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Isolating a Cell Maximally Secreting Acetylcholinesterase

Annual Report

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SUMMARY

Basic methods were developed for the isolation of subpopulations of cells which are high producers of a desired secreted cell protein. The ultimate goal of this research is to isolate a cell line maximally secreting human acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7). This positive selection system, termed the Cell Isolation Technique (CIT), has evolved in two different directions to screen individual cells trapped within agarose beads. Both approaches rely on a specific ligand-receptor or antigen-antibody interaction to capture the desired secreted protein and immobilize it within the beads. In both cases, the cells secrete product, product binds to immobilized specific reagents and thus accumulates within the beads. Beads with high densities of desired product are identified and physically isolated so as to enrich for subpopulations of high producer cells. The two approaches differ with respect to how they identify and sort the beads with desired cells. In the original method, secreted products are captured by reagents immobilized on the surfaces of sheep red blood cells co-encapsulated within the same beads. Subsequent introduction of specific monoclonal antibodies plus complement results in the lysis of erythrocytes within beads containing high levels of secreted protein. Hence, simple density gradient centrifugation is sufficient to separate beads with lysed red blood cells from the vast majority of beads with intact cells. The second approach uses a fluorescence activated cell sorter to screen beads which have accumulated captured secreted cell product now identified with fluorescently labeled antibodies. Although a cell line secreting high levels of AChE has not yet been developed by these methods, model studies have been encouraging. This report will therefore focus on the basic research behind this technology and its application for screening cells transfected with total genomic human DNA.

Keywords: Rhabdomyosarcoma cells; (RT)

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals:, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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I. Cell Lines Secreting Human Acetylcholinesterase (AChE)

Among the various cell lines screened for secretion of AChE, the A-204 human rhabdomyosarcoma muscle cell line (described previously) has proven to be the most consistent secretor of enzyme (2-4 munits/ml/day at confluence) and stable in culture. Cell lines of neuronal, glial and lymphocytic origins were also tested for secretion of AChE (Figure 1), but most initial cell isolation experiments were performed with the RMSC cells.

Cell lines were screened for the production of AChE by assaying the cell culture supernatants using a radiometric assay developed by Johnson and Russell (1) as modified by Rotundo and Fambrough (2) (described below). To avoid the high background levels of specific and nonspecific esterases present in serum, cells were adapted to growth for limited periods of time (3-4 days)in a defined media or media supplemented with a low molecular weight filtrate of fresh bovine lymph. The filtration of the lymph excludes proteins of molecular weights greater than 30,000 daltons and thus eliminates interfering esterases or immunoglobulins. Generally, the adherent cells were grown up in more complete fractions of bovine lymph or 10% fetal bovine serum supplemented media, rinsed for a day in defined media, and then maintained in the esterase free media for several days to accumulate secreted AChE (Fig 2). Levels of AChE activity for one experimient is presented below.

Supernatants were taken from near-confluent (80-90%) cultures of A-204 cells at 3 days in the media indicated.

*A-204 cells in medium supplemented with low mol.wt. lymph filtrate + transferrin	7.5 munits/ml
*A-204 cells in medium supplemented with N-2 defined medium	4.3 munits/ml
Low mol.wt. filtrate medium only	<0.05 munits/ml
N-2 defined medium only	<0.05 munits/ml
10% FBS supplemented medium	4.9 munits/ml
50% submicron filtrate of lymph	4.7 munits/ml

* = Identical flasks seeded at 0.25 million cells/ml

Note that both the submicron filtrate of fresh bovine lymph as well as fetal bovine serum have substantial levels of esterase activities making them unsuitable for culture media while cell supernatants are being assayed for secreted AChE. What these data imply is that if we are successful at culturing adherent cell lines such as A-204 in defined media or with low mol.wt. filtrate of lymph plus fibronectin, laminin or other adhesion factors, we should be able to assay secreted AChE in the same medium in which the cells actually proliferate. Alternatively, we could deplete serum of its endogenous esterase activity using inhibitors or antibodies coupled to affinity columns.

Adaptation of AChE Secreting Cells to Suspension Culture

Initial attempts to adapt adherent cell lines, such as the A-204 rhabdomyosarcoma line, to growth in defined media plus fibronectin (as an adhesion factor) have been unsuccessful. Even if these cells are first cultured in serum supplemented medium and then the medium is changed to one containing fibronectin (5 ug/ml), the cells lift off the plates and stop dividing. We are about to investigate other potential adhesion factors such as laminin (+/- collagen treatment of flasks), but in the meantime, we are attempting to adapt these cells to suspension culture using the established soft agar cloning technique and by growing them in AChE-depleted serum in spinner flasks. The hope is that new clones will grow in suspension culture and still secrete normal levels of AChE, which would greatly simplify our cell isolation methods since we will no longer have to accomodate adhesion dependent cells inside beads.

Radiometric Assay for Acetylcholinesterase

The most sensitive and reliable assay we have developed for monitoring AChE levels in cell culture supernatants has been the radiometric assay adapted from the procedures of Rotundo and Fambrough (2). We developed the assay to be sensitive enough for detecting AChE in raw, unconcentrated cell culture supernatants. The assay measures the amount of radioactive acetate which partitions into the organic phase of a liquid scintillation cocktail following the enzymatic hydrolysis of 14C-ACh. The assay is linear with respect to both time (Figure 3) and enzyme concentration (Figure 4) under the conditions employed. AChE activity is expressed in terms of milliunits per milliliter. One unit of AChE is operationally defined here as the amount of enzyme activity required to hydrolyze 1 micromole of 14C-ACh per hour at 37 Deg.C. at pH 7.0. The sensitivity of the assay was improved by increasing the specific activity of the 14C-ACh substrate. This was achieved by using little or no additional cold ACh carrier with the stock radioactive substrate, thereby raising the specific activity as high as 58 mCi/mmole (Rotundo and Fambrough worked with 14C-ACh at 2.3 mCi/mmole). Obviously, the trade off it that the net substrate concentrations drop tremendously (into the tens of micromolar range). Hence, a study was carried out to determine the optimum dilution of radioactive 14C-ACh with cold substrate. It was found that keeping the final concentration of ACh at 0.1 mM gave the highest signal to noise ratio, even higher than obtained when using the concentration (1.2 mM) required for maximum enzyme rate A further improvement in this assay occured when we developed the Radiometric Immunoadsorbant Solid-phase Assay (RISA), described in a later section.

A sensitive visual assay for spot checking small samples for AChE activity (such as sucrose gradient fractions) was also developed by applying methods described by Karnovsky and Roots (3) for in situ histologic staining of tissue slices.

Initial Characterization of AChE Released by Cultured Human Cells

AChE inhibition studies were employed to demonstrate that the esterase activity found in cell culture supernatants of the various cell lines was indeed true acetylcholinesterase. For the rhabdomyosarcoma cell line esterase activity, inhibition curves for the true AChE inhibitor ("BW284C51", 1,5-Bis-4-allyl dimethyl ammonium phenyl pentane dibromide) were identical to those obtained for crude human erythrocyte AChE and very similar to those reported for rat brain AChE by Pavari, Pecht and Soreq (1983) using their microfluorometric assay or in previous studies using the Johnson and Russell (1975) radiometric assay (Soreq, Parvari and Silman, 1982). In the presence of specific inhibitor (BW284 C51) and of non-specific inhibitor (iso-OMPA), at log -4 Molar, 92% and 40% of the esterase activity was inhibited, respectively, similar to values reported by Pavari et al. (4) for the rat brain enzyme. The concentration of specific inhibitor required for 50% inhibition (Ki) was appoximately log -7 Molar (Figure 7). For the non-specific esterase inhibitor, Iso-OMPA (Tetraisopropyl pyrophosphoramide), the Ki was above log -4 Molar indicating that our enzymatic hydrolysis of ACh was due to true AChE.

Enzyme kinetics studies revealed that the AChE activity recovered from the cell culture supernatants of the A-204 rhabdomyosarcoma line also behaved as expected for true AChE. The concentration of substrate required to give maximum rates of hydrolysis (Vmax) was found to be 1.2 mM ACh using the radiometric AChE assay (Figure 5). The AChE activity from RMSC cells exhibited the characteristic substrate inhibition at concentrations of ACh above 1.2 mM. The Michaelis constant, Km, was calculated to be 0.4 mM by analysis of a Lineweaver-Burk plot (Figure 6). Both of these figures compare favorably with values in the literature, such as the Rotundo and Fambrough (1979) study of chick embryo leg muscle AChE (Km = 0.6 mM), the Parvari, Pecht and Soreq (1983) study of rat brain AChE (Km = 0.417 mM) and earlier studies reviewed by Silver (6).

Preliminary sedimentation studies using 5-20% sucrose gradients indicate that the majority of AChE activity behaves as a broad peak moving with a sedimentation coefficient of around 10s and a separate peak with a value of around 16s (Figure 8). This study utilized our colorimetric assay derived from the Karnovsky and Roots (3) histological staining technique.

Freeze-thaw studies indicated the enzyme was relatively sensitive to freezing, so we store concentrated samples of AChE from cell supernatants at 4 Deg.C. or in 50% glycerol at -20 Deg.

Finally, we have confirmed that the AChE activity we detect in cell supernatants binds with high affinity to the AE-1 monoclonal antibody developed by Fambrough et al.(1982) using a novel enzymatic/antigenic coupled assay (RISA) described below.

As we further characterize the AChE released by human cells in culture, we will analyze the forms of enzyme on both native and denaturing electrophoresis gels using 3H-DFP binding, 125-I-antibodies and precipitation of Karnovsky stain (potassium ferricyanide) by the active enzyme to label the protein bands.

Radiometric Immunoadsorbant Solid-phase Assay (RISA) for AChE

A solid-phase version of the radiometric assay for AChE was developed which simulataneously applies two independent criteria for measuring levels of true AChE released into cell culture supernatants: specific enzymatic properities and specific antigenic properties. The correct antigenic AChE protein must bind to a specific monoclonal antibody (AE-1, described below) immobilized on a microtiter plate; after washing, the bound enzyme is then detected by radiometric assay, and tested for correct substrate specificity, Km, and inhibitor specificity. The AE-1 monoclonal IgG is adsorbed onto 96-well flexible PVC plates to capture AChE; antibody binding does not interfere with the enzymatic activity of AChE. Test samples or standards for AChE are then incubated in the wells for 2 hr at 37 Deg.C. The plates are washed and incubated overnight at 37 Deg C. with 14-C-ACh substrate. Hydrolysis rates are determined from the levels of 14-C-Acetate released into the reaction medium (Figure 9) Although we can use impure preparations of human enzyme (crude human erythrocyte AChE), we will be soon receiving a sample of immunoaffinity purified human AChE to standardize the assay, and enable us to estimate the absolute amounts of AChE protein present in the cell culture supernatants.

II. Production, Purification and Characterization of Mouse Monoclonal AE-1 Antibody

Large amounts of antibody specific to human AChE will be needed for cell isolation experiments with AChE-secreting cell lines. Fortunately, we have access to the mass culture system at Bio-Response which routinely produces gram quantities of monoclonal antibodies for commercial contracts. The mouse hybridoma AE-1 line, generated by Fambrough et al. (1982), was obtained from the ATCC cell repository and adapted to mass culture for research purposes. The cells were grown in a small scale research hollow fiber unit to high densities in regular tissue culture medium supplemented only with a low molecular weight filtrate of fresh bovine lymph and transferrin. A schematic diagram of the Bio-Response Mass Culture Technique (MCT) is shown in Figure 10 along with a brief description.

1.35 grams of monoclonal IgG were harvested continuously from the single unit over a period of 25 days. Mouse IgG1 represented at least 50% of the total protein collected in raw culture supernatants. The antibody concentration range for the unit ranged from 7.8 to 63 ug IgG1 per ml during the production phase of the culture. During this time the average cell density was maintained at 5-10 x 10^7 cells/ml. The harvested media was concentrated and pooled for subsequent purification.

Purification of the monoclonal antibody was performed through a research collaboration with Dr. Hector Juarez-Salinas and Timothy Brooks at Bio-Rad Laboratories. They further concentrated the antibody, precipitated it with ammonium sulfate and isolated nearly 400 mg of IgG1 from a single pool of medium using preparative hydroxylapatite high performance liquid

chromatography. A chromatogram and details of this procedure appear in Figure 11. The results of this collaboration were recently presented at the Fourth Annual Hybridoma Congress on February 6, 1985 in San Francisco.

Characterization of the AE-1 monoclonal antibody by SDS polyacrylamide gel electrophoresis (Figure 12) revealed that, aside from the added transferrin, the only major species of protein were the heavy and light chains of immunoglobulin. Purity of the antibody in the concentrated raw culture medium was at least 50%. Ammonium sulfate precipitation then quantitatively removed all of the transferrin, leaving a surprisingly clean preparation of antibody (see Lane 3, Figure 12). Subsequent passage through the preparative hydroxylapatite HPLC column separated the main IgG peak from apparent contaminating immunoglobulin light chains known to also be secreted by the hybridoma (SP 2/0 hybridoma fusion partner). However, it would appear that additional light chains were still present in the IgG peak since the intensity of this band is greater than expected relative to the staining of the heavy chain band (see MOPC 21 standard in Lane 1, Figure 12).

The presence of additional light chains in our purified AE-1 IgG was confirmed by Isoelectric Focusing Gel Electrophoresis (see Figure 13). Heterogeneity of the intact immunoglobulins was also apparent, both in our purified AE-1 antibody and in the MOPC-21 myeloma protein, but this seems of little consequence as evidenced by the functional activity assays.

Various fractions of the AE-1 antibody taken from different stages of purification were tested with our RISA assay (described above; see Figure 15). As purity of the antibody increased, the competition for binding to limited sites on the PVC plates by contaminating proteins was decreased. Nonetheless, pooled cell culture supernatant and ammonium sulfate precipitated antibody bound enzyme to the wells at 85% and 95%, respectively, the level obtained for the HPHT-purified material.

The AE-1 monoclonal has since been biotinylated and used to assay for AChE with the ELISA technique. The assay appears to work at very high levels of AChE activity (hundreds of milliunits per ml), which corresponds to ng/ml quantitities of enzyme. Unfortunately, this assay is several orders of magnitude less sensitive than is required to detect AChE in unconcentrated samples of cell culture media. On the other hand, it confirms that the same monoclonal antibody can be used in a "sandwich" type assay or capturing scheme to trap AChE released by cells, as is required in one version of our cell isolation technique. In the meantime, the biotinylated AE-1 has been used to couple avidin conjugated fluorescent probes for sorting RMSC cells expressing AChE antigens on their surfaces using FACS (discussed below).

Additional monoclonal antibodies to human AChE have been obtained through a researh collaboration with Novo Laboratories in Denmark for characterization of antigens found in the different forms of the enzyme secreted by various cell lines. These antibodies may later be useful for capturing or labeling AChE secreted by human cells inside beads for the CIT.

III. Bead Technology for the Cell Isolation Technique

For the first half of the year, attempts were made to improve on the original methods used to entrap single cells inside bead structures. The beads were made by mixing cell suspensions with solutions of sodium alginate and then forming them into droplets using a variety of techniques ranging from extrusion through fine needles to spraying them out of vibrating nozzles. All of these approaches relied on the addition of calcium to gel the alginate droplets into sturdy beads which could then be coated with polylysine. Subsequent dispersal of the alginate with citrate left our cells trapped inside the polylysine shell and ready for cell isolation experiments. However, it was difficult to obtain uniform beads in the right size range (50 - 100 um) and clumping as well as inconsistent results continued to hamper progress with CIT.

A breakthrough occured when it was discovered that suitable beads for trapping free cells could be made from a derivitive of agarose. This new material has the property of forming very stable beads in oil/aqueous emulsions. These conditions do not appear to harm cells; cells have been observed to remain viable and actually divide when maintained inside bead structures for up to 5 days. Soon after making this discovery, it was shown that secreted cell proteins as large as antibodies could be released into the medium outside beads, confirming that the material is semi-permeable to macromolecules. This was a crucial point since our CIT schemes depend on the introduction of specific antibodies and other reagents to bind to AChE accumulated inside.

Beads are now routinely made and then sorted by sieving to produce a population with a mean diameter of 60 um. These beads are used to encapsulate cells which secrete products of interest, such as AChE, for experimentation with the cell isolation technique described below.

Model Studies for the Hemolytic CIT Using Beads Filled with Sheep Red Blood Cells - Introduction

The original CIT scheme as proposed over a year ago involves the encapsulation of a high density of sheep red blood cells (SRBC) along with the individual human cells secreting AChE. The erythrocytes are derivitized with either a specific ligand or a specific antibody which serves to capture the enzyme as it is secreted by the single human cell within the bead. Higher rates of secretion translate into greater amounts of accumulated enzyme on the SRBC surfaces over time. Introduction of specific antibodies followed by complement would then lyse a high percentage of SRBC's in beads containing desirable cells which secrete AChE at a higher rate than the vast majority of cells in the starting population. Figure 15 depicts the various steps involved in the fabrication and sorting of beads containing secretory cells and red blood cells. Photographs of human cells inside beads (stained with fluorescein diacetate) and beads packed with sheep red blood cells are included in Figure 16.

Model studies for the CIT began with the encapsulation of mouse myeloma cells (as controls) vs. mouse hybridomas which secreted IgM against sheep RBC's. These experiments proved that it the secretion of an antibody which would bind specifically to the surface of co-encapsulated SRBC's could lead to significant lysis of the erythrocytes in the presence of complement and to the isolation of the resulting bouyant beads by density gradient centrifugation. By mixing beads containing hybridomas secreting antibody which would deliberately lyse red blood cells with beads containing control myeloma cells and intact red blood cells, it was possible to show that the isolation system could select one "positive" bead from at least 1,000 "negative" (intact SRBC) beads. The following data come from one such preliminary experiment.

5 x 10^6 SP-2 myeloma control cells encapsulated

plus 5 x 10^3 NS-2.1 anti-SRBC hybridomas in beads;

Following Complement induced lysis of SRBC's,

Density Step Gradient interface = 5 x 10^4 Beads

Containing 4.5 x 10^3 viable cells

(90% recovery)

Hence, the sensitivity of the approach is adequate for detecting high producers of AChE which could arise, for example, by spontaneous gene amplification events. For reference, in mammalian cell lines the frequency of such events is about log -3 to log -4 amplifications/gene per generation (see Schimke, 1982) (8). Separation of "positive" beads from beads with intact red blood cells can be achieved using a density step gradient. As few as 100 viable cells can be recovered at the interface using this technique, so that if we start with one hundred million cells, theoretically we should be able to isolate "positive" cells which occur at frequencies of around one out of a million.

The next step in this procedure was to derivitize the SRBC's with a reagent which would capture the AChE secreted by RMSC or other human cell lines. Two approaches to binding AChE to red blood cells have been investigated: conjugating a specific ligand which would bind AChE irreversibly and conjugating a specific monoclonal antibody which would bind AChE with high affinity.

Derivitization of Sheep Red Blood Cells with Ligand for AChE

As a first attempt to generate a capturing mechanism for AChE inside the beads, a procedure was developed to conjugate an irreversible inhibitor of the enzyme onto the surfaces of the SRBC's. The inhibitor chosen, p-nitrophenyl methylphosphono-chloridate, binds covalently to the esteratic site on the enzyme and has been used to capture AChE by affinity chromatography.

The compound was synthesized from p-nitrophenol and methyl-phosphonic dichloride (De Roos, 1959)(9). The chemistry of conjugation was modified to allow a heterobifunctional reagent, N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), to be used as the covalent linker between the inhibitor and activated thiol groups on proteins on the SRBC's (Jou et al. 1983)(10)(see Figure 15 flow sheet). The first batch of inhibitor synthesized has given mixed results when tested for direct binding of AChE to red blood cell surfaces via SPDP. Freshly derivitized red blood cells were mixed with diluted RMSC AChE to accumulate enzyme on their surfaces. The SRBC's were then incubated with dilutions of anti-AChE (AE-1 monoclonal antibody), developing antibody (adsorbed rabbit anti-mouse IgG) and complement (adsorbed guinea pig serum). Using a simple evaluation of the size of the SRBC pellets, it was determined that close to 90% of the SRBC's were lysed by the complement when the appropriate layers of ligand, enzyme, antibody and developing antibody were included (see Figure 17). Controls lacking the enzyme layer gave little (up to 20% lysis at high antibody concentrations) or no lysis.

Three experimental runs of the CIT were tried using the ligand conjugated SRBC's to capture AChE released by encapsulated RMSC cells. RMSC cells were trapped in beads so that most beads contained one or no beads along with hundreds of SRBC's. These experiments start with 10 million secretor cells, but it is expected that it will be easy to scale up at least three fold. The encapsulated cells were incubated overnight in AChE-free medium to allow them to secrete AChE which then binds to the derivitized SRBC's. The beads were then incubated with the AE-1 monoclonal antibody followed by secondary rabbit anti-mouse IgG. Exposure of the beaded SRBC's to adsorbed guinea pig complement induces hemolysis of those erythrocytes that have captured significant amounts of AChE. The beads with many lysed red blood cells were then harvested by density gradient centrifugation (see Figure 18). The bouyant beads contained the presumed positive high secretor cells, representing 1% to 3% of the starting population of cells. Beads from each interface in the step gradient were then cultured separately in microtiter wells. As the cells divided, they eventually broke out of the beads and adhered to the plastic substrate. Once the cells were well established as a monolayer, the medium was changed to a defined medium for assaying AChE. Unfortunately, no significant change in overall AChE activity on a per cell basis was detected among the different populations harvested from the grandients. Conceivably the high secretor cells don't divide at a fast rate and could be overgrown by lower secretors; alternatively, the selection process is not refined enough to distinguish dead cells releasing enzyme from live cells secreting enzyme. These experiments need to be repeated with RMSC cells adapted to suspension culture in AChE-free medium.

Since those experiments were performed, the ligand has been shown to be very impure and is now being re-synthesized with a more rigorous protocol. The new ligand will also be useful for generating affinity columns to purify AChE from our cell culture supernatants or to deplete serum of AChE for culturing RMSC cells which are dependent on adhesion factors for attachment.

Derivitization of Sheep Red Blood Cells with AE-1 Antibody

Purified fractions of AE-1 monoclonal antibody to AChE were conjugated to SRBC's using the same heterobifunctional coupling reagent, SPDP (Jou et al. 1983). Preliminary tests indicate that there is sufficient antibody coating the SRBC's to capture AChE at the levels it is secreted. However, the lysis with subsequent antibody, developing antibody and complement has been discouraging. Direct ELISA model studies for the sandwiching of AChE between AE-1 adsorbed to microtiter plates and biotinylated AE-1 (plus avidin-HRP) suggest that the level of AChE which binds is too low to get significant amounts of antibody-antigen complexes formed on the red blood cell surfaces.

Two other approaches to capturing AChE on the surfaces of SRBC are being investigated. First, a hapten such as DNP can be used to derivitize the antibodies which bind to the captured AChE so that additional anti-DNP antibodies can be introduced to amplify the sensitivity for complement induced lysis. Preliminary experiments have been encouraging but problems of non-specific binding of intermediate layers of developing antibodies have to be eliminated. Nonetheless, we have been able to get extensive lysis of SRBC's above background lysis using a sandwich composed of SRBC-AE-1 conjugate plus AChE, DNP-AE-1 and rabbit anti-DNP. It has been estimated that only 1000 molecules of antibody-antigen complexes per cell are required for effective complement induced lysis. The second approach would be to generate new hybridoma cell lines secreting IgM directed to the human AChE; theoretically, only one IgM per SRBC is required to induce complement dependent lysis of the erythrocytes.

Model Studies on the CIT Using Hybridoma Cells

In order to test the efficacy of the red blood cell lysis approach for separating positive beads from large mixed populations, we have focused more of our attention on models using hybridomas which secrete mouse IgG as the cell product of interest. AE-1 cells secrete IgG into culture medium at levels of around 20-30 ug/ml, which is six to seven orders of magnitude higher than the levels of AChE measured in the culture supernatants of RMSC cells. Obviously it would be a lot easier to optimize the CIT system using cells which secrete (in suspension cultures) product at these high levels and then later refine the methods to increase sensitivity hopefully to the point where even AChE could be detected and the positive cells isolated. These experiments are now in progress; a polyclonal rabbit anti-mouse IgG fraction has proven to be the most effective means of capturing low concentrations of secreted mouse antibody. This raises the possibility that a polyclonal antibody preparation may be preferable for designing a SRBC conjugate for capturing AChE; as soon as purified human AChE is obtained, we will attempt to generate a rabbit antiserum to test this hypothesis. Figure 19 illustrates this approach using a hybridoma model system.

IV. The Cell Isolation Technique Using the FACS Approach

Fluorescence activated cell sorting offers the potential for quantitative selection of cells based on the amount of fluorescent signal generated by accumulated cell product within a bead. In the past 3 months, we have demonstrated that the agarose beads can be analyzed and sorted by our modified FACS III instrument. Figure 20 demonstrates some of the early evidence we had for the compatability of the FACS instrument with our bead technology. In model studies using glutaraldehyde fixed chick red blood cells or standardized fluorescent microspheres, the beads behave as if they are fairly transparent with respect to emitted fluorescence. The apparent intensity of the fluorescence signal is attenuated 2 to 4 fold. Light scatter by the beads, however, is extensive and somewhat unpredictable. As a result, when sorting beads containing fluorescent material, we have found it necessary to set gates and trigger on fluoresence only.

In initial sorting trials, fluorescent microspheres and fixed chick red blood cells were encapsulated in beads and mixed together. The mixed population was then gated to exclude most of the low level CRBC fluorescence and trigger on the microsphere fluorescence. The sorted beads were analyzed after one run and found to have been enriched 13x for microspheres.

The next experiments were designed to investigate the signals generated by human cells which were labeled with defined fluorescent reagents and encapsulated inside beads. The results of some early trials using Hut-78 (human T cell line) cells are shown in Figure 20. Viable cells were incubated with fluorescein diacetate which provides a consistent strong signal for analysis and sorting. As before, the fluorescent signal was partially attenuated by the beads but still strong enough for detection. In a preliminary sorting experiment, the labeled cells were encapsulated, analyzed and sorted as shown in Figure 20. The high fluorescent signal beads were collected and analyzed by light microscopy. The ratios of total cells vs. total beads was compared and shown to be 9.5 x higher after sorting, indicating about a ten fold enrichment for viable cells in one pass. We should be able to improve this enrichment factor by further diluting the beads in the sorting fluid to lessen the risk of false positives caused by empty beads travelling in the same droplets as the beaded cells. In actual CIT runs, the frequency of high intensity positive fluorescent signals will be low which should lessen the probability of false positives.

Many of the subsequent experiments have been designed to improve our analysis of the fluorescence signals and not actually sort the beads. For example, considerable effort has gone into overcoming the problem of inherent auto-fluorescence with many cell lines, particularly the AChE secreting lines which appear to have high concentrations of catecholamines as well. The problem has been greatly diminished by modifying our FACS and using the new phycobiliproteins, such as phycoerythrin (PE), to label cells and tag their secreted products. These dyes offer the great advantage of sharing the same excitation wavelength as fluorescein, but emitting at much higher wavelengths. Although this would be advantagous for two color sorting (Fig. 22), for

our immediate purposes, it permits us to ignore the strong autofluorescent background. In a recent preliminary experiment, PE-avidin was used to label biotinylated AE-1 antibody which had been bound to the surfaces of RMSC cells. A modest PE signal was detected after the autofluorescence of the cells had been eliminated from the analysis. This result is the first indication we've had that AChE might be detectable on the outer surfaces of RMSC cells, and lends credence to the notion that one day we may be able to sort these cells (without beads) for high secretors of AChE if there exists a strong correlation between rates of AChE secretion and phenotypic expression of the enzyme on the cell surface.

Immobilization of Antibodies or Ligands in Beads for Capturing Secreted Products such as AChE

As in the hemolytic approach for CIT, a product-capturing mechanism is essential to the FACS approach for isolating a bead containing a cell which secretes high levels of that product. We have investigated a variety of substrates which would be easy to derivitize with a specific antibody or ligand, easy to co-encapsulate within the bead and small enough to have high surface to volume ratios, but large enough to be retained within the bead for extended periods of time. Our first choice, which is still only in the initial phase of investigation, is to use colloidal gold particles. Other particles have been considered such as latex beads and polystyrene microspheres, but the gold is attractive because the particles are very small (we prepare them at around 50 nm in diameter), and they readily bind proteins under very mild conditions.

Preliminary experiments to measure the signal generated by FITC-proteins adsorbed to colloidal gold inside the beads indicate that the fluorescence is quite low. It is hoped that by increasing the density of particles in the bead, sufficient product (such as antibodies released by hybridomas, and, eventually, AChE released by RMSC cells) will accumulate to permit the introduction of a fluorescent antibody to tag those beads which had accumulated high levels of product during a fixed incubation period. It is assumed that, as in cell sorting, the sorting of beads with fluorescent signals will be quantitative enough to isolate the upper few percent of the population. Experiments of this type are in progress at the time of this writing using model systems such as hybridomas secreting antibodies to optimize conditions for sorting (see Figure 23).

One possiblity for increasing the signal generated by bound AChE inside a bead would be to take advantage of known fluorogenic substrates to impart a strong fluorescent signal in those beads which had accumulated the most enzyme. These and other possible methods are being explored in an effort to overcome the obvious problem of very low levels of AChE secreted by any of the cell lines investigated to date. The sensitivity required of a CIT scheme as described here would have to be a million times greater for AChE secreted by RMSC cells than it would for antibodies secreted by our model hybridomas.

V. DNA Transfection Experiments in Progress

A major portion of the research effort is now directed toward the transfection of genomic DNA into recipient human cell lines to enhance the expression and secretion of AChE. Normal human embryonic muscle cells (obtained from Dr. Helen Blau at Stanford University) are currently being used as a source of high molecular weight DNA. These cells are known to be high secretors of the enzyme. The high molecular weight DNA will be restricted such that the gene for dihydrofolate reductase (dhfr) can be ligated to 60 kb fragments. These fragments will then be transferred into recipient cells using either the conventional calcium phosphate precipitation methods or possibly using electroporation. If the AChE gene is transferred successfully, we should be able to isolate a subpopulation of high secretor cells by virtue of their co-transfection with the dhfr gene. Hence, we would then select for cells secreting high levels of AChE. This AChE selection would be based on the CIT methods described above, using immobilized AE-1 antibody to the enzyme or specific irreversible inhibitors to capture enzyme secreted by the cells and fluoresceinated antibody to then label those beads which have accumulated higher amounts of AChE.

At the time of this writing, the basic methodologies required for this approach are being worked out. Preliminary transfection experiments using model systems have confirmed our capabilities for transfering genomic DNA into recipient cell lines.

VI. Comment on AChE Secretion Rates

Lastly, we would like to once again express our concern over the very low levels of AChE secreted by the various human cell lines and even the normal embryonic muscle cells. Although the enzymatic activity is clearly detectable by our RISA and radiometric assays, the absolute AChE protein levels are quite low by our calculations. The very high substrate turnover rate for this enzyme (circa 10,000 molecules of ACh per monomer per second) is deceiving since the secreted AChE levels in cell culture media (several munits/ml) really corresponds to only tens of picograms/ml of AChE protein. Hence, even a 100-fold increase in AChE secretion rates in any of the cell lines we have studied would probably result in only nanogram/ml production levels of the enzyme. Nonetheless, if we can successfully develop the basic selection technology, we will be in a strong position to select for genetically engineered cells which are true high secretors of the enzyme.

One possible way around this problem of low detection signals for AChE is to take advantage of the enzyme's unusually high specific activity and utilize a fluorogenic substrate to label beads which had accumulated quantitatively higher amounts of AChE from encapsulated cells. Theoretically, if we were successful in immobilizing the enzyme released by the high secretors in the beads, then we should be able to introduce a dye which would generate a strong fluorescent signal by reacting with the AChE hydrolysis product. If the level of fluorescence was great enough, and the activated dye did not quickly diffuse out of the beads, we might be able to sort the "positive" beads on our FACS instrument. Such fluorogenic dyes exist; Pavari et al. (1983)(4) used N-(4-(7-diethylamino-4-methylcoumarin-3-yl) phenyl)maleimide (CPM) (Molecular Probes, Inc.) to develop a sensitive microfluorometric assay for AChE. The dye becomes highly fluorescent when it reacts with thiocholine generated by the enzymatic hydrolysis of the substrate analog, acetyl-thiocholine. We are currently investigating the possiblity of using such dyes to greatly improve our detection system for the Cell Isolation Technique using the FACS approach.

Conclusions

The fundamental methods for the Cell Isolation Technique are being developed to provide a basic research tool which should enable us to select for cells maximally secreting human acetylcholinesterase from among natural or genetically engineered cells producing the enzyme. The combination of the bead technology with our FACS instrumentation looks most promising as a means of isolating high secretors of AChE. In the meantime, considerable effort is being invested in model systems such as the isolation of high secretors of specific antibodies from hybridomas to establish working protocols. Transfection experiments and immortalization experiments are just getting underway, but their success at deriving a high secretor of AChE will also depend heavily on the effective application of the basic cell isolation technique described in this report.

LITERATURE CITED

1. Johnson, C.D. and Russell, R.L. (1975)
   Anal. Biochem. 64, 229-238

2. Rotundo, R.L. and Fambrough, D.M. (1979)
   J. Biol. Chem. 254, 4790-4799.

3. Karnovsky, M.J. and Roots, L. (1964)
   J. Histochem. Cytochem. 12, 219-221.

4. Pavari, R., Pecht, I. and Soreq, H. (1983),
   Anal. Biochem. 133, 450-456.

5. Soreq, H., Pavari, R. and Silman, I. (1982)
   Proc. Natl. Acad. Sci. USA 79, 830-834.

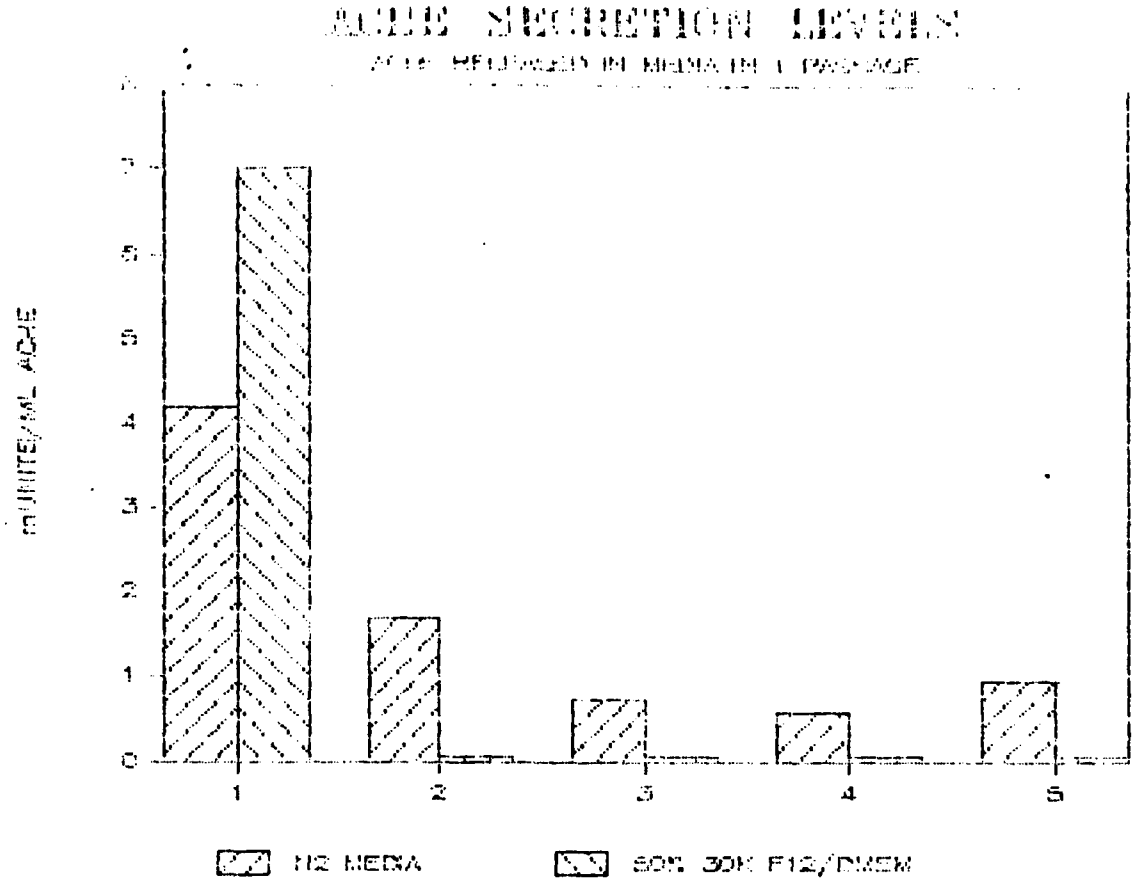
6. Silver, A. (1974) The Biology of Cholinesterases,
   Elsevier/North-HOlland, New York/Amsterdam, pp. 59-67.

7. Fambrough, D.M., Engel, A.G., and Rosenberry, T.L.
   (1982) Proc. Natl. Acad. Sci. USA 79, 1078-1082.

8. Schimke, R.T. (1982), Gene Amplification,
   Cold Spring Harbor Laboratory, N.Y.

9. de Roos, A.M. (1959) Rec. Trav. Chim. 78, 145.

10. Jou, Y.H., Mazzaferro, P.K., Mayers, G.L. and
    Bankert, R.R. (1983) in Methods in Enzymology,
    Vol. 92, Academic Press, pp. 257-276.

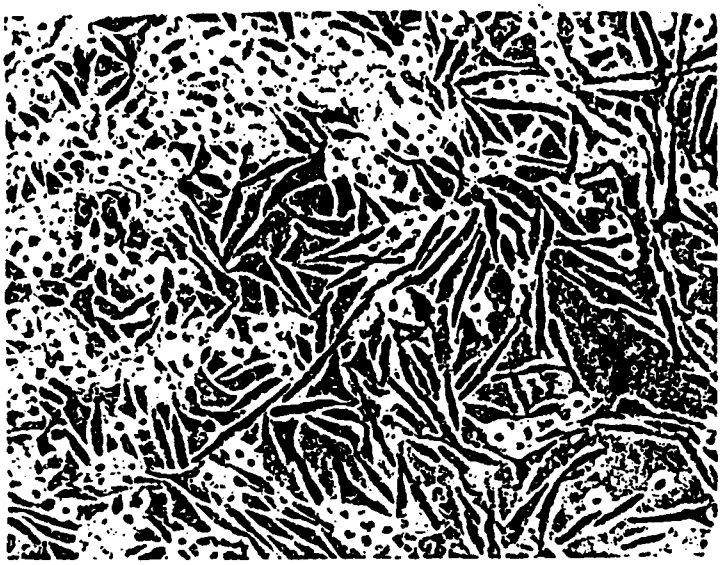


LEVELS OF HUMAN ACETYLCHOLINESTERASE SECRETED

BY VARIOUS ESTABLISHED CELL LINES

Among the various cell lines screened for the secretion of human AChE, the A-204 rhabdomyosarcoma muscle lined proved to be the most consistent high secretor of enzyme. To avoid high background levels of esterase activities in serum, cells were adapted to culture for limited periods of time in N2 defined medium or medium supplemented with a low molecular weight filtrate of fresh bovine lymph. Cell culture supernatants were then screened at confluence for AChE production using the radiometric assay described in Figures 3-4. Human cell lines tested here were:
1) A-204 rhabdomyosarcoma; 2) SK-N-MC neuroblastoma;
3) SK-N-SH neuroblastoma; 4) U-87-MG astrocytoma;
5) U-138-MG glioblastoma.

FIGURE 1



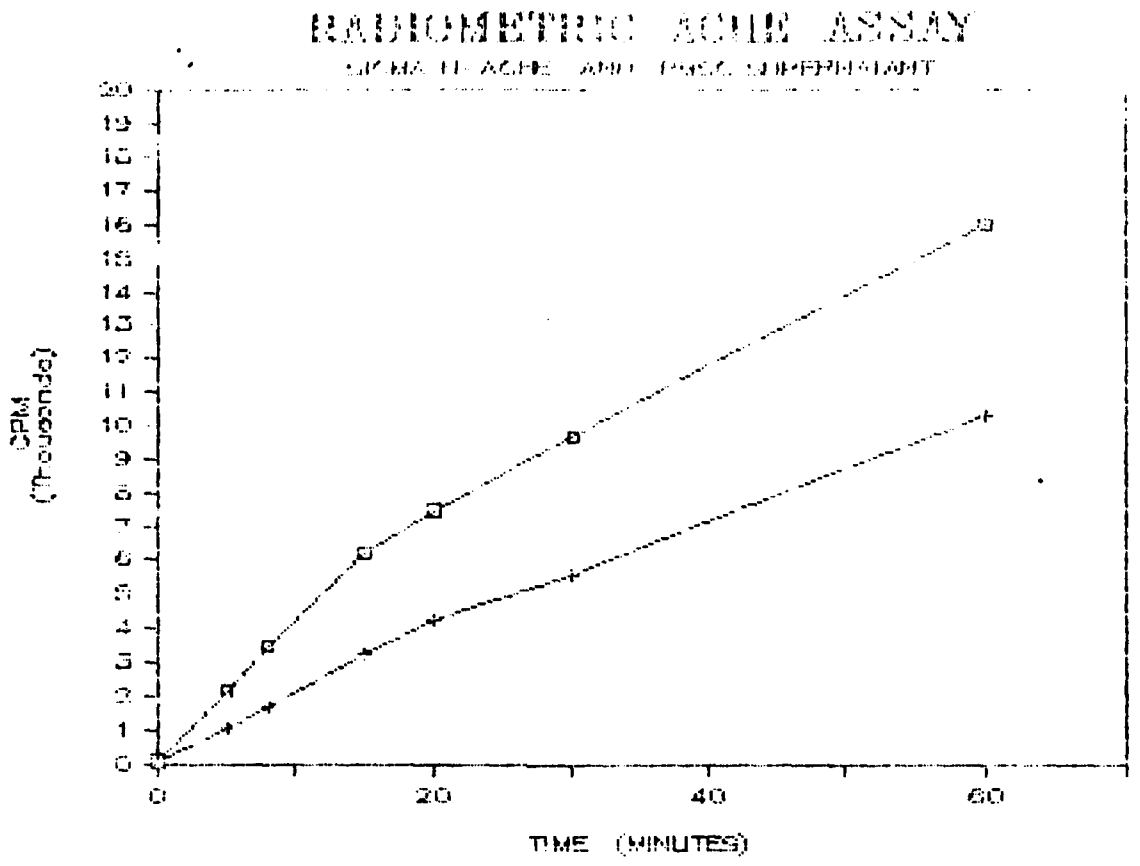
A-204 RHABDOMYOSARCOMA MUSCLE CELL LINE IN CULTURE

RMSC line photographed as cells approached confluency in 15% fetal bovine serum supplemented Coons-F12:DMEM (1:1).

These adherent cells require attachment factors present in serum to proliferate and remain attached to their substrate over a long period of time. In order to avoid high backgrounds of AChE found in serum supplemented medium, cells are cultured in alternative serum-free media after they reach confluency (1 x 10^5 cells/cm^2) After 24 hours, the cells are rinsed in PBS and maintained for 48-72 hours in the serum-free media to collect secreted AChE. AChE levels typically reach 4-7 munits/ml after 2 days.

Doubling time in 10-15% FBS medium is approximately 40 hr; cells will continue to divide through one more cycle after switching to defined medium or medium supplemented with the low molecular weight filtrate of fresh bovine lymph. After 48 hours they begin to detach from their substrate and cease to divide.

FIGURE 2

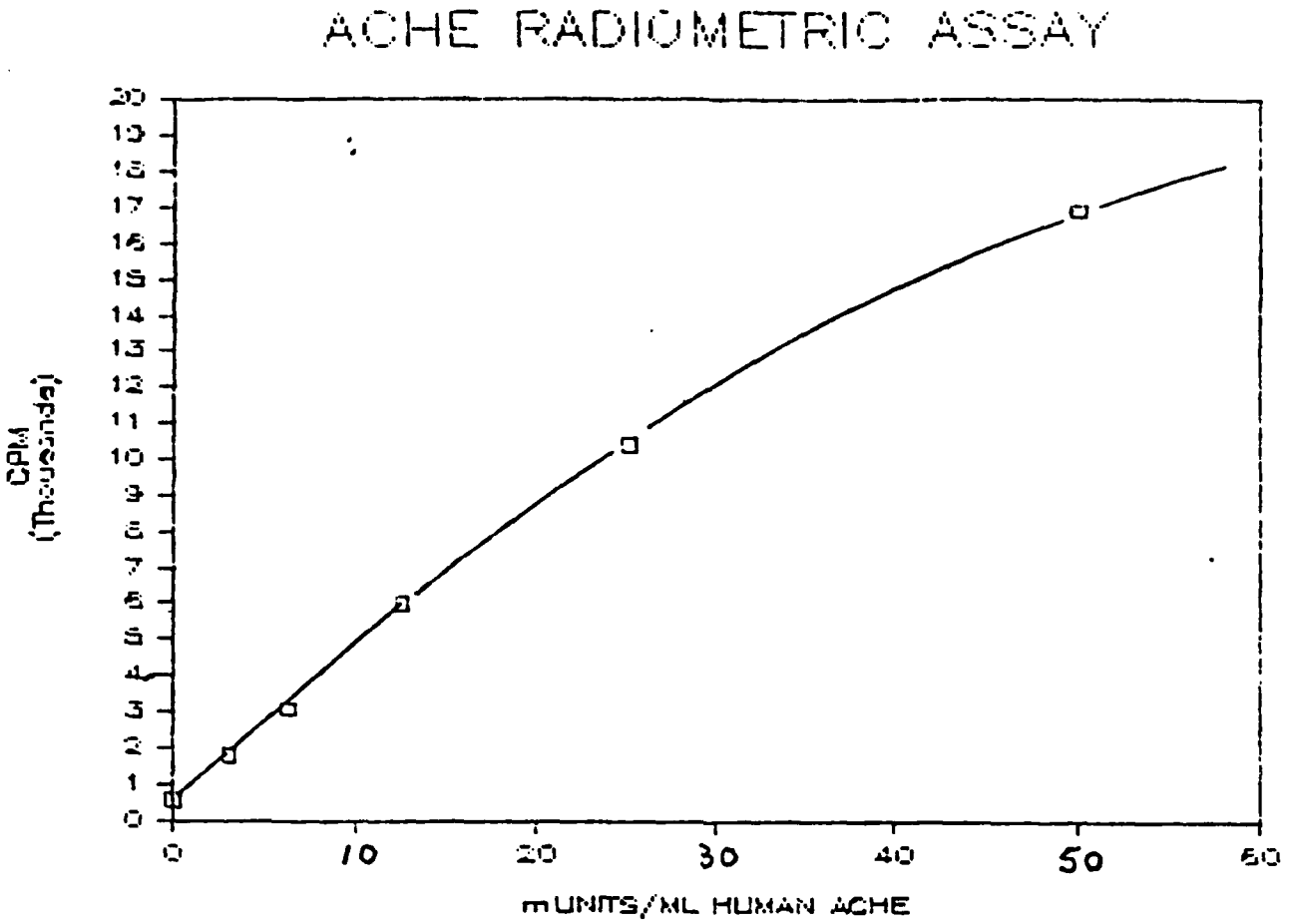


RADIOMETRIC ASSAY ADAPTED FOR SCREENING HUMAN AChE

RATE VS. TIME

Radiometric assay for AChE modified from Rotundo and Fambrough (1979) to increase sensitivity for detecting AChE in cell culture supernatants. Human erythrocyte AChE (upper line) at 10 munits/ml vs. test supernatant from rhabdomyosarcoma cell culture supernatant (lower line). Reaction performed in 0.1 ml final volume as described in Figure 4 for standard curve.

FIGURE 3

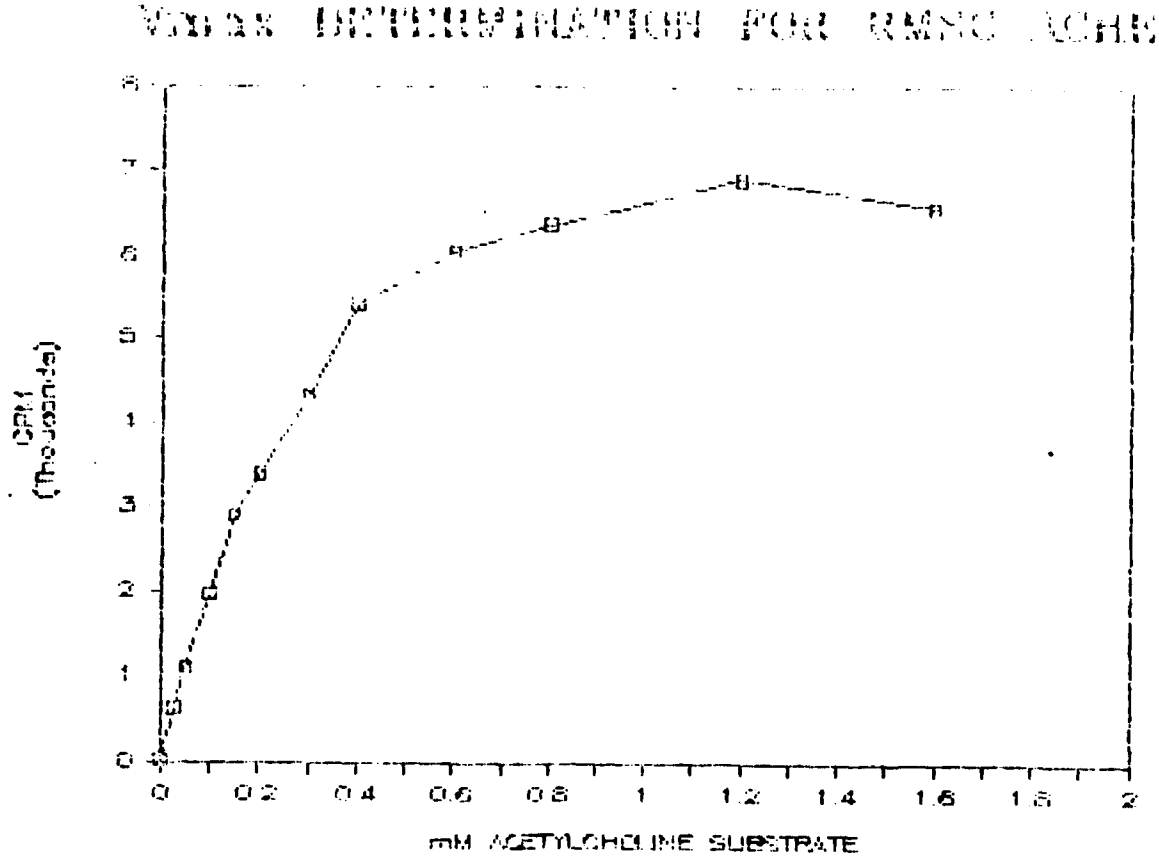


STANDARD CURVE FOR HUMAN AChE RADIOMETRIC ASSAY

RATE VS. [ENZYME]

Radiometric assay modified from Rotundo and Fambrough (1979) to increase sensitivity for detecting human AChE in cell culture supernatants. Standard curve using crude human erythrocyte AChE. 14-C-ACh at 4.8 mCi/mMole; final [ACh] = 0.1 mM in reaction medium consisting of 0.1 M sodium phosphate, 0.15 M NaCl, 0.25 mM EDTA, 1% Triton X-100 and 0.02% sodium azide, final volume = 100 ul. 2 hr reaction at 37 Deg.C. Reaction stopped with addition of 2 ml 50 mM glycine-HCl, 1 M NaCl pH 2.5 and combined with a 5 ml scintillation cocktail designed to extract the protonated form of released 14-C-acetate. The mixture was centrifuged and the upper 4 ml (80%) solvent layer removed for counting in a Beckman scintillation counter. Spontaneous 14-C-ACh hydrolysis accounted for most of the background counts (500 cpm).

FIGURE 4

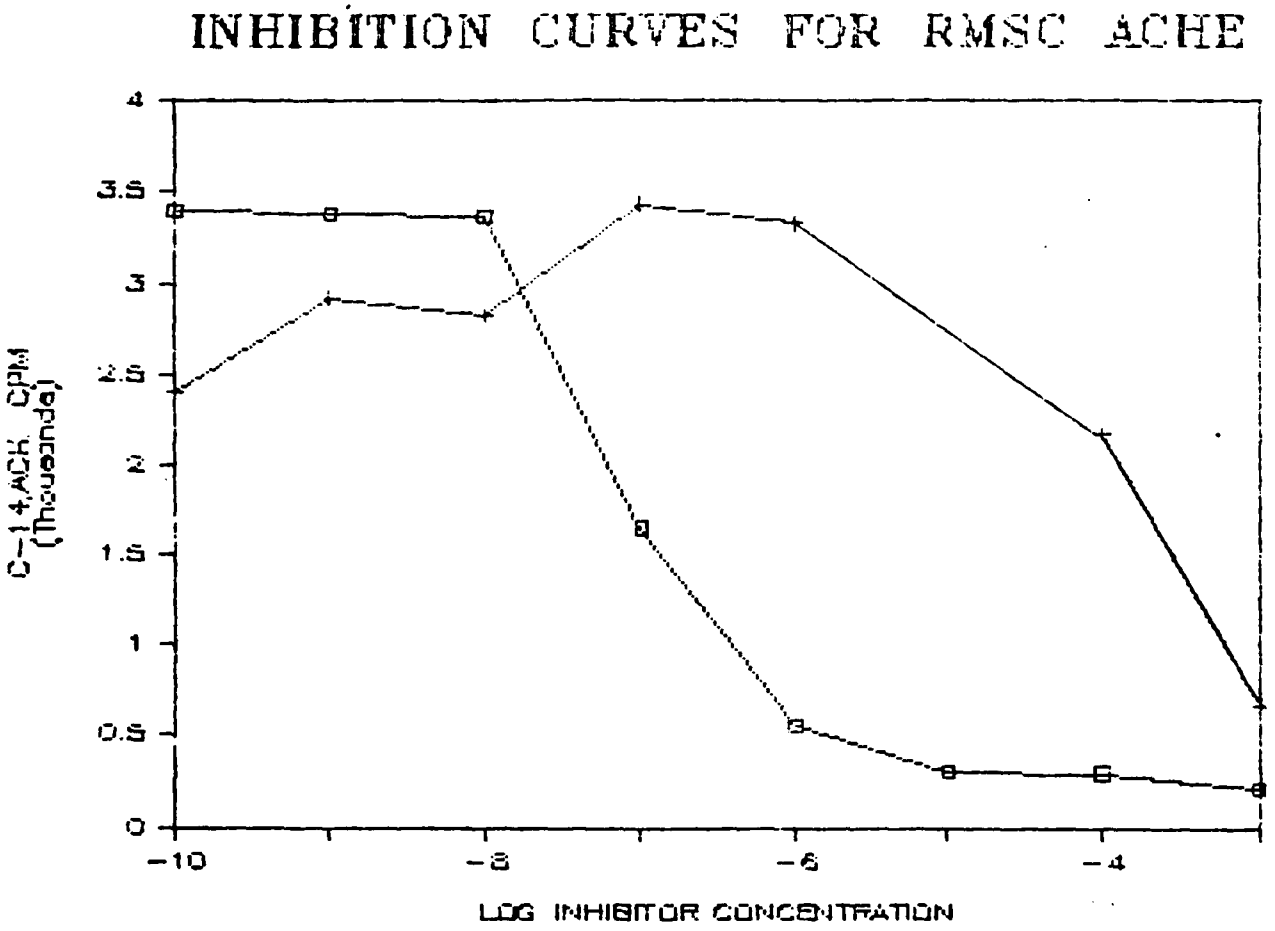


RADIOMETRIC ASSAY FOR HUMAN ACHE SECRETED FROM RMSC CELLS

RATE VS. [SUBSTRATE]

Using the assay described in the previous figures, the concentration of ACh substrate required to give maximal rates of hydrolysis (Vmax) was found to be 1.2 mM. The AChE activity from RMSC cell supernatants (shown here) began to exhibit characteristic substrate inhibition at concentrations of ACh above 1.2 mM.

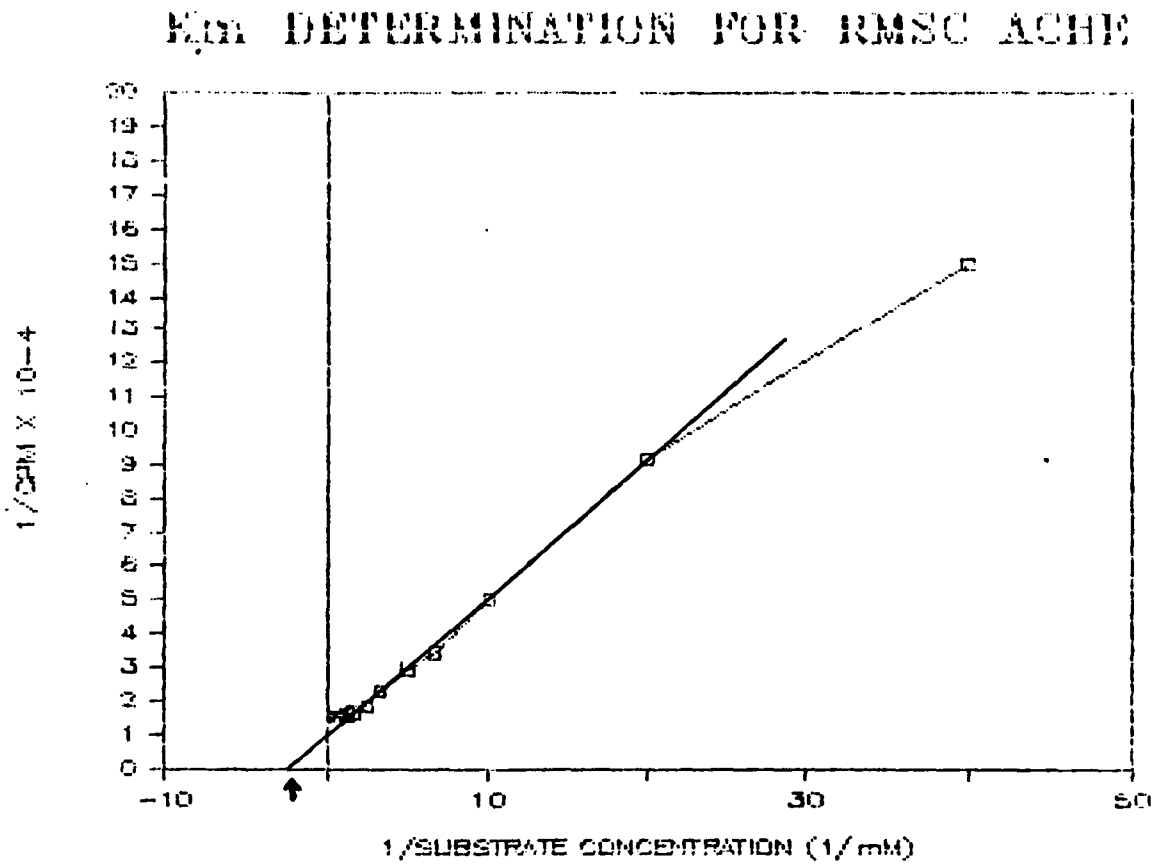
FIGURE 5



INHIBITION CURVES FOR HUMAN AChE SECRETED BY RMSC CELLS

Using the radiometric assay described in the previous figures, RMSC supernatant AChE activity was tested for inhibition with the indicated concentrations of BW284 C51, a true AChE inhibitor ([]) and Iso-OMPA, a non-specific esterase inhibitor (x). The concentration required for 50% inhibition with BW284 C51 was 10^-7 molar, whereas for the non-specific esterase inhibitor, the apparent Ki was around 10^-4 molar. Both values are in close agreement with those reported in the literature for AChE isolated from other sources (see text).

FIGURE 7

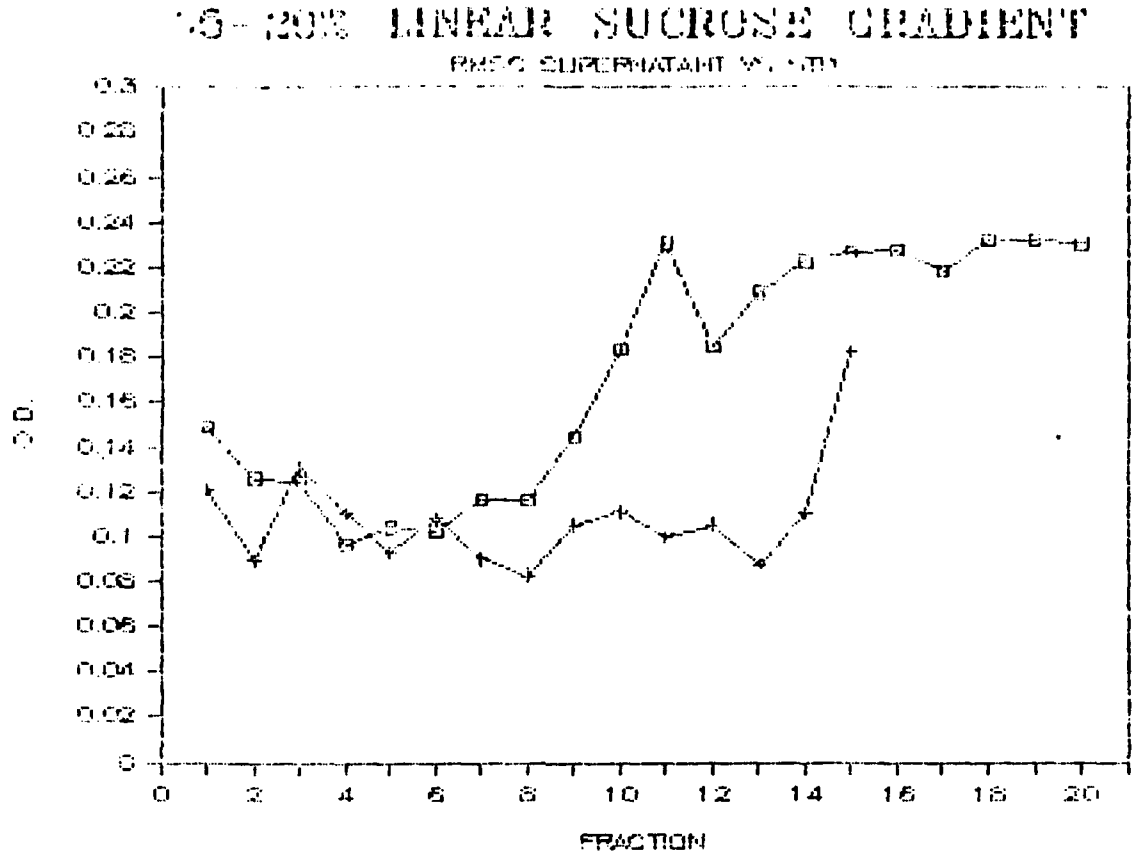


RADIOMETRIC ASSAY FOR HUMAN AChE SECRETED BY RMSC CELLS

LINEWEAVER-BURKE PLOT

The Michaelis constant, Km, for the AChE detected in the supernatants of RMSC cell cultures was calculated to be 0.4 mM by analysis of this data generated with the radiometric assay. This value is in close agreement with values obtained for AChE isolated from rat neuroblastoma cultures and chick skeletal muscle cultures.

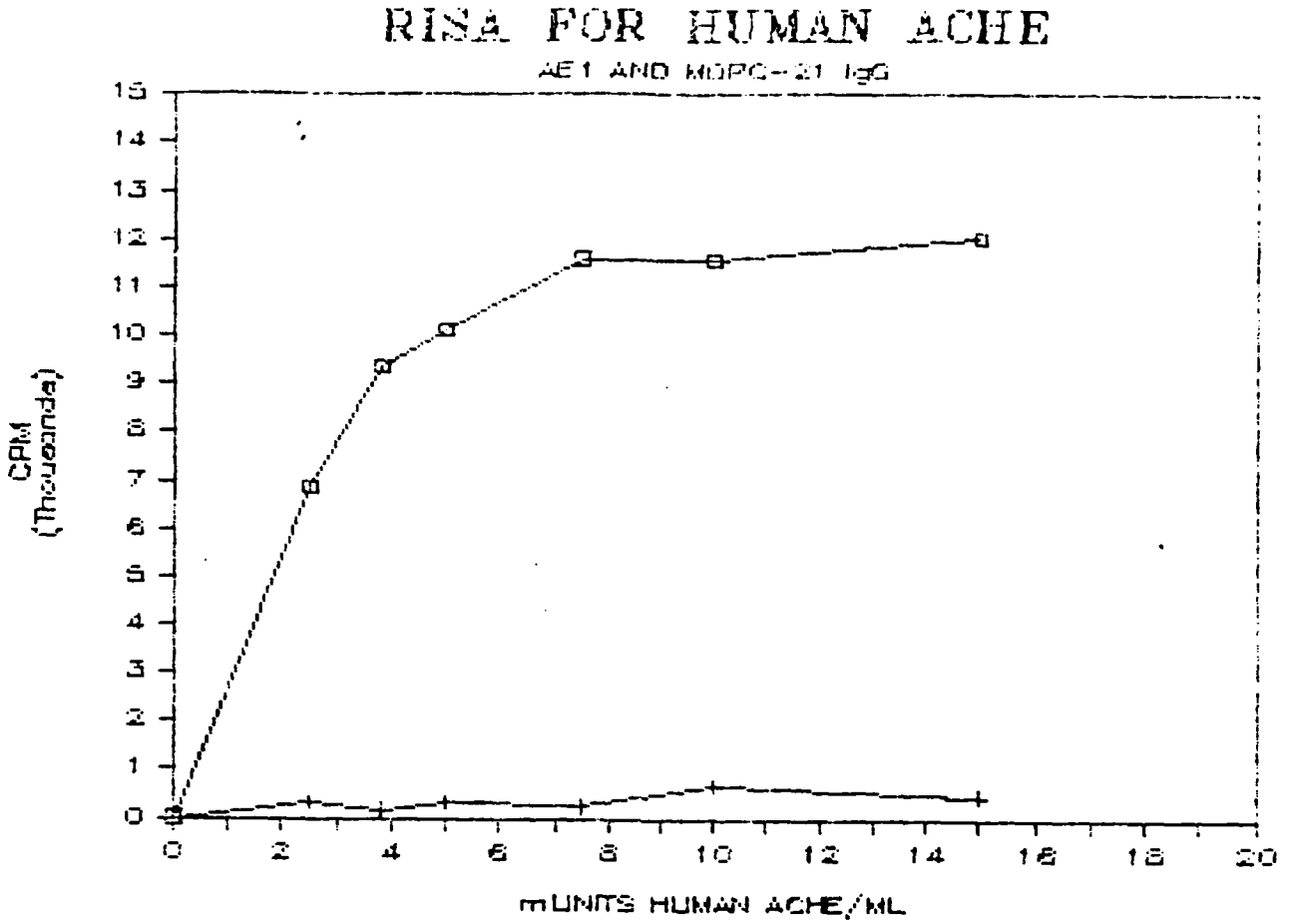
FIGURE 6



SECRETED RMSC AChE ANALYZED ON A SUCROSE GRADIENT

Concentrated supernatants from RMSC cells were run on a 5-20% linear sucrose gradient in PBS for 13 hr at 37,000 rpm. Fractions were analyzed by a colorimetric microtiter assay developed from a histochemical staining procedure described by Karnovsky and Roots (1964). Upper trace ([]) shows AChE activity detected for the RMSC supernatant sample. Lower trace (+) shows AChE activity from Triton X-100 solubolized human erythrocyte enzyme. The prominent peak for RMSC AChE has a sedimentation coefficient value of approximately 10s.

FIGURE 8



RADIOMETRIC IMMUNOADSORBANT SOLID-PHASE ASSAY (RISA)

A novel assay was developed to employ both enzymatic and immunological criteria for analyzing human AChE released by cells in culture. Purified monoclonal antibody to human AChE (AE-1) was adsorbed to wells in a 96-well microtiter plate (flexible PVC, Dynatech). After washing and blocking the remaining non-specific sites with 0.5% BSA/PBS, test samples of RMSC supernatants or dilutions of human erythrocyte AChE were incubated in the wells for 2 hr at room temperature. After washing away unbound enzyme, the wells were incubated with 14-C-AChE in a reaction medium identical to our routine AChE radiometric assay described in previous figures. Using an overnight incubation of the radioactive substrate enhanced the sensitivity of the assay without appreciably increasing the background.

The above data show the binding of RMSC AChE to plates which had been incubated with AE-1 vs. MOPC-21 as an negative antibody control.

FIGURE 9

SCHEMATIC DIAGRAM FOR MCT SYSTEM

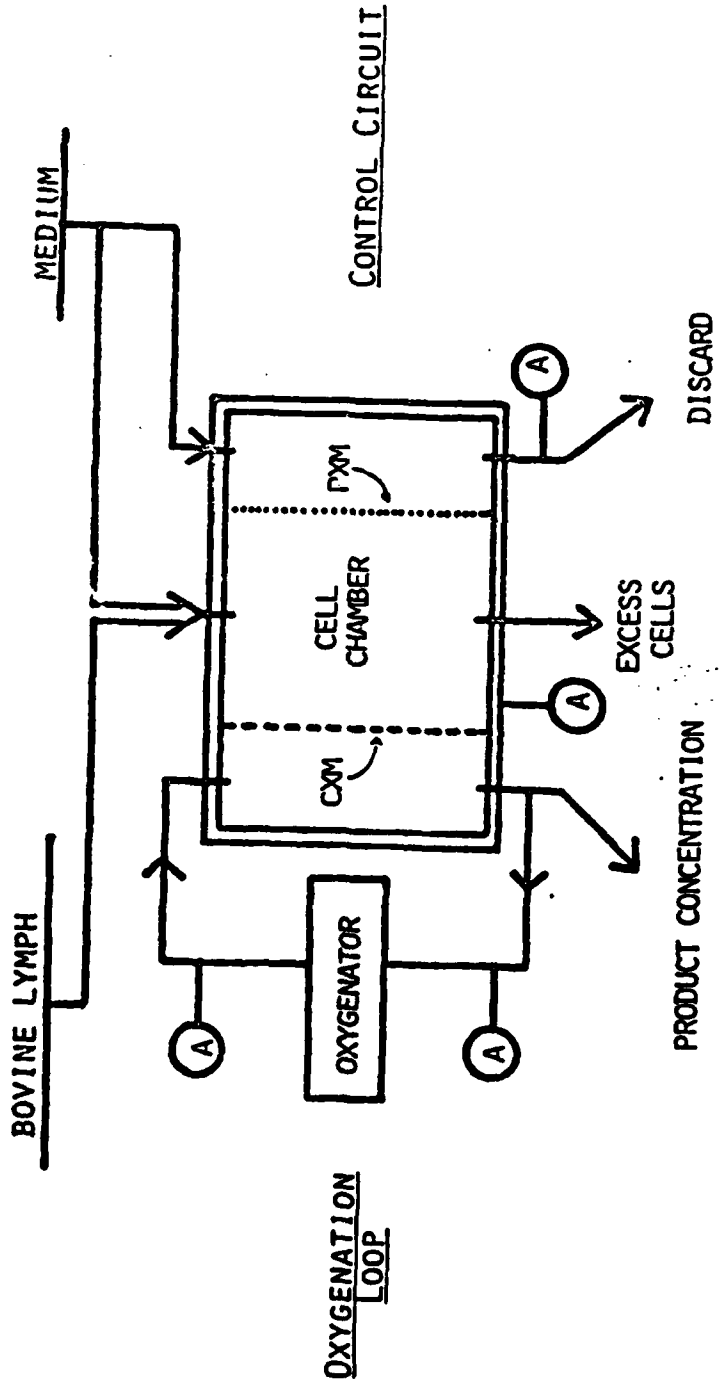


FIGURE 10

Schematic Diagram for MCT System

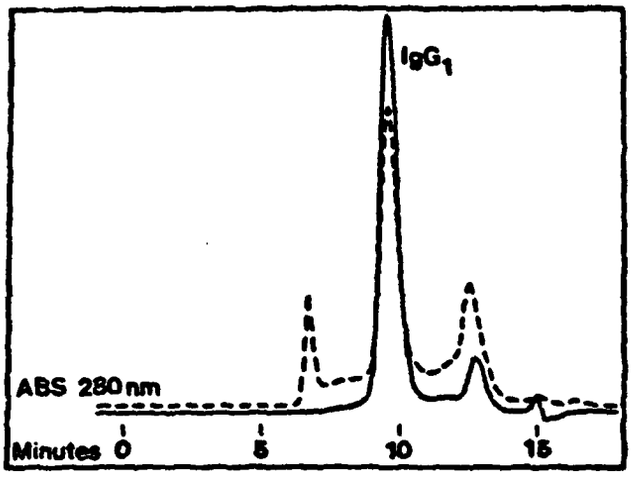
Each hollow fiber culture unit is divided into three functional compartments by two semipermeable membranes: the CXM (cell exclusion membrane)—a microporous membrane that prevents cell migration; PXM (product exclusion membrane)—an ultrafiltration membrane that retains the product (MAb). Within the enclosed volume surrounding the hollow fibers is the cell growth chamber.

Media is perfused directly into the chamber and is withdrawn along with cell-free secreted product across the CXM. Diffusion across the PXM removes lactic acid and other low molecular weight metabolites and replenishes vitamins, cofactors, and amino acids which have been depleted. The PXM also isolates cells and cell products from the rest of the system.

Both the PXM and the CXM not only permit cells grown in suspension culture to be run continuously in a pseudochemostat mode, but they also enable on-line removal of cell-free secreted product. Access to the growth chamber enables the periodic removal and sampling of cells through sampling ports "A". This particular research project, generating MAb for in-house use, employed a small-scale research culture unit, not an industrial scale unit.

FIGURE 10

Preparative High Performance Hydroxylapatite Monoclonal Antibody Purification



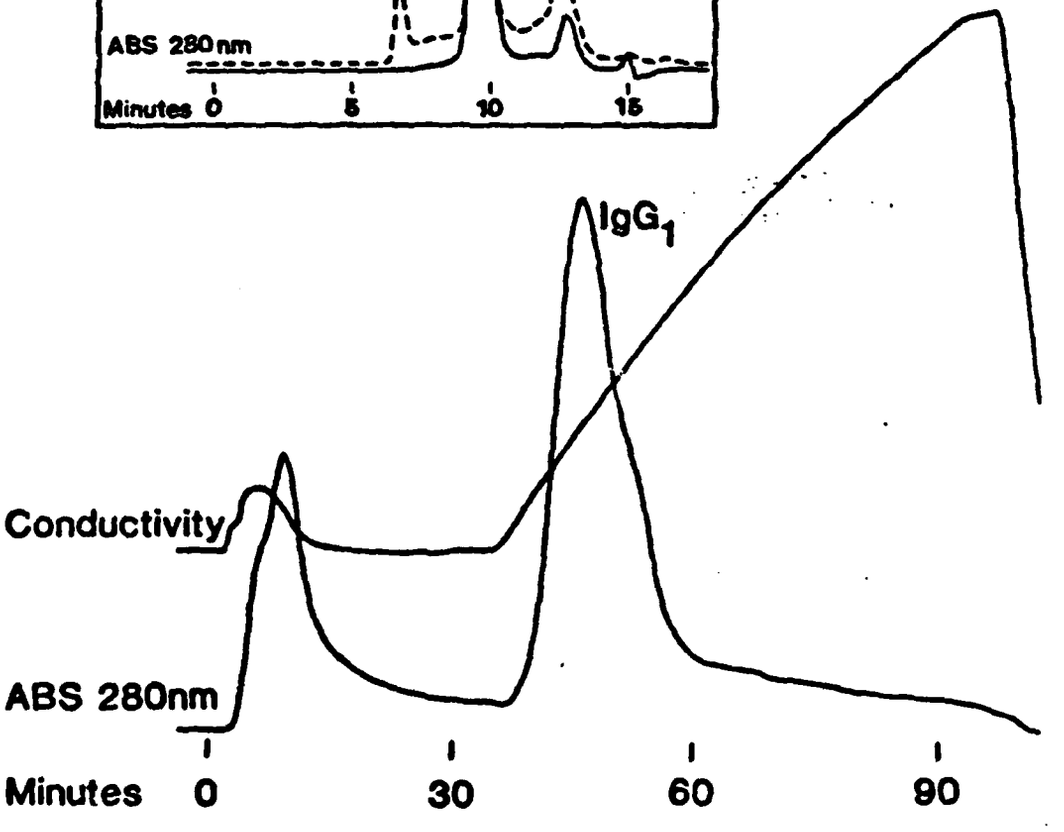


FIGURE 11

PREPARATIVE HIGH PERFORMANCE
HYDROXYLAPATITE MONOCLONAL ANTIBODY
PURIFICATION

System:	Bio-Rad Laboratories MAPS/Preparative HPHT (50 × 21/5mm)
Sample:	AE-1 Mouse monoclonal IgG_1/lymph supplemented culture media
Preparation:	Ultrafilter (10L to 1L); AS precipitate; ultra-centrifuge; diafilter vs 15% B (≈ 50mM); 0.2μm filter
Injection:	33 ml
Flow rate	5.0 ml/min
Gradient:	Isocratic at 15% B/30 min; 15-100% B/60 min

The ability of HPHT to purify preparative amounts of monoclonal antibody produced in tissue culture is illustrated using an IgG_1 directed against human acetylocholine esterase. 10 liters of a defined culture media containing the antibody was concentrated to ≈ 33 ml (≥700 mg protein) and loaded onto preparative HPHT at 5 ml/min. Following injection, isocratic elution at a slightly elevated ionic strength allowed the contaminants to pass unbound through the matrix, increasing the capacity for antibody and yielding essentially baseline resolution when the IgG was eluted by the linear gradient that followed. 98% of the loaded protein was recovered, and the IgG peak contained between 350-400 mg of antibody, depending on the assay (absorbance or IgG-specific ELISA). Size exclusion chromatography (see insert) shows the difference between the load sample (dashed line) and the HPHT purified material (solid line), with an estimated purity of >90%.

FIGURE 11

SDS-Polyacrylamide gel electrophoresis (under reducing conditions)

Lane 1:	Molecular weight standards: transferrin, bovine serum albumin, mouse IgG_1 heavy and light chains (MOPC-21)
Lane 2:	Pooled raw culture medium supernatants; note prominent transferrin and immunoglobulin bands
Lane 3:	Ammonium sulfate precipitate of crude culture medium (lane 2), now enriched for immunoglobulin
Lanes 4, 5, 6:	Unbound proteins which passed through hydroxylapatite column, including additional light chain material
Lane 7:	Leading edge, IgG_1 peak
Lane 8:	Trailing edge, IgG_1
Lanes 9, 10:	Remaining proteins eluted at high phosphate concentration

Nearly 400 mg of antibody were recovered in the single IgG_1 peak

FIGURE 12

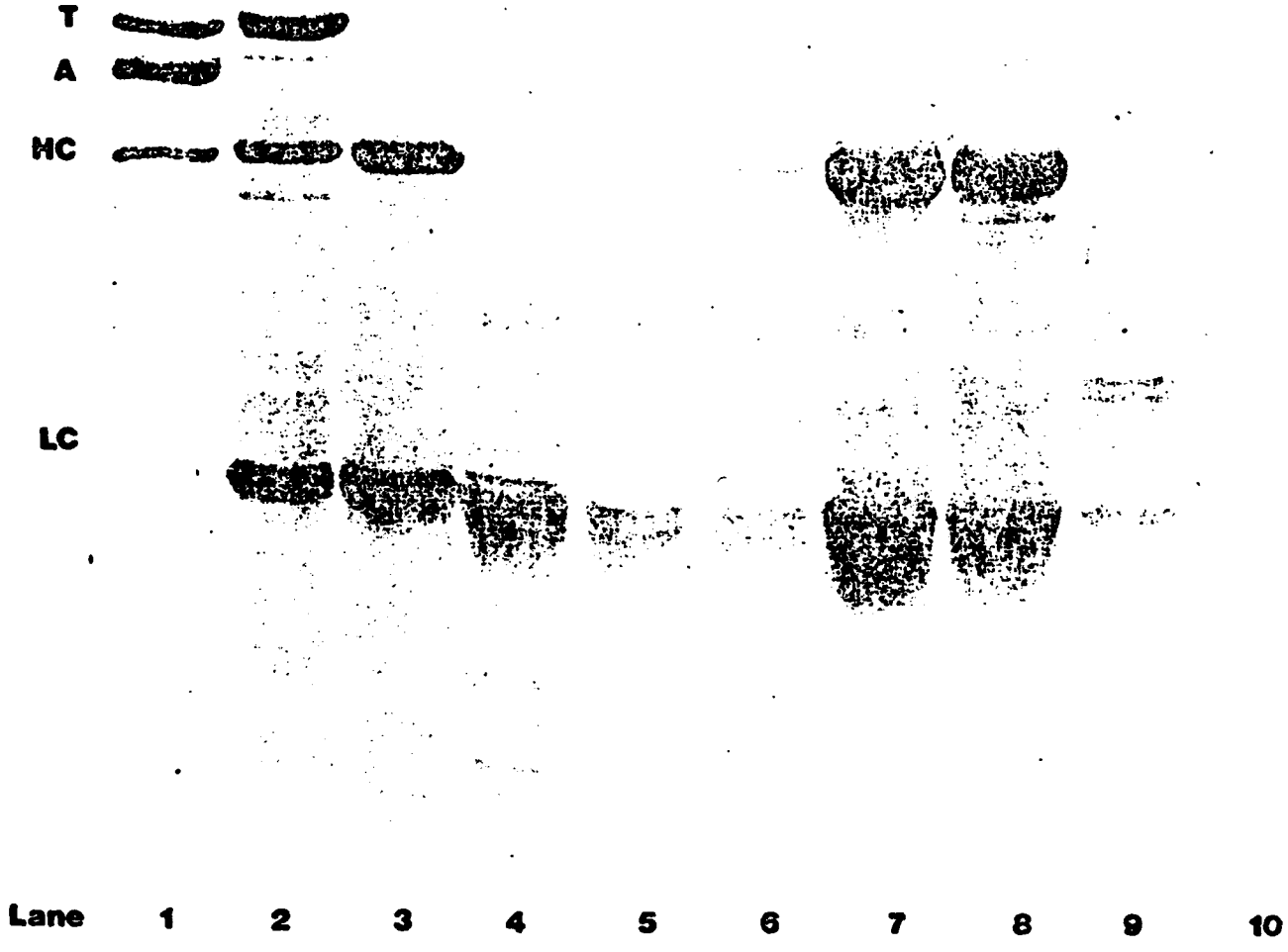
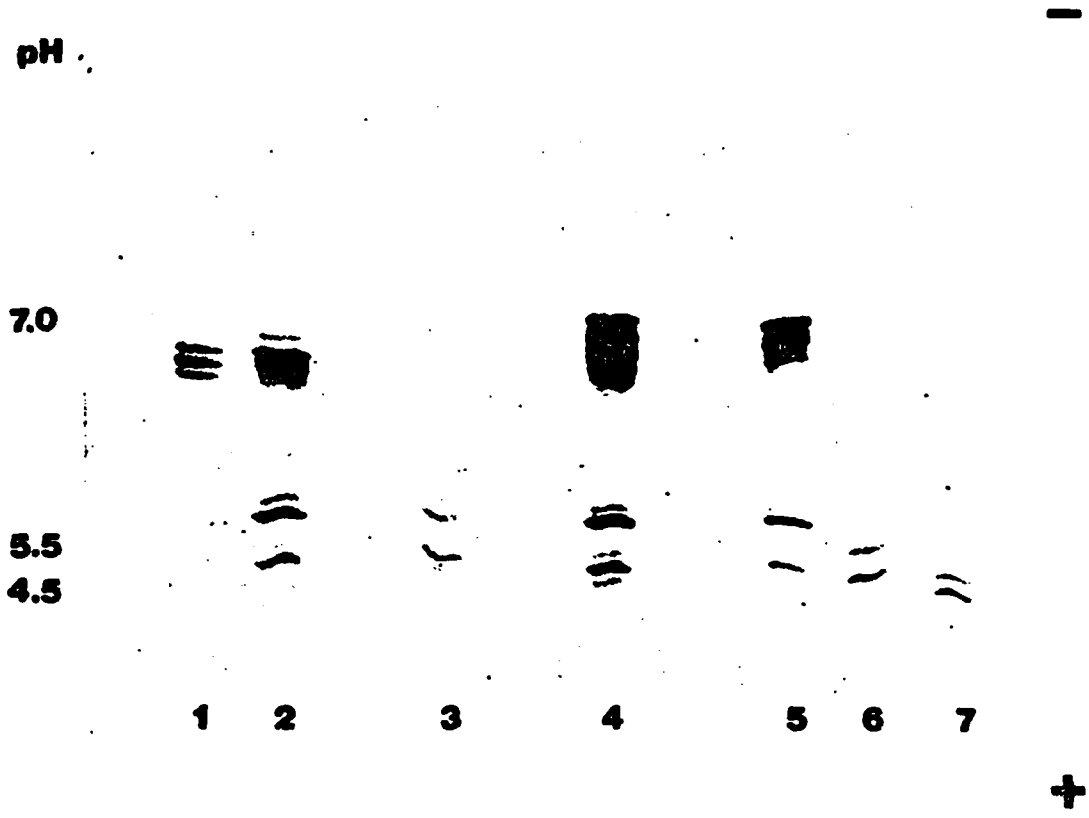


FIGURE 12



Isoelectric Focusing Gel
(LKB Ampholine PAGplate, pH 3.5-9.5)

Lane 1: Mouse IgG Standard (MOPC-21)

Lane 2: AE-1, ammonium sulfate precipitate

Lane 3: AE-1, concentrated raw culture medium

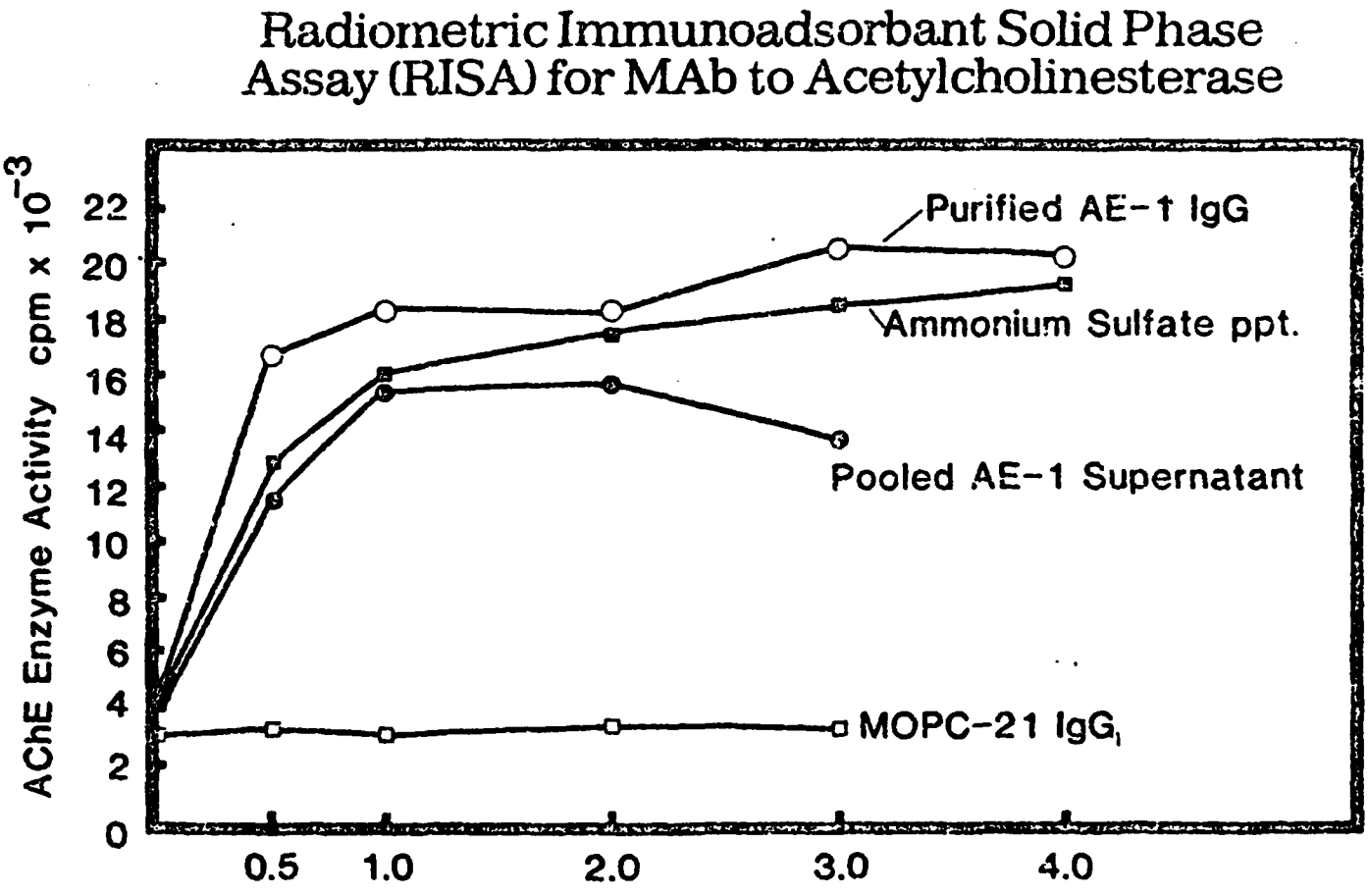
Lane 4: AE-1, HPHT, IgG_1 peak, leading edge

Lane 5: AE-1 HPHT IgG_1 peak, trailing edge

Lane 6: Mouse Lamda chain standard (RPC-20)

Lane 7: Mouse Kappa chain standard (MOPC-41)

FIGURE 13



The RISA assay, described in Figure 9, was used to evaluate the abilities of different fractions of the AE-1 monoclonal antibody to capture human AChE present in the incubation media. Hydrolysis rates were determined from the levels of 14-C-Acetate released into the reaction medium by the captured enzyme.

FIGURE 14

CELL ISOLATION TECHNIQUE - HEMOLYTIC APPROACH

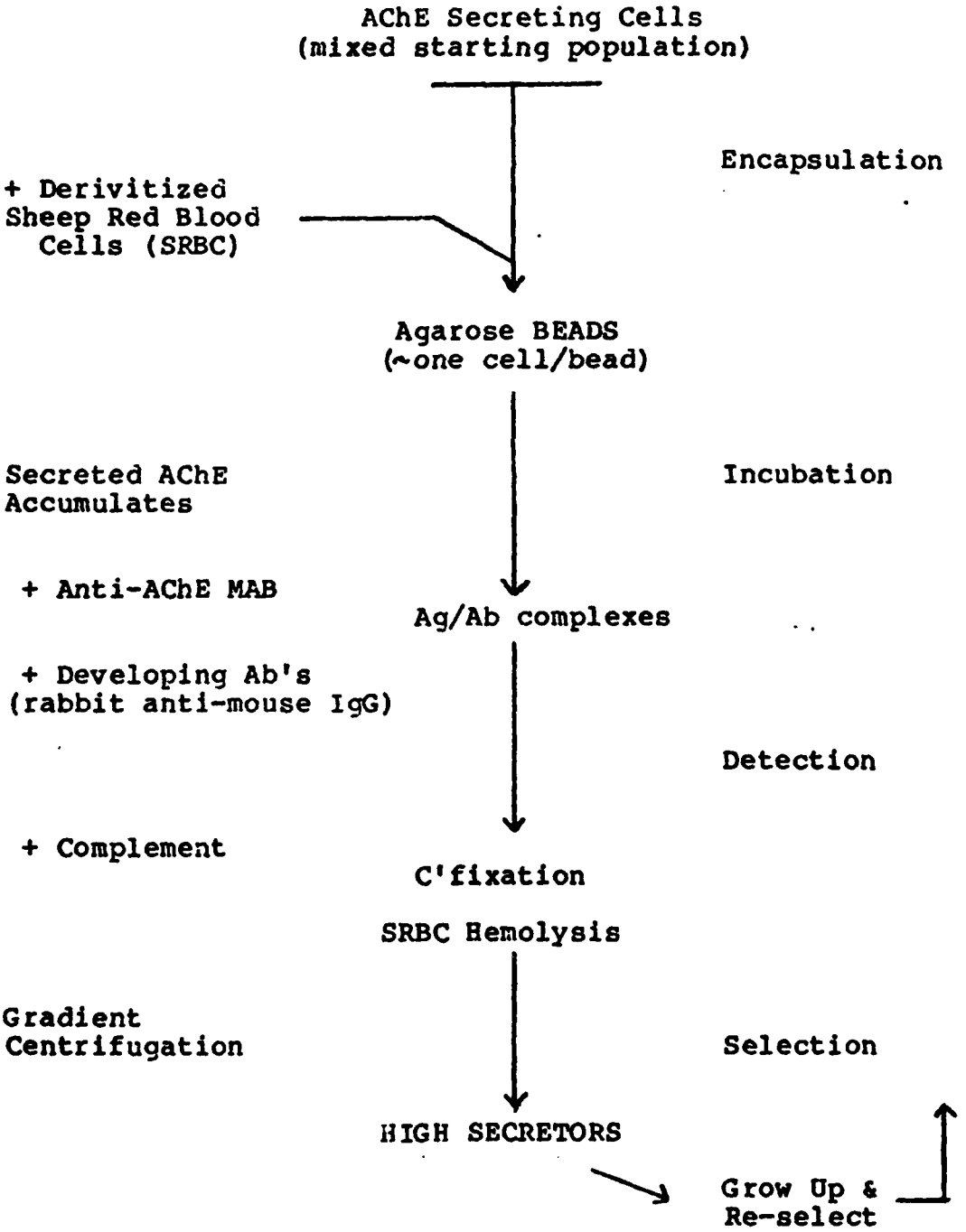
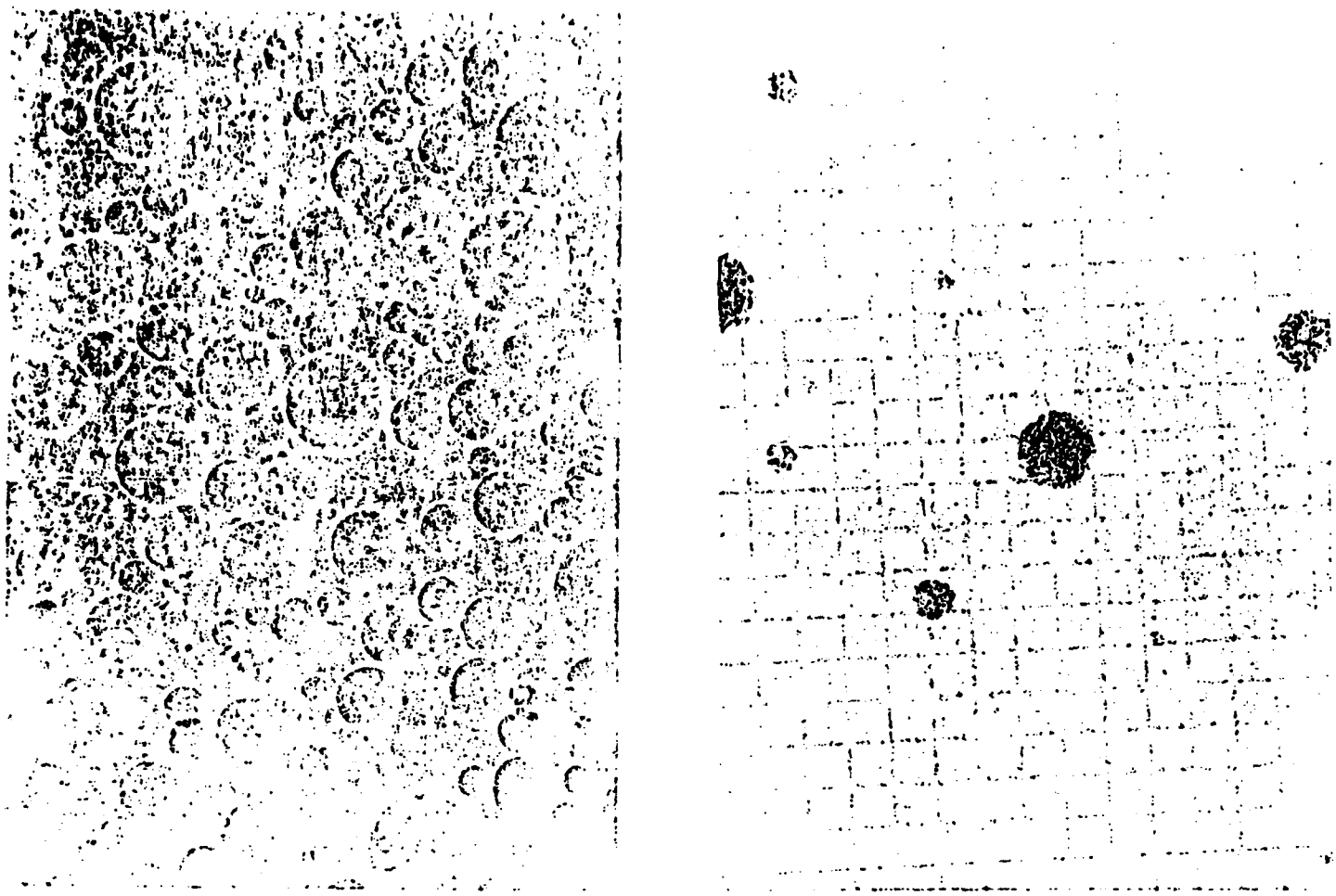


FIGURE 15



A

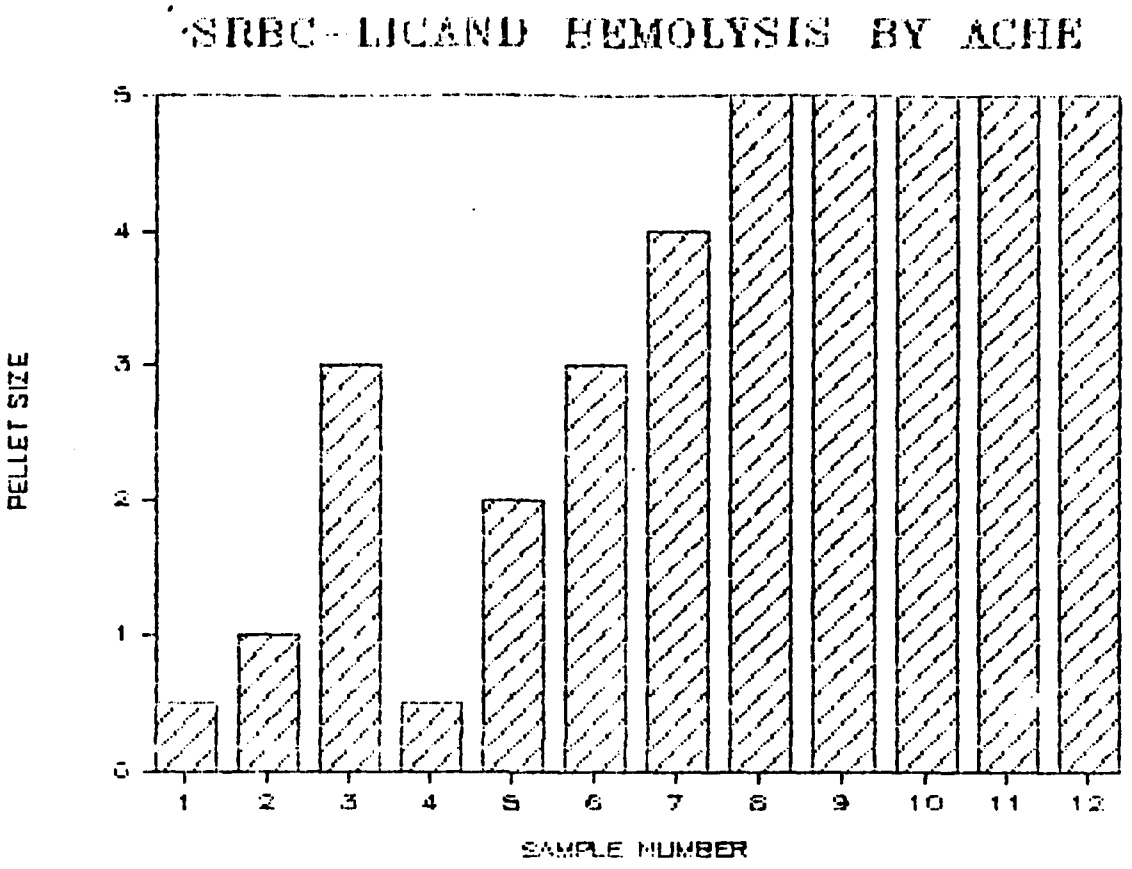
Hybridoma cells

B

Derivitized Sheep
Red Blood Cells

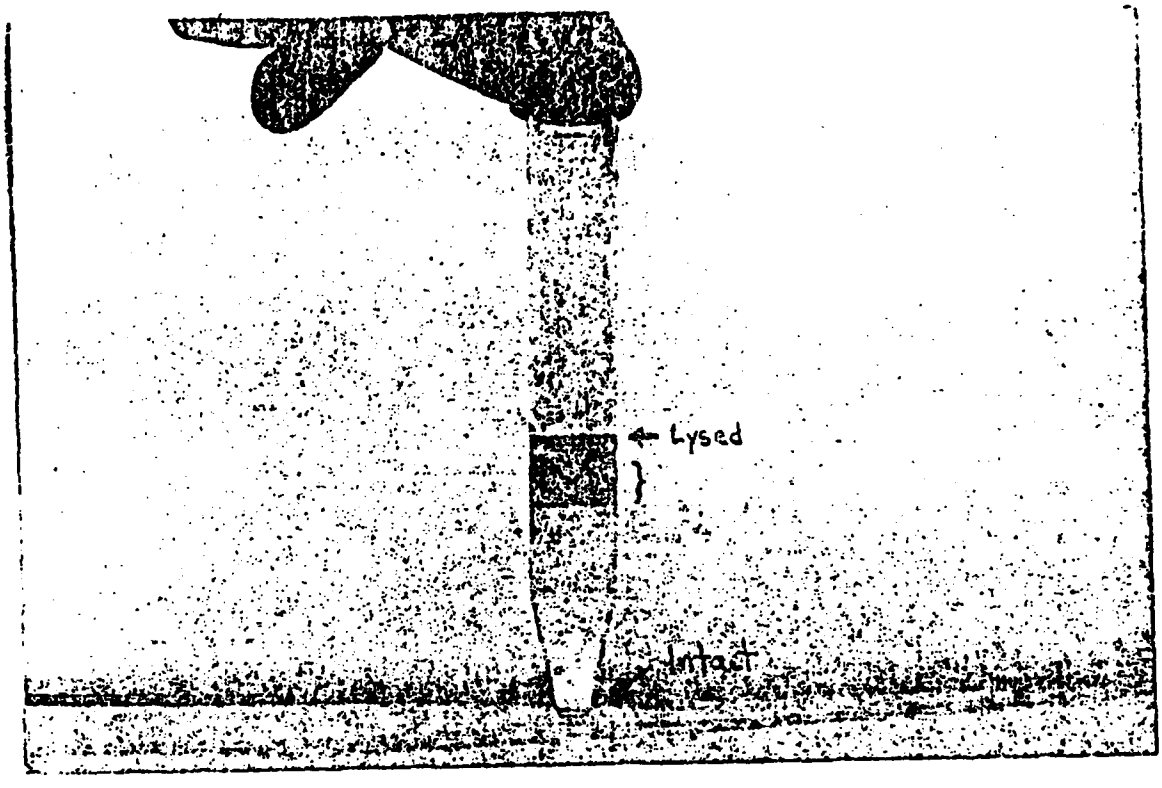
EXAMPLES OF CELLS TRAPPED IN AGAROSE BEADS

FIGURE 16



Sheep red blood cells were derivitized with a ligand which binds AChE (a specific inhibitor of the enzyme). The derivitized SRBC's were incubated with supernatant from RMSC cell cultures, washed and then incubated with monoclonal antibody to human AChE (AE-1) pre-adsorbed with SRBC. Test groups were then incubated with the developing antibody layer, rabbit anti-mouse IgG (adsorbed), washed and then exposed to guinea pig complement. Hemolysis occurred where all components were included (samples #1,2,4,5 in different concentrations of primary or secondary antibodies). Samples lacking the developing antibodies showed partial hemolysis of the SRBC population (#3,6). Sample #7 represented the no antigen control with very high levels of AE-1 antibodies, suggesting some cross reactivity with the endogenous AChE on the surfaces of the SRBC. Samples #8-12 were the remaining controls lacking AChE antigen. All incubations were carried out at 37 Deg.C. for 30 min.

FIGURE 17



SEPARATION OF POSITIVE BEADS IN HEMOLYTIC APPROACH FOR CIT

A preliminary experiment in which agarose beads containing completely lysed SRBC's (top band, colored with free hemoglobin) in beads could be separated from beads containing partially lysed populations of SRBC's (intermediate band). The pellet would have contained all the beads remaining with intact SRBC's (negative population of cells).

FIGURE 18

CELL ISOLATION TECHNIQUE - FACS APPROACH

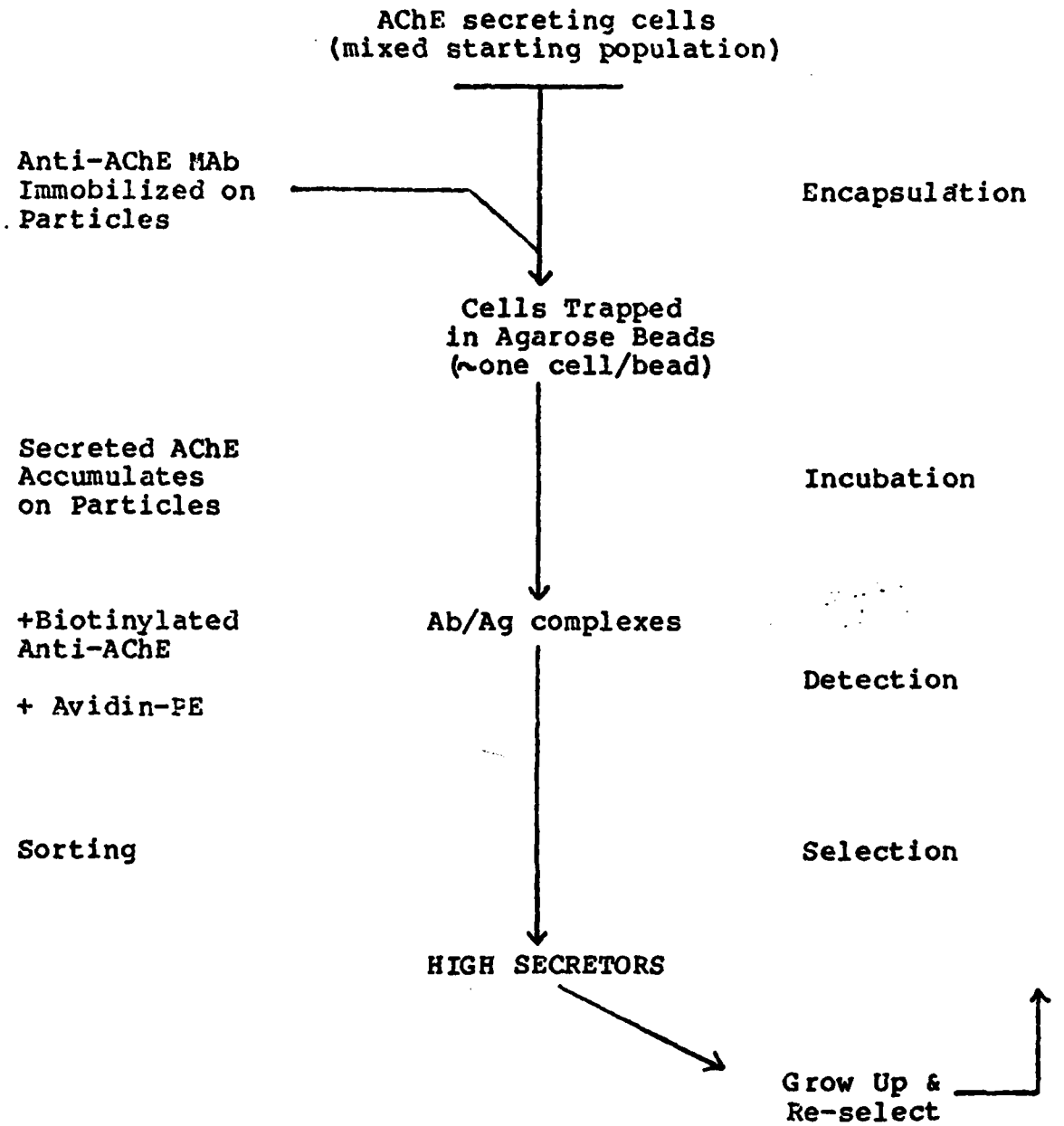
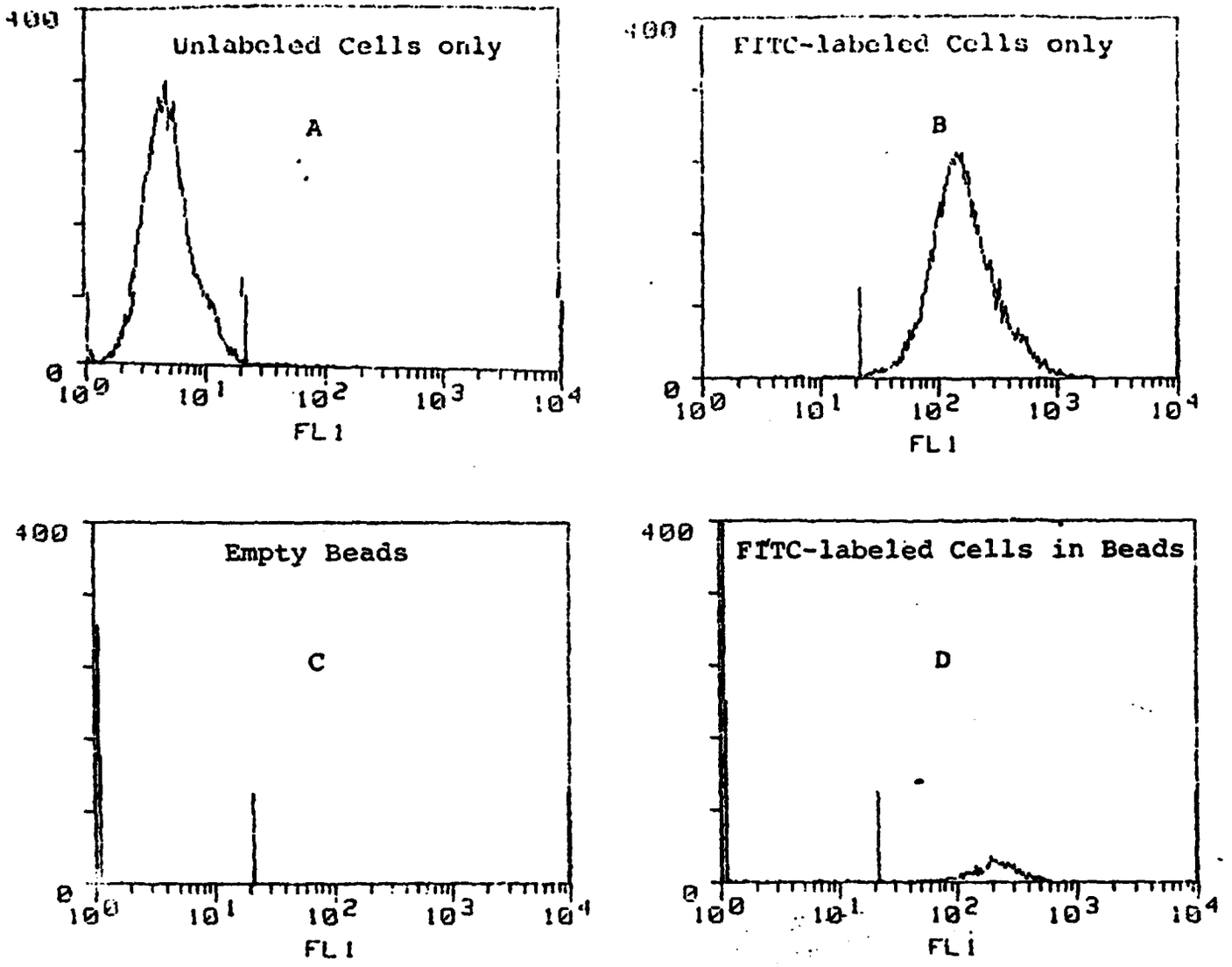


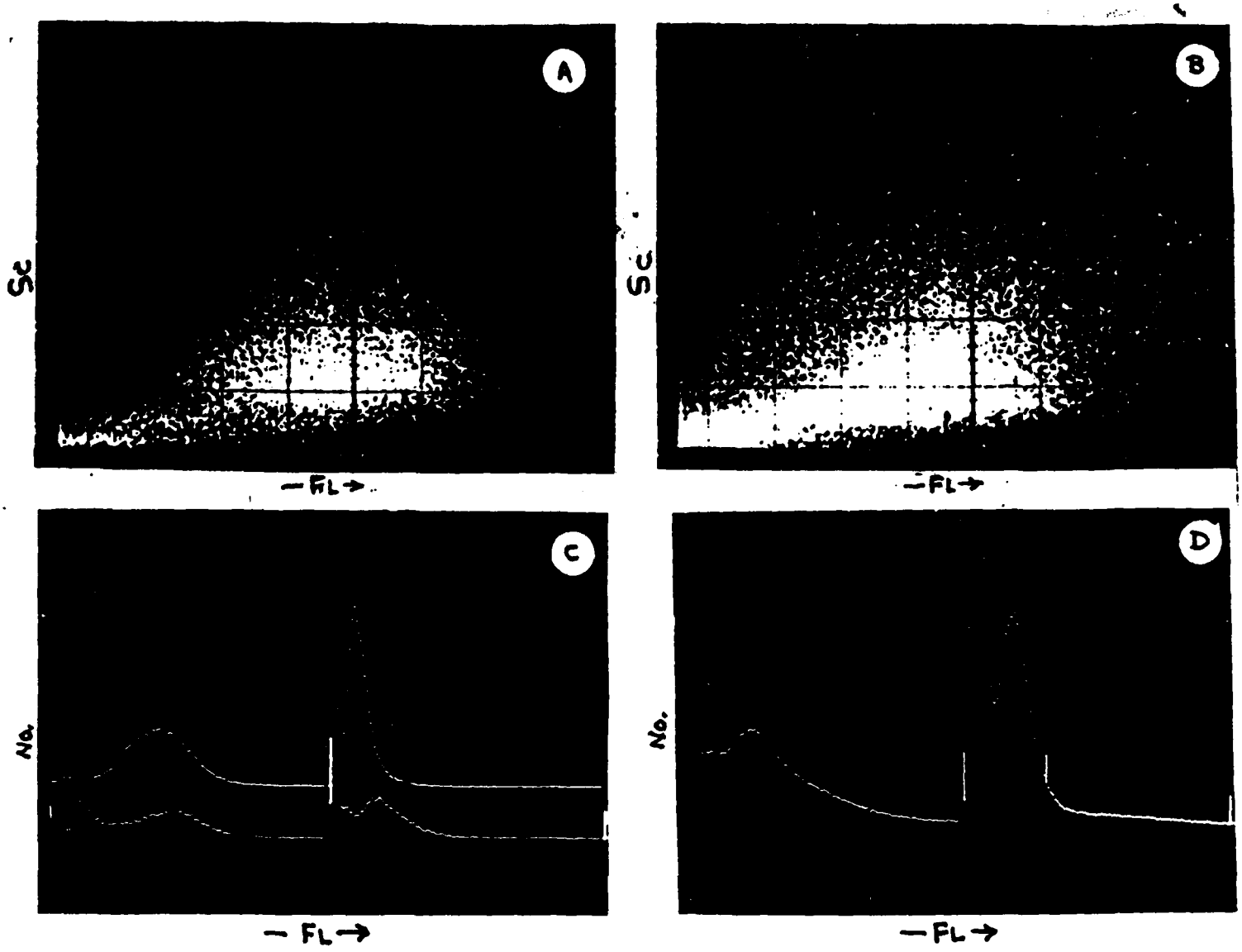
FIGURE 19



HUMAN CELLS (HuT-78) IN AGAROSE BEADS ANALYZED BY FACS

Cells were labelled with FITC-anti-human transferrin receptor monoclonal antibodies and encapsulated in agarose beads. Control cells were unlabelled. Both free and encapsulated cells were then analyzed on a FACS 440 to generate these fluorescence histograms. Note that although there were few cells analyzed in beads, the few that were there appeared to have about the same fluorescence intensity.

FIGURE 20

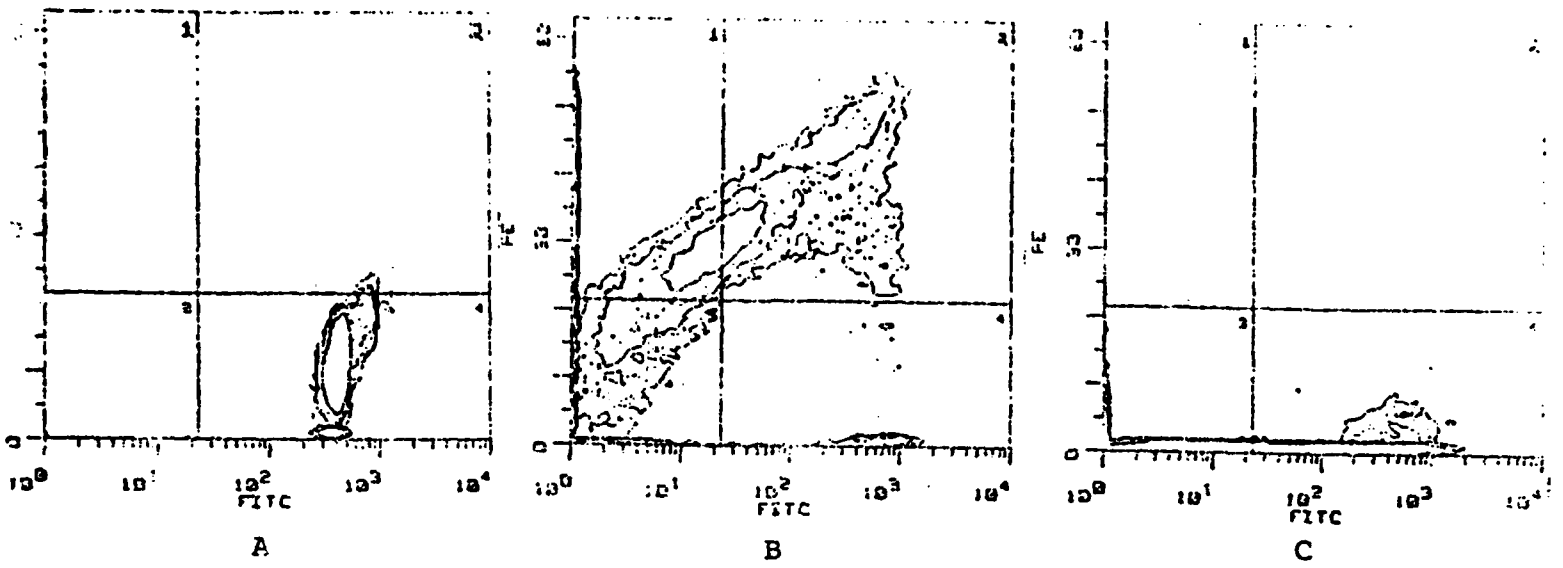


FLUORESCENCE VS. SCATTER PROFILES FOR

SORTING OF HUMAN CELLS IN BEADS

HuT-78 cells, a human T-cell line, were labelled with FITC-anti-human transferrin receptor and encapsulated in agarose beads. Beads were sized, gradient purified and analyzed on a FACS III instrument. Control signals were generated with free cells (not encapsulated) labelled with the same surface marker. Encapsulation in beads seems to have little effect on the fluorescence and scatter properties of the labelled cells. Figure D indicates sort window in fluorescence used to isolate beads containing the most fluorescent cells.

FIGURE 21



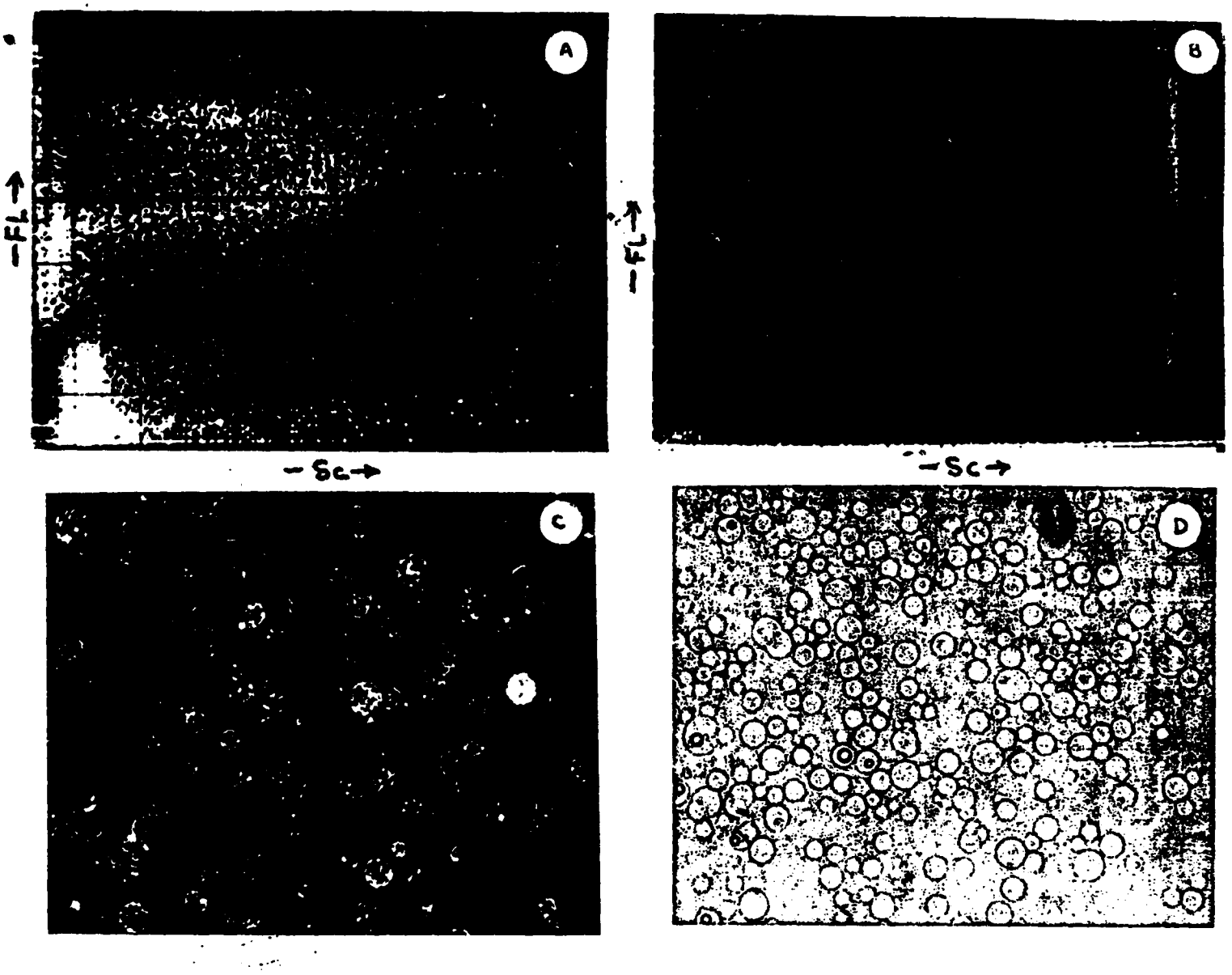
A B C

DOUBLE LABELING EXPERIMENTS TO DETECT CELLS IN BEADS

BY TWO COLOR FACS ANALYSIS

HuT-78 cells were labelled with phycoerythrin-anti-human transferrin receptor and mixed with an equal number of FITC labeled latex microspheres of about the same diameter. The mixture of FITC labeled microspheres and PE labeled cells was encapsulated in agarose beads, sized, gradient purified and analyzed on a FACS 440 to determine if both signals could be detected in the same "events". Figure A is a control sample of only FITC-microspheres trapped in beads; nearly 100% of the singal is confined to quadrant 4. Figure B shows that upon introducing PE-labeled cells, the FITC and PE signals now coincide in the same sorting events. 34% of all the events have both signals (quadrant 2), indicating that about one third of the beads detected by fluorescence have both cells and microspheres inside of them. Figure C shows the same experiment now performed with an isotype control antibody labeled with PE; all the PE signal has disappeared leaving only the FITC microsphere signal.

FIGURE 22



FACS ANALYSIS OF FLUORESCENT PARTICLES

TRAPPED WITHIN AGAROSE BEADS

FITC labeled latex particles (100 nm) were encapsulated in agarose beads as a model for the detection of fluorescent antibodies bound to AChE captured by particles in beads (see flow sheet for FACS approach to CIT). Figure A shows the fluorescence vs. scatter histogram for beads with FITC particles shown in the fluorescence micrograph below (C). Control empty beads are shown on the right: Fluorescence vs. scatter in B and light micrograph of empty beads in D.

FIGURE 23