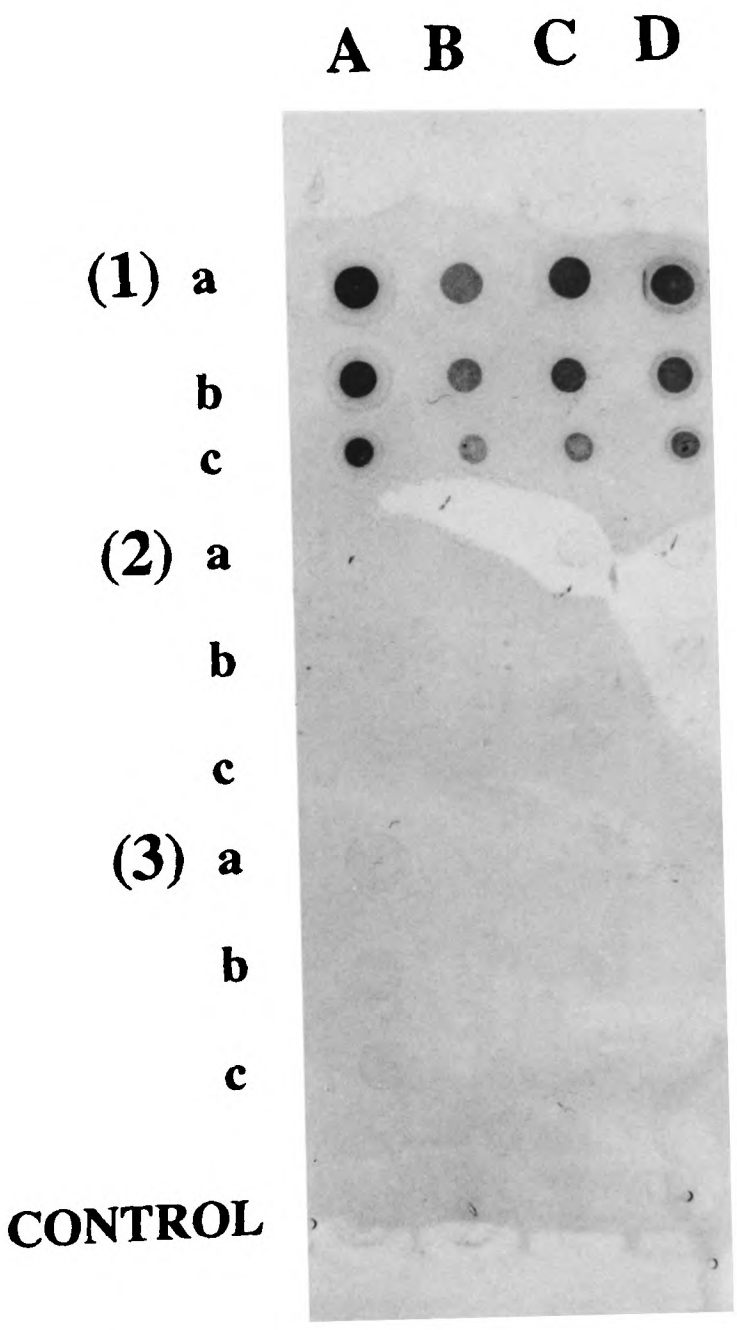


FIGURE 2. Dot-blot immunoassay for the crossreactivity of the four mAbs on (1) "soluble" caudate nucleus (2) erythrocyte (3) "membrane-bound" caudate nucleus AChE. The mAbs tested were (A) BMS-5 (B) BMS-6 (C) BMS-7 (D) BMS-8, and the amounts of AChE applied were (a) 10 mU (b) 7mU (c) 4 mU. In the control column, BSA at 20 μg was used.

TABLE I. ELISA titres of the four mAbs.

MAbs	Caudate Nucleus AChE ED_{50} +/- S.E.M.	Erythrocyte AChE ED_{50} +/- S.E.M.
BMS-5	1.5 x 10-3 +/- 0.281	-ve
BMS-6	2.3 x 10-3 +/- 0.349	-ve
BMS-7	3.9 x 10-3 +/- 0.126	-ve
BMS-8	3.1 x 10-3 +/- 0.348	-ve

-ve, negative response
ED50, dilution of neat tissue culture supernatant containing mabs which yields one-half of maximum AChE activity

nucleus AChE centrifuged as a single symmetrical peak. A significant proportion of caudate nucleus AChE which had been preincubated with each of the four mAbs sedimented through a sucrose gradient at a much faster rate than that preincubated with just control media (Figure 3A). Based on sedimentation profiles, it was apparent that BMS-7 shifted the gradient more markedly, relative to the position of the control. Hence, the degree of antigen-antibody complex formation is BMS-7 > BMS-8 > BMS-BMS-6 > BMS-5. The shifted peaks were still reasonably symmetrical in appearance, without any evidence of intermediate peaks or any residual AChE showing sedimentation properties of the native enzyme. The sharp, symmetrical peaks suggest that single type complexes predominated, as may be expected from mAbs binding to single epitopes.

Similar experiments were performed with erythrocyte AChE and the four mAbs (Figure 3B). In a control gradient, AChE activity from erythrocytes centrifuged as a symmetrical peak. In the presence of each mAb, no shift in the sedimentation profile was evident. Taken in line with the results from the earlier immunoblots, the present finding would further suggest that the four mAbs do not cross-react with any epitope from erythrocyte AChE. Finally, it is relevant to mention that the symmetrical peaks obtained in the foregoing experiment strongly contrasted with that which is observed with polyclonal antibodies. The latter give rise to asymmetrical peaks with

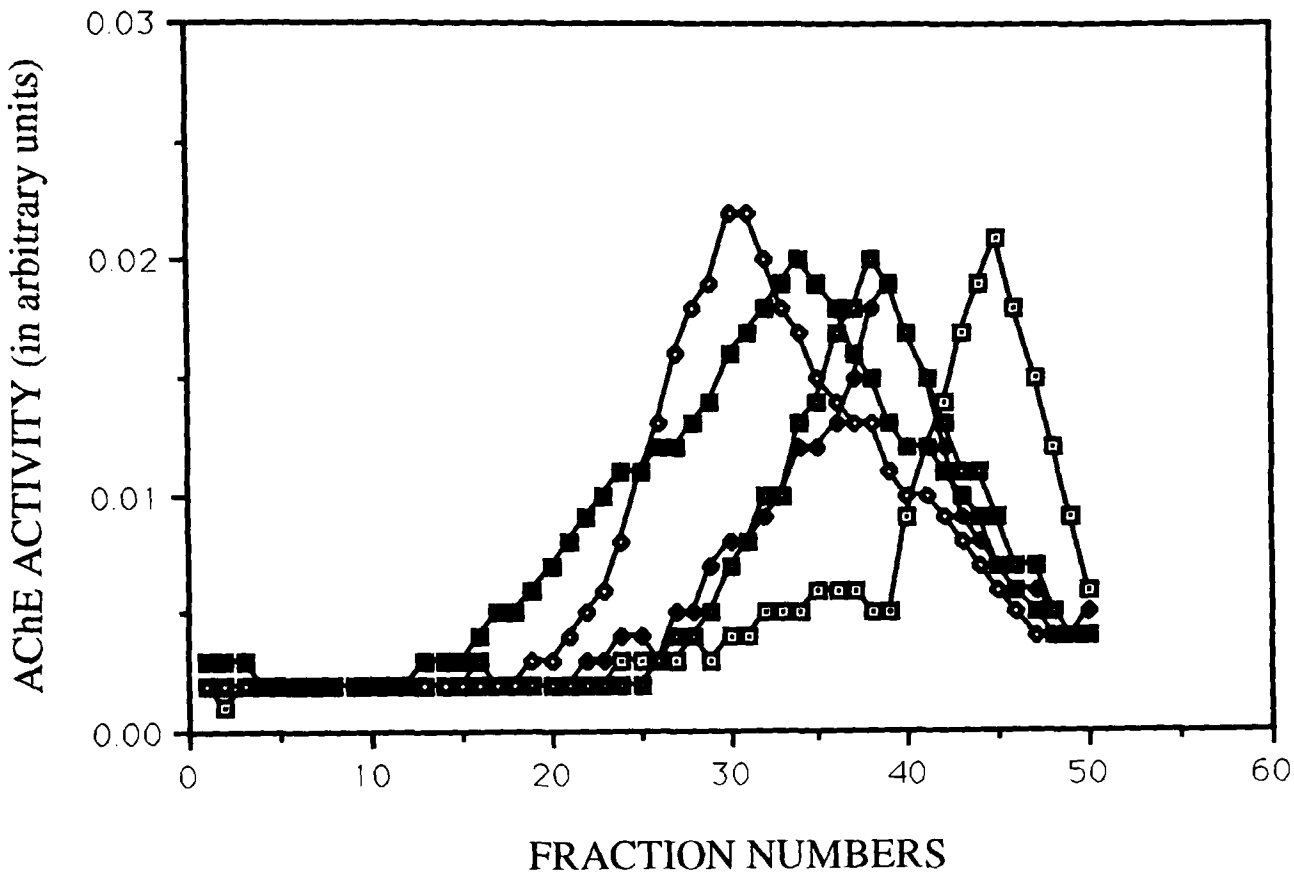


FIGURE 3A. Effect of preincubation of caudate nucleus AChE with control tissue culture medium (open square), BMS-5 (filled diamond), BMS-6 (partially-filled square), BMS-7 (open diamond), and BMS-8 (filled square) on the subsequent sedimentation of the antigen-antibody complex through a sucrose density gradient.

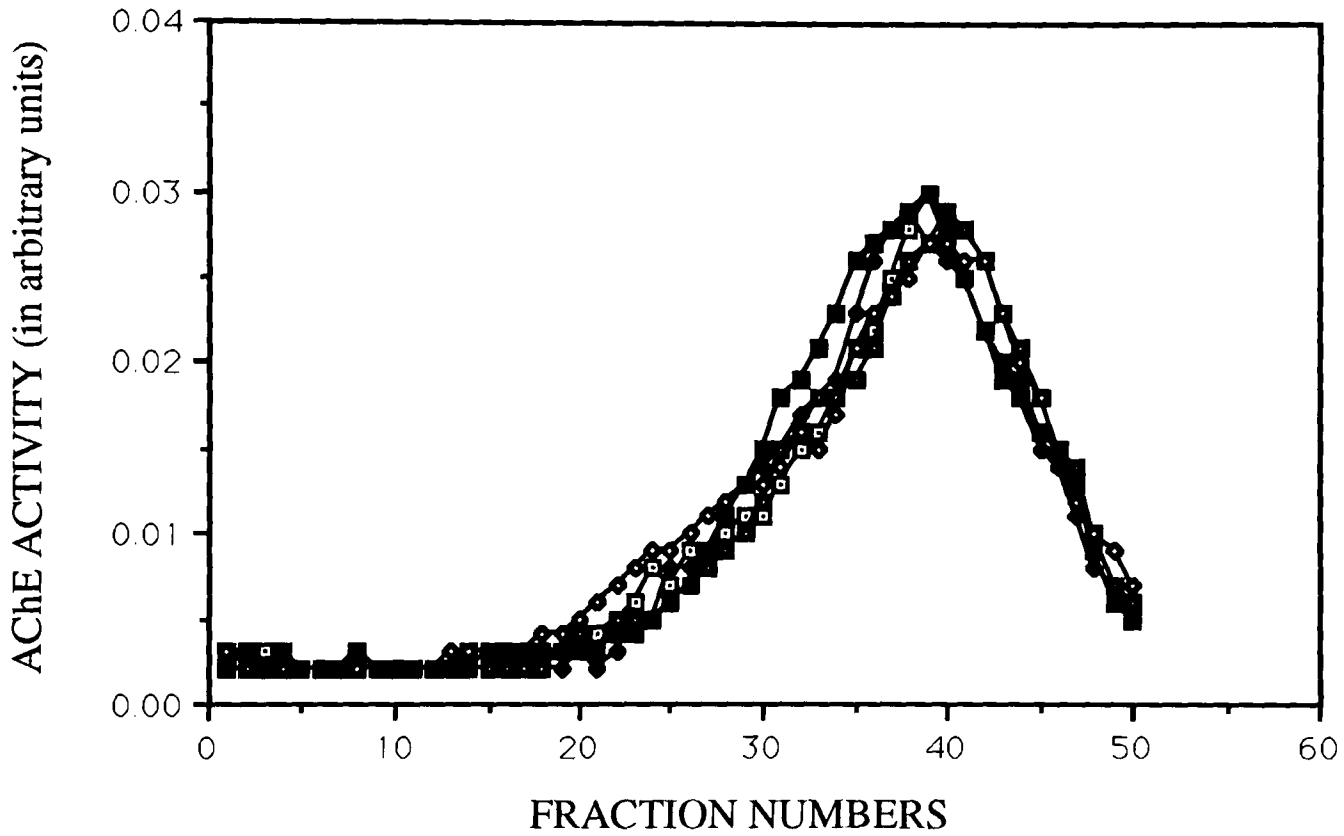


FIGURE 3B. Effect of preincubation of erythrocyte AChE with control tissue culture medium (open square), BMS-5 (filled diamond), BMS-6 (filled square), BMS-7 (open diamond), and BMS-8 (filled square) on the subsequent sedimentation of the antigen-antibody complex through a sucrose density gradient.

AChE activity sedimenting at the bottom of the gradient tubes.

It has been previously mentioned that AChE exists as isoforms. In order to elucidate the binding preference of these mAbs to the different AChE isoforms, an immunoassay of these mabs were carried out on electroblotted isoelectric focussing (IEF) gels (Figure 4). All of the four mAbs showed strong positive binding to foetal bovine serum AChE and cerebrospinal fluid AChE. A minimal reaction can be detected for caudate nucleus AChE, with mAbs BMS-7 and -8 showing weak positive binding. In contrast, no binding was detected for both "membrane-bound" caudate nucleus and erythrocyte AChE. The isoforms that are identified by these mAbs are compatible with those detected on an activity-stained IEF gel. From this experiment, a salient feature that can be gathered is that the four mAbs display greater positive binding to CSF AChE vis-à-vis caudate nucleus AChE. Although AChE from both sources is of the G_4 form, it may be possible that the C-terminus of CSF AChE is more readily exposed than that of the caudate nucleus, hence allowing unrestrictive binding by the respective mAb.

In order to determine whether the four mAbs are able to recognize the anomalous AChE band (Navaratnam *et al.*, 1991), ante-mortem samples of lumbar cerebrospinal fluid (CSF) from control and clinically-diagnosed Alzheimer's disease (AD) patients were used (Figure 5). The AD samples were previously run on IEF gels and demonstrated to contain

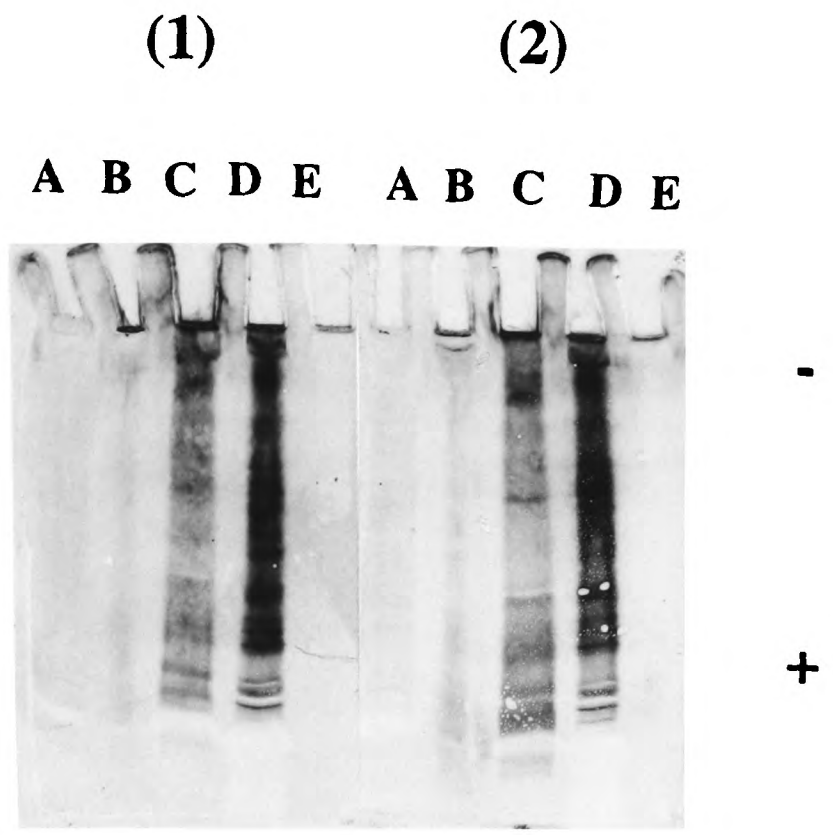


FIGURE 4. Western blots showing binding of mAbs to AChE which were subjected to isoelectric focussing (IEF). Isoforms of AChE from (A) Triton-extracted caudate nucleus, (B) caudate nucleus supernatant, (C) cerebrospinal fluid, (D) foetal bovine serum, and (E) erythrocyte were separated by IEF and electroblotted onto nitrocellulose, as described in the Materials and Methods section. These were then incubated with mAbs (1) BMS-5, (2) BMS-6, (3) BMS-7, and (4) BMS-8.

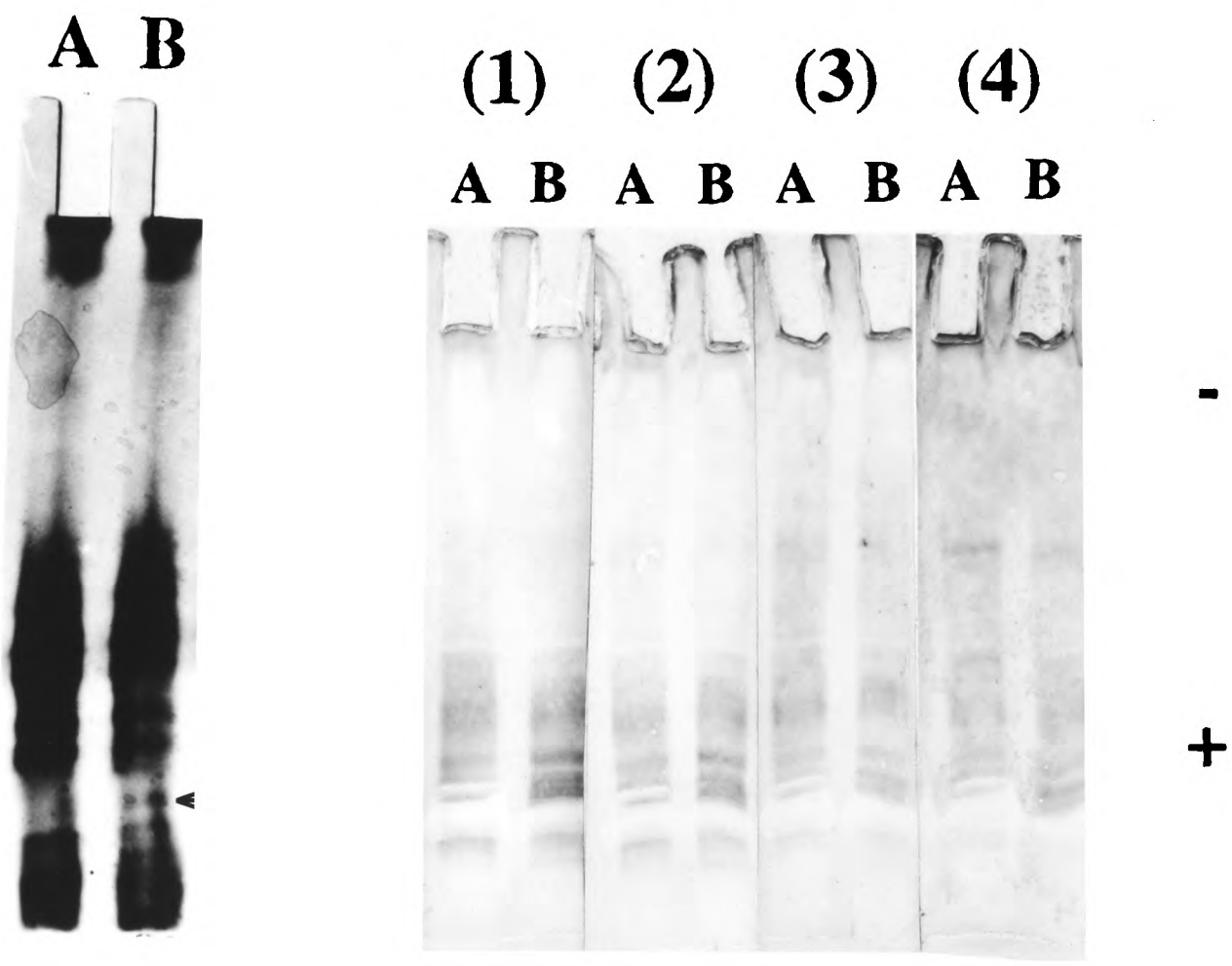


FIGURE 5. Binding of mAbs to cerebrospinal fluid (CSF) AChE which were subjected to isoelectric focussing (IEF). Isoforms of AChE from (A) control ante-mortem CSF and (B) ante-mortem CSF from an Alzheimer's disease patient were separated by IEF (left figure) and electroblotted onto nitrocellulose (right figure), similar to that done in Figure 5. Arrow shown on tract B points to the position of the anomalous AChE isoform. The nitrocellulose strips were then incubated with mAbs (1) BMS-5, (2) BMS-6, (3) BMS-7, and (4) BMS-8.

this anomalous band (with an isoelectric point between 5.3 - 5.5), when activity stained. Interpretation of findings is somewhat complicated due to the conflicting results obtained. In some cases, it was actually possible to see a faint anomalous band identified by mAbs BMS-5 and -6. However, due to the ephemeral nature of nitrocellulose immunoblots, much of this positive reaction to the anomalous band disappears when brought to photography. Also, in other blots, no trace of this anomalous band can be seen. In view of these results, it is not possible to conclude that the present mAbs are unable to identify this anomalous band.

Finally, in order to assess the affinity of the four mabs to denaturing and reducing conditions, AChE from various sources were subjected to SDS-PAGE. Immunoblotting of the four mAbs to SDS-reduced AChE from caudate nucleus, foetal bovine serum, and erythrocyte is shown in Figure 6. The four mAbs bound to two distinct bands at MW 62 and 50 kDa for both caudate nucleus and FBS AChE. For the latter, additional bands found in the region of 29 - 45 kDa were visible. In contrast, the four mAbs failed to identify the catalytic subunits of erythrocyte AChE.

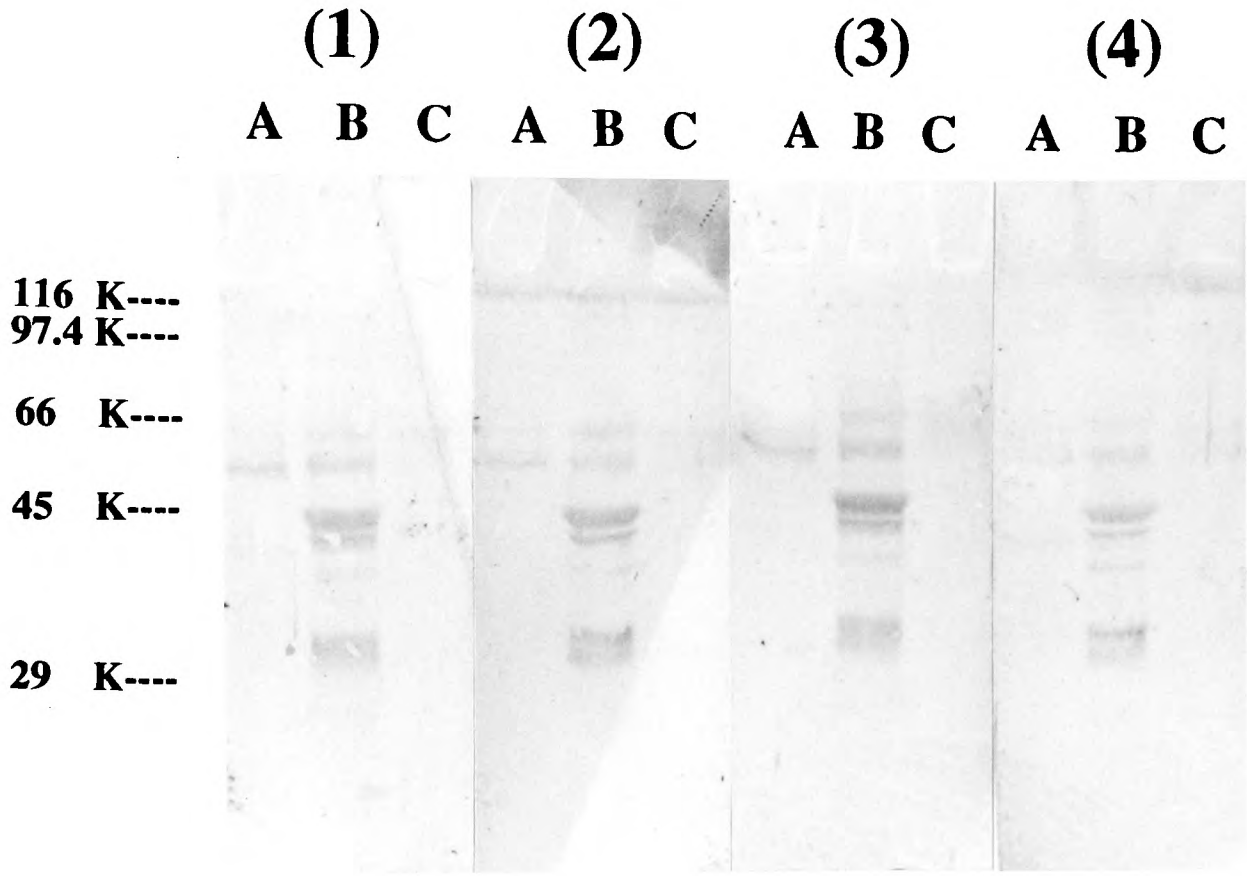


FIGURE 6. Western blotting to show binding of mAbs to AChE under reducing conditions. AChE from (A) caudate nucleus (B) foetal bovine serum, and (C) erythrocyte were separated by SDS-PAGE and electroblotted onto nitrocellulose strips. These were then incubated with mAbs (1) BMS-5, (2) BMS-6, (3) BMS-7, and (4) BMS-8.

DISCUSSION

In order to further raise monoclonal antibodies (mAbs) with higher specificity against the human brain form of acetylcholinesterase (AChE) and to test the applicability of a new technology, I immunised mouse spleen cells in culture with a preparation of a synthetically-produced peptide corresponding to the C-terminal 16 amino acid residues of the human brain AChE sequence. The technology of in vitro immunization is a fairly novel one and has several advantages over that of the classical in vivo (i.e. intrasplenic) mode of immunization.

These advantages include, to wit: (1) The optimal conditions for in vitro immunization is a 5-day antigen stimulation period, in contrast to the several weeks needed for in vivo immunization (Reading, 1982). (2) It is possible to elicit strong antigenic response against highly conserved or weakly immunogenic determinants which are normally impossible in vivo because the normal cellular control of the immune response (viz. suppression or tolerance) to "self-antigens" does not function in vitro. As such, it has been possible to raise mAbs against murine albumin and murine haemoglobin using in vitro immunization of murine splenocytes (Borrebaeck and Moller, 1986). (3) Comparatively small amounts of the antigen are needed and it is possible to maintain defined concentrations of the antigen during the immunization. In one report, as low as 1 ng of sperm whale myoglobin was required to induce an immune response. In general, the recommended dosage seems to be 1-2 μg (Borrebaeck, 1984).

(4) The effects of defined concentrations of monokines and lymphokines can be tested periodically, and every step of the in vitro immunization can be monitored during the entire stimulation period. (5) The population of antibody-secreting splenocytes is significantly higher because of preferential survival of in vitro activated cells (Borrebaeck, 1983). And finally, (6) this technique can also be used to generate antibodies against some antigens that are toxic in vivo yet have negligible toxicity in vitro. An example would be bungarotoxin which is toxic to neurones, but does not have any effect on splenocytes in vitro (personal communication: Dr. C. Popham).

In view of the fact that peptide synthesis is a fairly expensive process and only a few μg quantities of the material can be prepared, this aspect makes the method of in vitro immunization invaluable. Indeed, this method offers an attractive approach when minute quantities of antigen are considered.

The use of peptide sequences as antigens is not an uncommon procedure for raising specific mAbs. However, not so much information on this topic has been documented. In trying to raise mAbs to peptide sequences (i.e. low molecular weight molecules), it is necessary to attach these to a carrier which is usually a protein. It is beneficial to use a substantially large protein carrier because this possesses thymus-dependent properties, and allows the induction and proliferation of T helper cells

which aids in eliciting an immunogenic response (Catty and Raykundalia, 1988). In most cases, the process of conjugating a hapten (e.g. a peptide sequence) to a carrier protein is quite straightforward, involving the spontaneous coupling of a hapten to the carrier protein's amino group. In a few cases however, haptens do not conjugate spontaneously to carriers. In these cases, it is necessary to carry out a chemical "conjugating" or "bridging" procedure. Several agents are available to facilitate conjugation and the use of these usually depends on the available functional groups of the hapten, its orientation and distance to the carrier, and retention of biological and immunological activity of the complex. Some of the most commonly used conjugating agents include glutaraldehyde, carbodiimide, and N,N'-O-phenylenedimaleimide. As for carrier proteins, the most commonly employed are heterologous gamma globulins, bovine serum albumin (BSA), thyroglobulin, keyhole limpet haemocyanin, and *Maia squinada* haemocyanin (Makela and Seppala, 1986).

It should be noted that in vitro immunization can be used to raise mAbs against synthetic peptides not coupled to a protein carrier. In one study, a synthetic peptide corresponding to amino acids 1-19 of thyroglobulin was successfully used to raise specific mAbs (de Boer *et al.*, 1987).

From the in vitro immunization performed, I obtained four stable cell lines and these were grown in three

limiting dilution steps. Sufficient concentrations of mAbs from neat tissue culture supernatants were yielded, thus allowing their characterization and use on immunoassays. Isotyping of these four mAbs revealed them to be of the IgM class. This is not surprising because one of the egregious disadvantages of in vitro immunization over its in vivo counterpart is that IgMs are predominantly generated. Initial reports on in vitro immunization suggested an almost equal distribution of IgG and IgM secreting hybridomas (Luben and Mohler, 1980). In recent years however, this claim has been refuted and it is almost a generally held view that IgMs are the main class secreted (Luben *et al.*, 1982). The fact that I obtained IgMs is not surprising since in vitro immunization is carried out over a few days compared to several months for in vivo immunization. Hence, IgM production in this case represented a primary phase of immune response. In comparison with other studies, the percentage of IgMs produced could be as high as 100% for anti-apolipoprotein mAbs (Erkman *et al.*, 1987), 80% for anti-calmodulin mAbs (Pardue *et al.*, 1983), or as low as 15% for anti-thyroglobulin mAbs (de Boer *et al.*, 1987). In the latter case however, it must be underscored that combined in vitro and in vivo methods were used.

The fact that the mAbs which I obtained are all IgMs is in contrast to the high IgG yield claimed by the manufacturers. I utilised a "Cell-Prime In Vitro Immunization" kit which allows immunization to be carried

out in two stages for a duration of 6 days. First, a monolayer of support cells was established and primed with the C-terminus peptide. Secondly, a splenocyte suspension was added to the support cells together with fresh C-terminus peptide antigen. Following this, it is claimed that the expected isotype analysis of reactive clones is 45-55% for IgM and also 45-55% for IgG. No IgA, IgD, and IgE isotypes were detected (personal communication: Immune Systems Helpline Staff). In my case however, it was not feasible to screen for the isotype of all the positive clones which I initially identified. Doing so would be overly pedantic. Nonetheless, the ten positive lines which I initially screened for their isotype all showed IgMs. From the initial 960 wells, where fused cells were plated into, a total of 33% of the hybridomas showed C-terminus specific activity. This compares well with the expected result of 27% claimed by the manufacturers.

The high percentage of IgMs raised most likely points to an increased fusion of immature splenocytes with myeloma cells, due to the short priming duration. Although the manufacturers did not wish to divulge information pertaining to the basis for claiming that their product provides a high IgG yield (on grounds that this is covered by an existing patent), I suspect that their extended immunization period may play a pivotal role. It is claimed that when splenocytes were exposed to secondary or tertiary in vitro primings, a ratio of roughly 50% IgM and 50% IgG was obtained (Pardue *et al.*, 1983). I could have perhaps

extended the immunization period for another day and restimulated the splenocyte culture, thereby allowing isotype class switching to take place. However, this would have been a risk which may result in death of a large population of plasma cells. As a matter of fact, attempts at such have reportedly been largely unsuccessful (Van Ness *et al.*, 1984). Finally, it can also be proposed that the IgM produced is due to the physical state of the C-terminus antigen or perhaps the low levels of lymphokines present during the antigen-specific B cell activation (Borrebaeck, 1986).

From the present experiments, the four mAbs obtained showed selective preference for brain and foetal bovine serum (FBS) AChE under IEF conditions. None of the four mAbs reacted with the erythrocyte material, as would be predicted because the G_2 form of AChE in erythrocytes does not contain this sequence of amino acids. The cross-reactivity with FBS AChE is not surprising because the last 16 amino acid sequence of the C terminus of both brain and FBS AChE is similar. As a matter of fact, the similarity extends up to 42 residues at positions 533 - 575 (Soreq *et al.*, 1990). It was previously mentioned that the amino acid sequence of FBS AChE showed overall homologies of 90% with human AChE, 60% with *Torpedo* AChE, and only 50% with human serum BuChE (Doctor *et al.*, 1990). In order to compare the last 16 residues of the AChE sequence from different sources, a table is presented in the next page.

KNQF-DHY-SKQDRCSDL	Human Brain AChE
KNQF-NDYTSKKESCVGL	Human BuChE
KNQF-DHY-SKQDRCSDL	Foetal Bovine Serum AChE
KNQF-DHY-SRHESCAEL	*Torpedo* AChE

Taken from Soreq *et al.* (1990).

The specificity of these four mAbs (BMS-5, BMS-6, BMS-7, BMS-8) to brain AChE is almost comparable to that of mAb 2G8 (Liao *et al.*, 1991). The only difference is that mAb 2G8 was raised against *Torpedo* AChE, does not crossreact with FBS AChE, and is directed against a carbohydrate residue epitope.

The use of this synthetic peptide certainly has increased the chance of raising antibodies targetted specifically against the C terminus of human brain AChE. This would not have been possible if purified brain AChE was used because mAbs produced from in vitro immunization demonstrated a much broader range of specificities, in comparison with mAbs obtained from in vivo immunization (Dahmus *et al.*, 1988). In one study, the use of a complex protein preparation (i.e. rabbit pox virus RNA polymerase II) precluded raising mAbs directed at a specific peptide (Morrison *et al.*, 1985). Hence, the advantage of using a synthetic peptide is that the majority of the mAbs raised are directed to the epitope of interest.

Under SDS-denaturing conditions, the four mAbs were able to recognise human brain and FBS AChE, but not erythrocyte AChE. This was consistent with the results obtained under nondenaturing conditions (i.e. dot blots

and IEF). The ability of these mAbs to recognize denatured AChE is almost similar to the HR mAbs raised against human cerebellum AChE. The only difference is that HR-2, -3, and -5 all crossreact with erythrocyte AChE with nearly equal affinity (Rakonczay and Brimijoin, 1988). It is reasonable to assume that since the four mAbs bound to denatured AChE, primary sequence determinants and not conformational determinants comprise the binding sites between the mAbs and AChE. This assumption is not too far-fetched, having been suggested by one investigator working on toxic shock syndrome toxin (Bonventre *et al.*, 1988).

Unfortunately, I was unable to demonstrate directly at which epitope these mAbs are directed to. Nonetheless, an attempt was made to determine whether the present mAbs recognised specifically the C terminus as its epitope. Using AChE recombinant material, this was chemically treated with cyanogen bromide in order to cleave the carboxyl side of methionine residues. Since an AChE molecule contains 8 methionine residues, complete cleavage ought to yield 9 fragments. Upon testing the four mAbs on these fragments, however, no visible binding reaction was noted. I cannot think of any coherent explanation to proffer on this finding, taken in line with the fact that since these mAbs were able to recognize denatured AChE, ipso facto, they should also recognise the C terminus fragment. It is likely however that the four mAbs recognise identical epitopes in view of their close titration curves and binding affinities. Indeed, even at a single epitope

level, mAbs may be clonally heterogeneous with a range of varying affinities (Catty, 1988).

In immunoblot experiments of IEF gels, I also observed that the four mAbs showed a tendency to bind stronger with CSF AChE as compared with brain AChE, on the basis of intensity of developed band patterns. This was consistently observed even when the concentration of samples applied were uniform. It is tempting to speculate that although brain and CSF AChE are similar in structure, it may be the case that the C terminus in CSF AChE is more freely available for the mAb to gain access to. This may be due to differences in orientation of sugars which gives rise to steric hindrance. Alternatively, a recent unpublished finding in our lab claimed that CSF samples may contain monomeric forms of AChE. It is reasonable to assume that a mAb would have better binding access to the C terminus of a monomer, as compared with that of a dimer or tetramer which may be partly occluded. At any rate, the above explanations are purely conjectural.

As mentioned in an earlier chapter, one of the reasons for raising mAbs against human brain AChE was to possibly have one against the anomalous AChE band (Navaratnam *et al.*, 1991). Using the four mAbs however, I was not able to unequivocally demonstrate any mAb specificity for the anomalous band. This however should not be taken in a discouraging light because in the long term, it may be possible to use these mAbs to immobilize AChE isoforms with the anomalous isoform being retarded and collected for

further characterization. Moreover, the ability of these mAbs to bind to SDS-denatured AChE may be a feature which will allow them to identify denatured forms of AChE as abnormal products of degenerating neurones. Indeed in some cases, inactive and partially degraded AChE are found in brain regions of degenerative neurological disorders (Rakonczay and Brimijoin, 1988).

In conclusion, four mAbs were raised against the C-terminus portion of human brain AChE using the relatively new technology of in vitro immunization. These mAbs recognize both non-denatured and denatured human brain AChE, and not human erythrocyte AChE. Hopefully, these mAbs may join the current library of anti-AChE mAbs which can be used for elucidating the localization, turnover, and fate of this enzyme in brain tissues.

Chapter 7.

IMMUNOCYTOCHEMISTRY OF ANTI-ACETYLCHOLINESTERASE MONOCLONAL ANTIBODIES

INTRODUCTION

Immunocytochemistry is a widely used method for elucidating the localization of an antigen of interest. Its chief advantage is that exiguous amounts of antigen can be detected in situ, and its signal can be greatly enhanced by the use of first layer monospecific antibodies. Currently, fluorescent or enzyme labelled antibodies are used for cell or tissue staining.

In an earlier chapter, I have documented literature on the production of monoclonal antibodies to AChE. In a few of these studies, attempts were made to elucidate the immunocytochemical properties of these mAbs. One example is the ZR (1 to 7) series of mAbs, which was raised against rat brain AChE (Brimijoin *et al.*, 1986). Under maximal fixation conditions, immunofluorescence staining (observed by a sensitive video system) was seen confined to neurone-rich regions in the striatum. The fluorescent staining was greatest at the cell surface. In the spinal cord, staining of neurones and associated processes was observed using these mAbs (Rakonczay and Brimijoin, 1986).

In another study, the immunocytochemical property of the AE (1 to 5) series of mAbs was tested. These mAbs were raised against human erythrocyte AChE. In particular, mAbs AE-1 and AE-3 stained neuromuscular junctions in human intercostal muscle sections. The staining distribution was mainly around the primary synaptic cleft and in the secondary folds of the neuromuscular junction (Fambrough *et*

al., 1982). The latter observation is consistent with the known distribution of AChE at neuromuscular junctions.

As for the other monoclonal and even polyclonal antibodies raised against AChE, I have not come across any papers in the literature pertaining to their immunocytochemical characterization. Majority of studies have mainly focussed on ELISA and Western blot immunological characterization of these mAbs.

As a pre-emptive remark, the apparent lack of immunocytochemical documentation of these mAbs may have been occasioned by difficulties in demonstrating positive tissue staining. I have similarly encountered such a problem with my pool of mAbs, and will elaborate further in the succeeding sections.

RESULTS

The present pool of mAbs were able to demonstrate moderately good binding affinities when tested on ELISA capture assays and on immunoblots. To further assess the immunological characteristics of these mAbs, immunocytochemical work was carried out on frozen sections of acetone/alcohol-fixed human post-mortem samples and rat brain striatum. A detailed explanation of the procedures used for the present immunocytochemical work is given in the Materials and Methods section.

Initially, it was essential to determine optimal fixation conditions. It is a commonly held rule of thumb that immunocytochemical staining is only as good as the tissue specimen used. Hence, with frozen tissues, if these are improperly fixed, staining of artifacts will occur. In the present experiment, several fixatives were tested such as acetone, alcohol, and 10% formaldehyde. Acetone appears to obliterate the nucleus, whilst alcohol fixation seems to cause slightly more distortions. 10% Formaldehyde is also not suitable because of the strong positive background staining which can be observed. In the final analysis, it was empirically obtained that a clearer picture can be seen with acetone/alcohol (40:60) fixative. Since fixation time is critical to prevent antigen masking, 10 min was empirically determined as the optimal time to achieve good morphology of the sections. It is unclear why such a combination of fixatives worked better than when used separately.

As a negative control experiment, caudate nucleus sections were incubated with a 1/5000 dilution of normal mouse serum in lieu of the BMS-6D6, and subjected to the secondary antibody and streptavidin-HRP. This was later on counterstained with Mayer's haemalum. In a typical section, counterstaining of the nuclei in majority of neurones is noticeable (Figure 1). These nuclei are widely distributed throughout the tissue section. The structure of the neurones per se is not well defined, brought about by the harsh conditions of freezing. Nonetheless, the white matter in the background is clearly evident.

As a positive control experiment, mAbs GFAP and 2F11 were used, at dilutions of 1/500 and 1/100, respectively. The latter stained neurofilaments, whilst the former was directed against glial fibrillary acidic proteins. As such, astrocytes and ependymal cells were stained by GFAP (data not shown).

The caudate nucleus is a part in the brain lying on either side of the lateral ventricles. Caudate nucleus sections were treated with purified mAb BMS-6D6 (concentration about 50 μg/ml) at several dilutions ranging from 1/100 to 1/2000. At an optimal dilution of 1/800, a good staining in the neuronal cytoplasm, possibly around the neuronal nuclear membrane, can be seen under low magnification (Figure 2A). Apart from neurones, high magnification also revealed staining of astrocytes and oligodendrocytes (Figure 2B). The latter are predominant in the white matter of the CNS and form myelin sheaths around

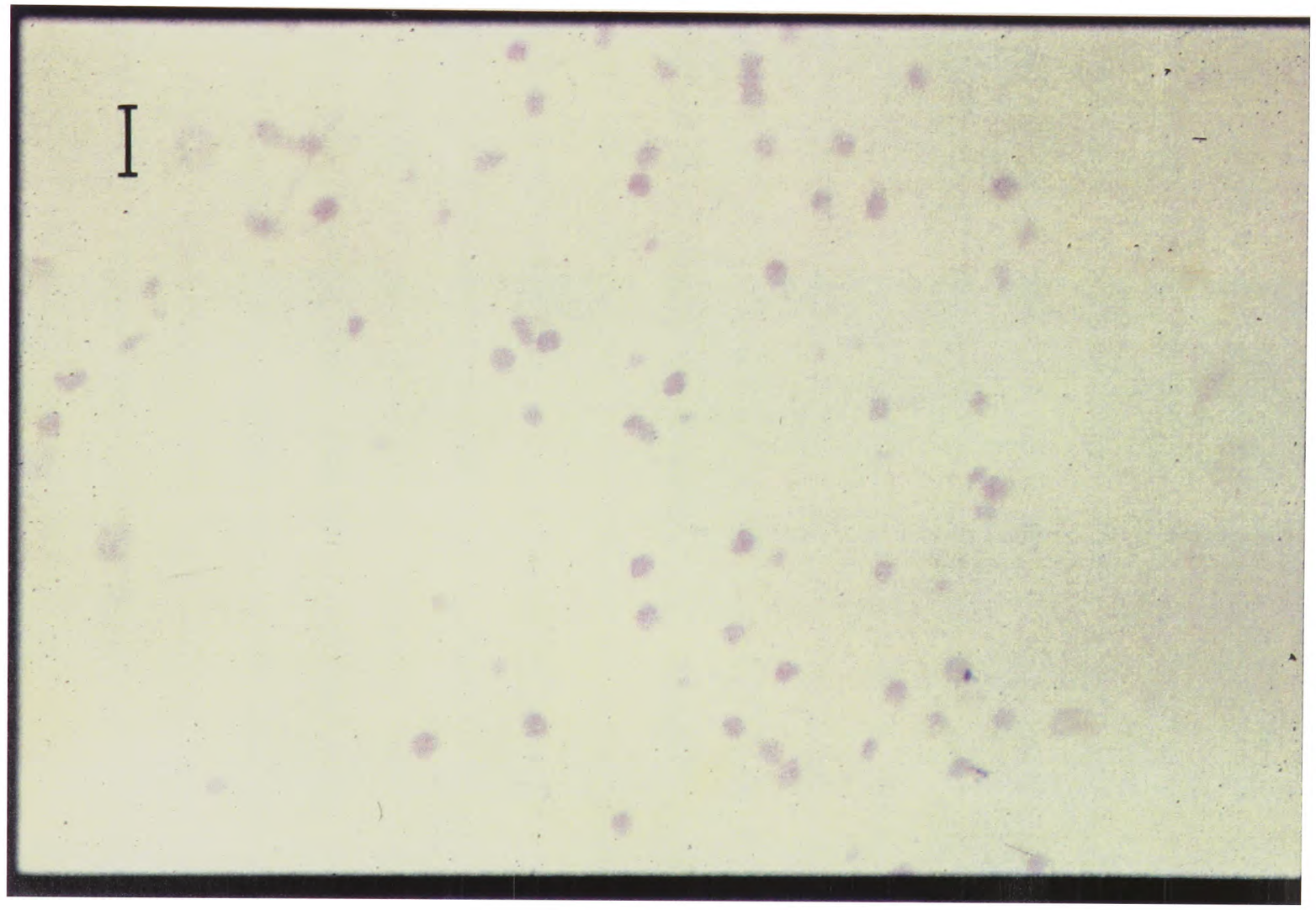

I

FIGURE 1. Immunoperoxidase staining of human caudate nucleus. Cryostat sections (6 μm) were sliced from alcohol/acetone fixed human caudate nucleus and treated essentially according to the procedure described in the Materials and Methods section. Sections were observed on a microscope with a x15 objective. Photographs were made directly from the microscope. The above is taken from a control counterstained caudate nucleus section, showing general staining of nuclei. Horizontal line on upper right-hand corner represents a scale of 20 μm.

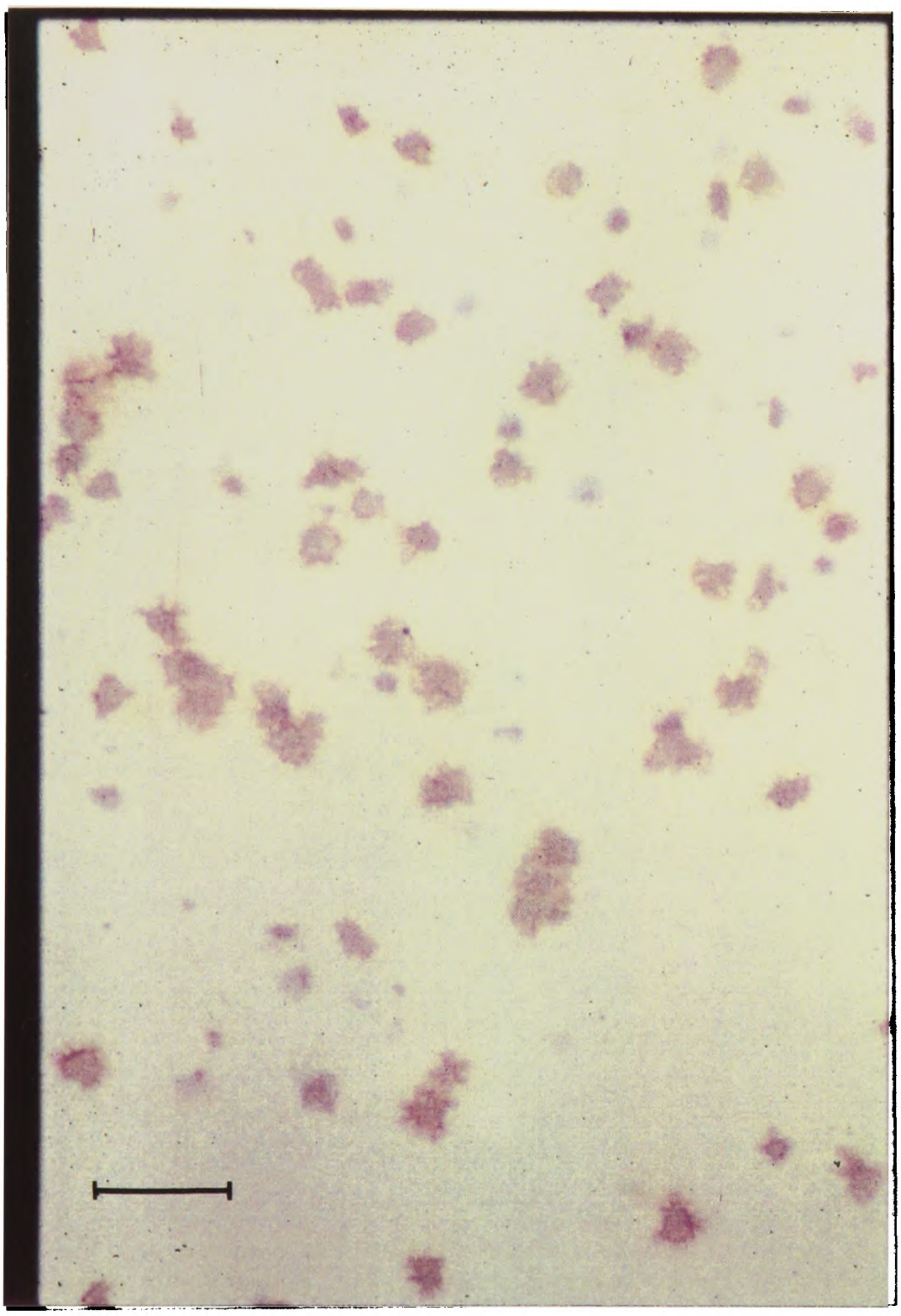


FIGURE 2A. Immunoperoxidase staining of human caudate nucleus. Sections were treated with mAb BMS-6D6 and photographed, as described in the legend to Figure 1A. Specific staining of nucleus of neurones and astrocytes after incubation with a 1:800 dilution of BMS-6D6. (x20 objective) Scale: 20 μm.

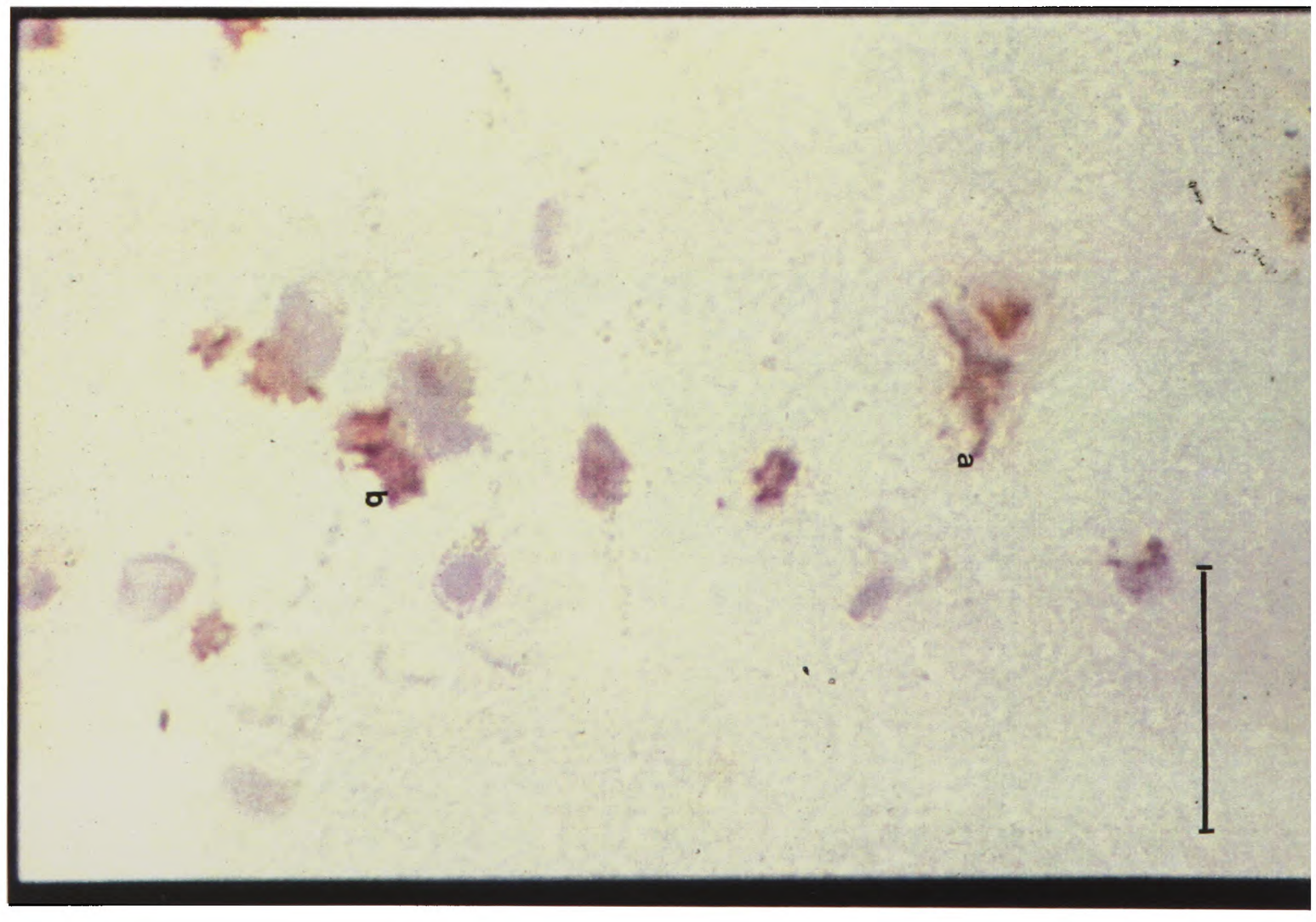

a
b

FIGURE 2B. Section from Figure 2A showing staining of (a) astrocytes and (b) satellite cells after incubation with 1:800 of BMS-6D6. (x40 objective) Scale: 20 μm.

axons, whilst the former are neuroglial cells which can be found in both the white and grey matter of the CNS.

Further experiments with BMS-6D6 at dilutions ranging from 1/100 to 1/2000 were undertaken on temporal cortex sections. At an optimal dilution of 1/500, high magnification revealed positive staining surrounding the nuclear membrane of most neurones, similar to that observed in the caudate nucleus (Figure 3).

When paraffin-embedded sections of hippocampus were used, however, a different picture appears. The hippocampus is an area of well-organized neuronal structure lying in the centre of the brain around the thalamus and slightly above the corpus callosum. Using paraffin-fixed sections of hippocampus, the effect of BMS-6D6 was also tested at dilutions ranging from 1/25 to 1/800. At an optimal dilution of 1/25, a slight staining can be seen in the cytoplasm of neurones in the hippocampus (Figure 4).

In another experiment, paraffin-embedded sections were treated with 0.1% trypsin or 0.1% protease to expose aldehyde linkages which mask tissue antigen and thereby preclude their identification by the primary antibody. One major obstacle is that enzyme digestion must be well controlled to prevent damages to the tissue and destruction of the antigen. As such, digestion time was empirically determined to be 10 min each for both trypsin- and protease-treated sections.

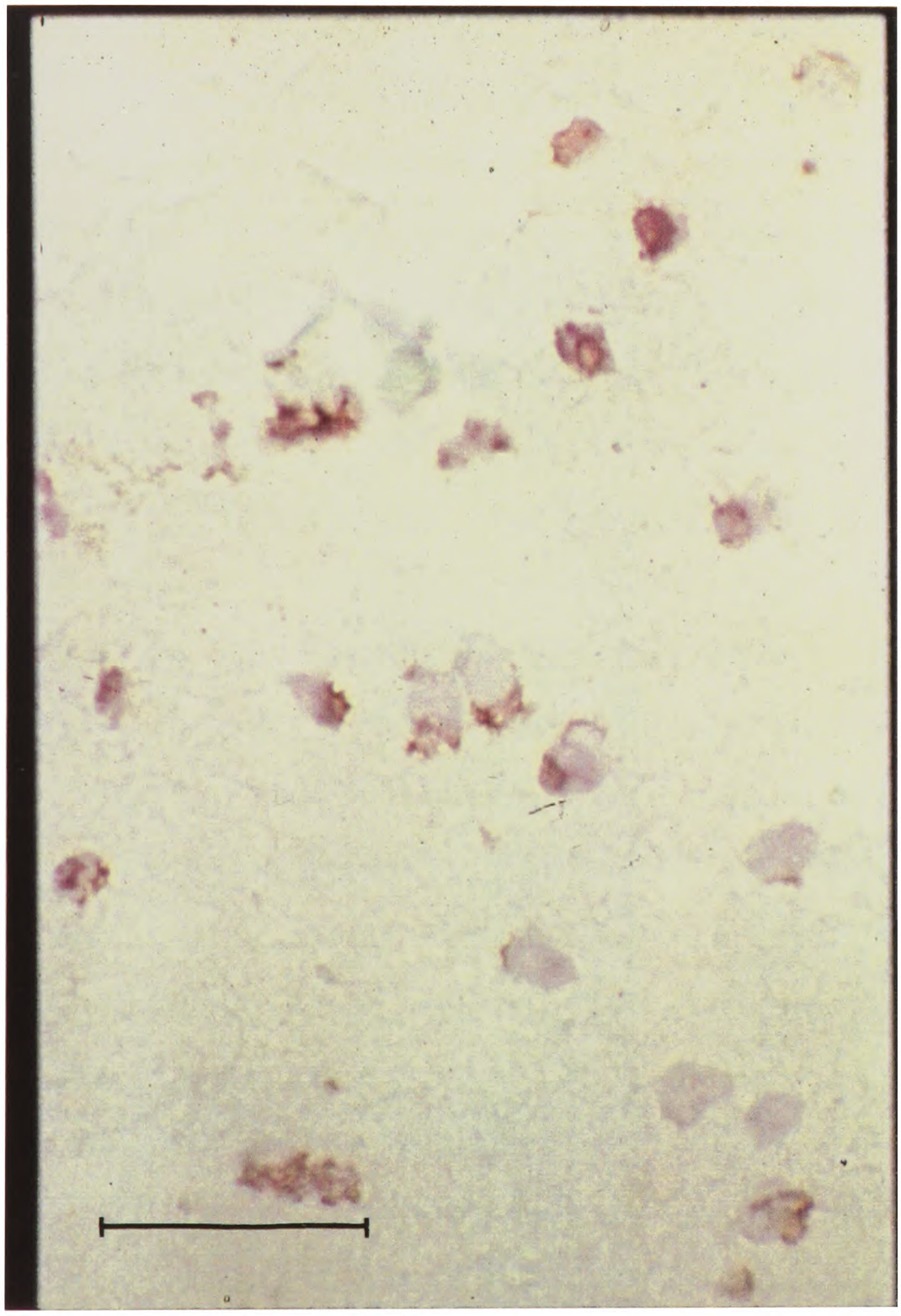


FIGURE 3. Section showing some neurones with peripheral staining after incubation with 1:500 of BMS-6D6. (x40 objective) Scale: 20 μm.

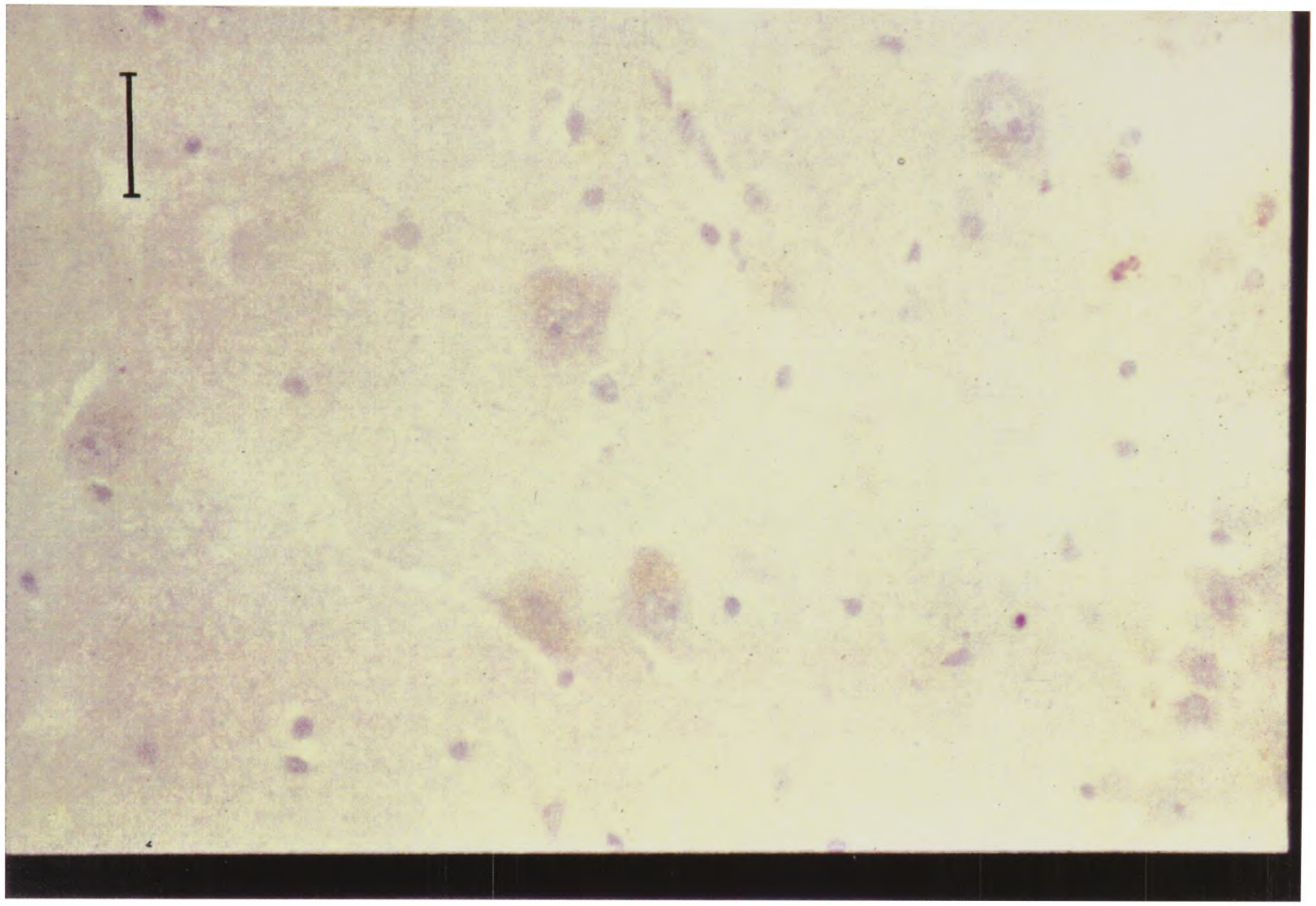


FIGURE 4. Immunoperoxidase staining of paraffin hippocampal sections. Sections (5 μm) from paraffin blocks were sliced on APTES coated slides and treated with a 1:25 dilution of BMS-6D6. Some degree of staining is evident in the cytoplasm of neurones. (x20 objective) Scale: 20 μm.

In trypsin-fixed hippocampal sections, BMS-6D6 at an optimal dilution of 1/25 displayed staining of the cytoplasm of neurones. (Trypsin incubation took place at pH 7.8 and 37^{o} C because, like any other enzyme, trypsin requires certain temperature and pH values for optimal activity.) Under high magnification, it was evident that some Nissl reaction in a number of large neurones existed (Figure 5). These Nissl substances are granular in appearance and are thought to be ribosomes.

The immunocytochemical property of the mAbs raised by in vitro immunization was also assessed. A dilution of 1:10 was used for all the four mAbs. In a typical section (Figure 6), BMS-7 demonstrated faint staining around blood vessels. All the other three mAbs displayed a similar effect. The results obtained for these mAbs are not in consonance with their erstwhile demonstrated binding affinities in immunoblots.

The conflicting results obtained in frozen human caudate nucleus sections pointed to the possibility of non-specific binding. Further, in most cases, the sections used have been stored for a protracted time period, thus possibly altering the antigenic structure of AChE. In order to solve this problem, further experiments on rat brain sections were carried out. Rat brains were used because these can be sectioned and studied immediately without any storage delay periods.

The same mAbs were tested on frozen rat striatum sections (data not shown). Although this area of the rat

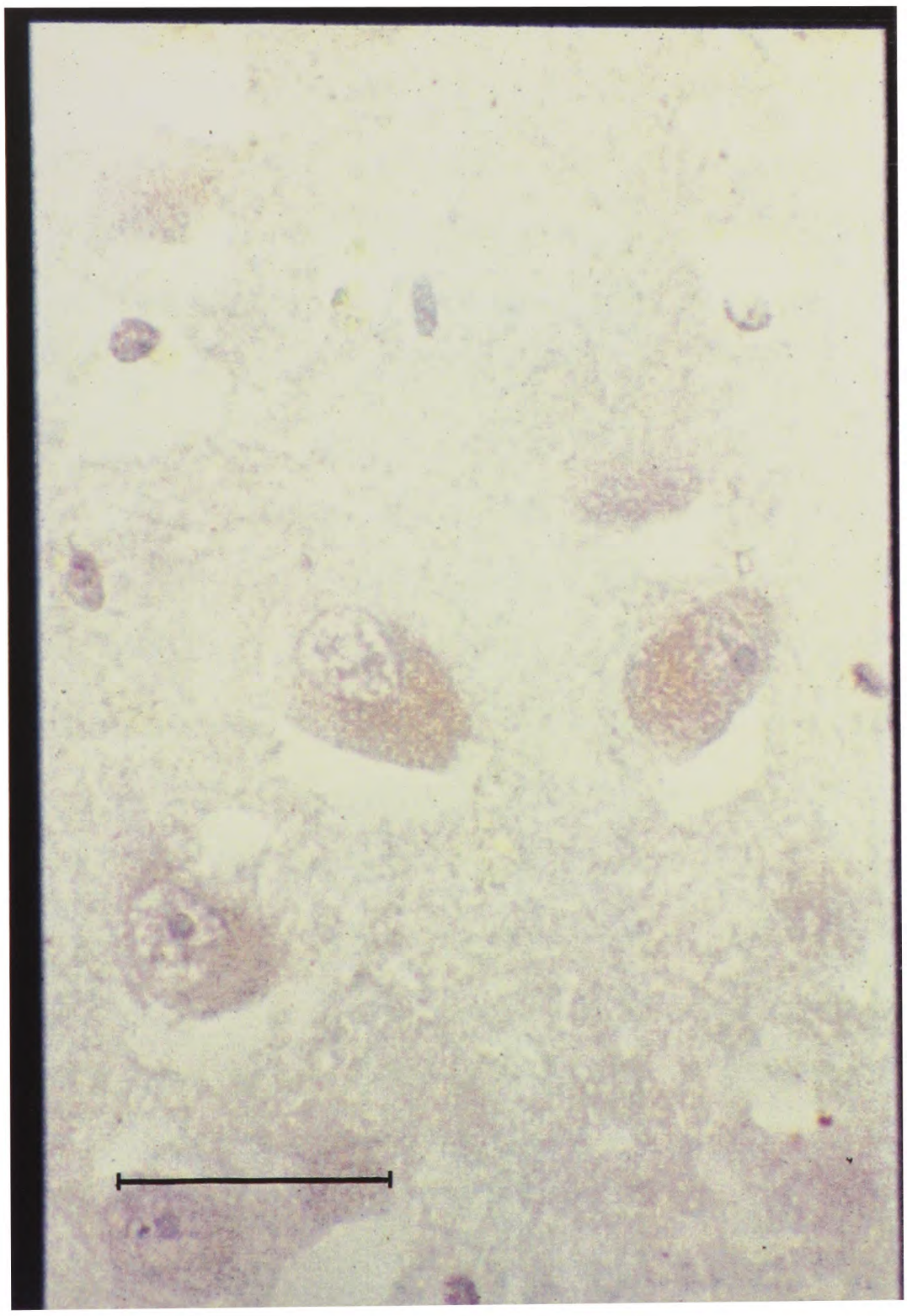


FIGURE 5. Immunoperoxidase staining of trypsin-fixed hippocampal sections. Sections (5 μm) of hippocampus were treated with a 1:25 dilution of BMS-6D6. This section shows specific staining of Nissl granules in some large neurones. (x40 objective) Scale: 20 μm.

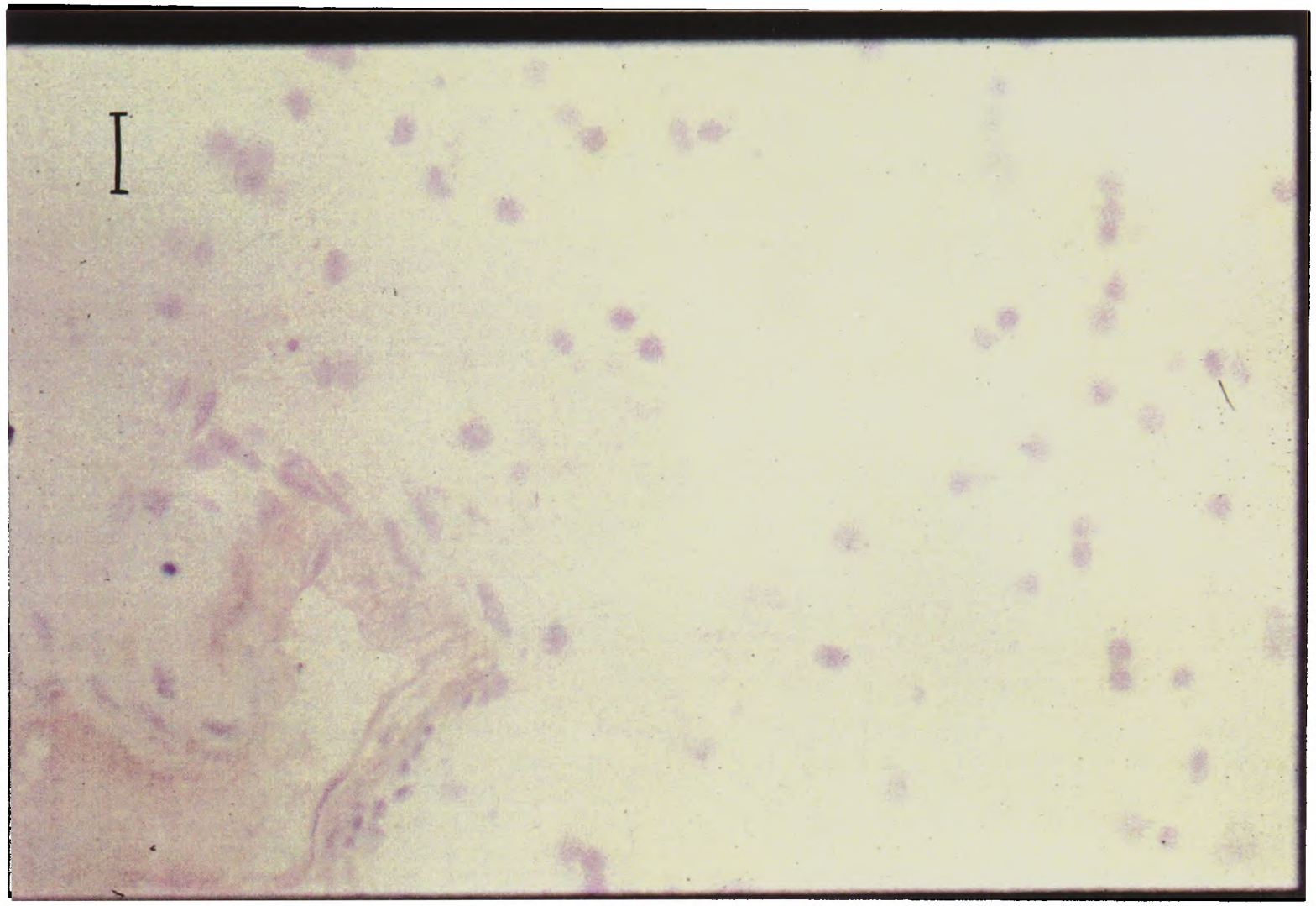


FIGURE 6. Immunoperoxidase staining of caudate nucleus. Sections (6μm) of caudate nucleus kept at -20° C and acetone/alcohol fixed were used. Dilution of mAb BMS-7 was at 1:10. No specific reaction was observed. (x20 objective) Scale: 20 μm.

brain is very rich in cholinergic neurones, no specific positive staining could be observed for any of the mAbs. In a preliminary experiment however, rat striatum were homogenized and soluble AChE fractions were extracted. These were blotted onto nitrocellulose paper and detected with BMS-6D6, and -7. The two mAbs cross-reacted with the soluble AChE fractions (immunoblot data not provided). In line with this finding, it is therefore difficult to assess the reason(s) for the lack of cross-reactivity in frozen sections. However, several propositions are suggested, and these shall be presented in the Discussion section.

DISCUSSION

In the present chapter, I have carried out work to demonstrate the immunocytochemical staining characteristics of the mAbs which I raised against AChE. Previous studies on the immunocytochemical analysis of AChE mainly focussed on trying to elucidate the distribution patterns of this enzyme. In these attempts however, very few immunological work was actually undertaken. The demonstration of AChE distribution was mainly carried out by enzyme histochemical means (such as by a modification of the Koelle copper thiocholine method) or quantitatively by assaying AChE activity in selected brain areas. As I earlier mentioned, in most of the papers dealing with production of anti-AChE mAbs, no further attempts at immunocytochemistry have been documented.

Indeed in my case, I was slightly hesitant about the applicability of my mAbs for immunocytochemistry, primarily because of the nature of their isotype (i.e. IgM). Nonetheless, there have been cases when IgMs were successfully used in tissue sections (personal communication: Dr. C. S. Morris). Although immunocytochemistry may provide definite answers to a mAb's specificity, it is usually the general rule of thumb that mAbs which behave well in assays and immunoblots may not necessarily perform well in tissue sections (personal communication: Dr. M. Esiri and Dr. S. Totterdell).

The findings presented earlier were mainly with those obtained with BMS-6D6. In preliminary experiments, BMS-

3E4 was demonstrated to have strong staining around blood vessel walls at dilutions 1/10 and 1/20 (data not shown). This was consistent with the result obtained with BMS-3E4 in ELISA capture assays (i.e. strong preference for erythrocyte AChE). As for mAb BMS-6D6, there was no apparent cross-reactivity with blood vessels seen in tissue sections. This is very much compatible with its behaviour in ELISA capture assays and immunoblots. In caudate nucleus sections, BMS-6D6 showed some staining in the nuclear membrane periphery of neurones, astrocytes, and oligodendrocytes. This area of brain was chosen in view of the fact that the caudate nucleus is known to contain a high distribution of cholinergic neurones. In fact, previous investigators have shown a high distribution pattern of AChE-positive neurones in the dorsolateral and ventral areas of the caudate nucleus (Hartz-Schutt and Mai, 1991).

The observation that staining was mainly around the periphery of the nuclear membrane is somewhat difficult to explain. Further, it is somewhat difficult to assess, even at high magnification, whether the staining is in the external or internal nuclear membrane. If staining is around the external nuclear membrane, then a likely theory would point to newly-synthesized AChE being pushed towards the nucleus. It is known that a large proportion of the synthesized AChE subunits may remain inactive. Some of these (both active and inactive AChE pool) are not transported to the cell membrane and are eventually

degraded in nonlysosomal compartments (reviewed in Massoulie *et al.*, 1993). Indeed, AChE activity in large secondary lysosomes has been previously demonstrated (Pow and Morris, 1989). This finding is actually almost similar to that obtained in canine tracheal smooth muscle where staining of intracellular AChE was associated with the perinuclear envelope (as well as the sarcoplasmic reticulum and Golgi apparatus). No explanation for this finding was proffered (Adler *et al.*, 1991).

Like most glycosylated proteins, AChE are synthesized first as inactive precursors, then acquire enzyme activity in the rough endoplasmic reticulum, and then translocated into the lumen where their signal peptides are cleaved. The glycans that are added in the endoplasmic reticulum are remodeled in the Golgi apparatus (Massoulie *et al.*, 1993). The translation of mRNA into inactive AChE precursors in the endoplasmic reticulum and the presence of transportable forms of AChE in the cytoplasm may support the finding that in paraffin and trypsin-fixed hippocampal sections, staining of the cytoplasm by BMS-6D6 is observed. Previous investigators have demonstrated that the hippocampus is richly distributed with AChE. In the adult mouse, a distinctly stratified AChE pattern was seen associated with intrinsic hippocampal connections (Slomianka and Geneser, 1991). As a matter of fact, AChE was shown to have a concentration gradient, increasing from dorsal to ventral in the rat hippocampus (Hortnagl *et al.*, 1991).

Unfortunately, such a gradient was not seen in the present hippocampal sections.

The observed staining within the nucleus of trypsin-digested temporal cortex is somewhat difficult to explain, in line with current knowledge on AChE's synthesis and assembly. It is likely that this finding is more artefactual than real. This may have been occasioned by the fact that the different brain areas used for this study were obtained from different patients. Hence, it is probable that different interim periods prior to cryopreservation were the cause of these discrepancies (i.e. structural or enzymatic destruction).

The findings obtained in human brain sections are somewhat equivocal in the sense that both cytoplasmic or nuclear membrane staining is demonstrated, and that a majority of neurones (rather than known cholinergic neurones) are stained. It is more likely that the present findings point to some degree of non-specific staining. Although such a staining was absent in negative controls, it is difficult to ascertain possible reasons for it.

In the hope of circumventing this problem, however, brains (frozen fixed and unfixed) from rats were used. Rat brain is deemed very suitable for immunochemical studies of this nature since the biochemistry, physiology, and distribution of AChE in this species are very much well documented (Rakonczay and Brimijoin, 1986). Furthermore, it was hoped that the problem of protracted storage time can be resolved because unlike human brain, rat brain can be

processed, sliced, and studied immediately, thus precluding antigen degradation.

Unfortunately, sections taken from the rat striatum did not reveal anything new that was unseen with human caudate nucleus sections. Most sections appear to possess non-specific staining.

With regard to the in vitro mAbs, immunocytochemical findings did not seem to tally with its observed specificities in capture assays. The present findings are redolent of mAbs AE-1 and AE-5 (raised against human erythrocyte AChE), which demonstrated binding with caudate nucleus and spinal cord AChE in sedimentation analyses. On the other hand, attempts at using these mAbs in immunoperoxidase staining of AChE-positive neurones in human caudate nucleus and spinal cord sections resulted in very weak to almost nil staining (Fambrough *et al.*, 1982).

Several reasons for the apparent lack of specific staining in brain sections can be suggested: (1) Since the mAbs were raised against soluble brain AChE, this may have been dissipated during the washes, thus rendering the concentration of antigen in sections too low to be detected by immunocytochemistry. (2) The antigenic site of brain AChE may be sensitive to the fixatives used, and is therefore denatured (personal communication: Dr. D. Wong). In this case, no attempts at assessing other forms of fixative were carried out, in view of technical considerations beyond my control. The only reason for using acetone/alcohol for this purpose was because it was

shown to be effective for mAb BMS-6D6. (3) The binding affinities for these mAbs are too low to suit delicate applications such as immunocytochemistry. The anti-AChE mAbs which I raised had weaker affinities for brain AChE (ED_{50} as high as 10^{-3} to 10^{-4} M), in contrast to the ZR series of mAbs which had affinities in the nM range (Brimijoin *et al.*, 1986). In this case, immunofluorescence might offer a more sensitive staining. (4) Finally, the very nature of the mAbs' isotype may have precluded their usage in immunocytochemistry. Although I mentioned earlier that IgMs may be successfully employed in immunocytochemistry, the antithesis can also hold true. This is perhaps the reason for the obvious lack of popularity of IgMs with immunocytochemists.

In the current work, I was unable to test the immunocytochemical features of these mAbs on brain from Alzheimer's disease (AD) patients. I surmised that since the present mAbs showed no demonstrable specific staining on age-matched control sections, I therefore had no reason to believe that their effect on AD brain sections would be any more different.

To recapitulate, mAbs raised against AChE were tested by immunocytochemistry on normal human brain tissue sections. MAb BMS-6D6 showed staining on either the nuclear membrane periphery or on cytoplasmic Nissl granules, which is believed to be a result of non-specific binding. In contrast, the mAbs raised by in vitro immunization did not demonstrate any staining that

corroborated their specificities in capture assays. Based on these findings, it may be concluded that the present in vitro mAbs share a similar property with the AE-1 and AE-5 mabs which worked in capture assays but showed weak to nil staining in tissue sections.

EPILOGUE:

Quo vadis?

SUMMARY AND FUTURE DIRECTIONS

At the onset of this project, I initially aimed to develop a diagnostic test where the enzyme AChE is used as a marker for neurological pathologies. In particular, my interest focussed on the possibility of developing an ante-mortem diagnostic test for Alzheimer's disease, based on an anomalous isoform of AChE which was discovered by a colleague (Navaratnam *et al.*, 1991) in lumbar CSF samples from histologically diagnosed cases. In tackling this endeavour, two points had to be taken into consideration. The first is to develop an optimum method for preparative purification of this anomalous isoform. Secondly, methods that would ensure production of specific mAbs had to be employed.

Using the procedure of chromatofocussing, no success in separating this anomalous isoform was achieved. Hence, other chromatographic procedures were used for preparative purification of AChE (both from control and AD brains), in the hope that a mAb against this anomalous isoform could be raised based on a random chance of probability.

The conventional method of in vivo immunization was first used, and this produced four mAbs (BMS-3E4, -6D6, -7G10 and -9F4). Of particular interest is BMS-6D6 which seemed to recognize brain G_4 AChE in ELISA capture assays, whilst the other three recognized only the G_2 form.

In another procedure, the novel method of in vitro immunization was employed using a synthetically-produced C-terminus peptide as antigen, in the hope that more specific mAbs with higher binding affinity to G_4 AChE may be raised. Four mAbs (BMS-5, -6, -7, -8) were successfully raised and demonstrated to bind specifically to brain AChE in both immunoblots and ELISA capture assays.

Unfortunately, when the two sets of mAbs were tested in immunocytochemistry, no specific staining has been observed. Several reasons for this had been proposed, but one factor which is of major significance is the fact that all these mAbs are of the IgM isotype. Indeed, IgMs have always been considered by immunocytochemists as treacherous mAbs that are of inconsequential value.

I, however, think otherwise! The present mAbs would certainly prove valuable in biochemical work and clinical diagnostics. Future work could be targetted on carrying out ELISA assessment of CSF from control and AD groups in order to compare AChE levels between the two. With the plethora of samples currently available from OPTIMA, I envisage that the present mAbs would certainly play the role of indispensable tools for AChE research.

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