Production of monoclonal antibody against alkaline phosphatase isolated from mouse lymphoid cell line SJL 46

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Atlanta University

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PRODUCTION OF MONOCLONAL ANTIBODY AGAINST ALKALINE
PHOSPHATASE ISOLATED FROM MOUSE LYMPHOID CELL LINE SJL 46

A THESIS
SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY
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DEPARTMENT OF BIOLOGY
ATLANTA, GEORGIA
May, 1985
ABSTRACT

BIOLOGY

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A.I.M.L.T. Institute of Medical Technology, 1975

PRODUCTION OF MONOCLINAL ANTIBODY AGAINST ALKALINE PHOSPHATASE ISOLATED FROM MOUSE LYMPHOID CELL LINE SJL46.

Advisor: Dr. Judith Rae Lumb

Master of Science degree conferred May 20, 1985
Thesis dated May, 1985

Alkaline phosphatase (APase) is a cell surface enzyme found to be associated with the membrane of lymphoblastic cells and some lymphoid cell lines. This enzyme appears and disappears in the early differentiation processes of the cells of the immune system (B and T cells). The appearance of APase in pre-T cells seems to precede that of terminal deoxynucleotide transferase (TdT). The specific role of APase in T cell differentiation pathway is not known. It has been hypothesized that APase acts as an early differentiation marker for pre-T cells.

The goal of this research is to produce a monoclonal antibody against APase using SJL-46 cells, a pre-T cell line, as the source of the enzyme. The monoclonal antibody can then be used to isolate and purify proteins with APase activity and detect cross-reacting material. At the cellular level, the monoclonal antibody can also be used to identify APase+ cells, remove APase+ cells and sort APase+ cells from a heterogeneous mixture of cells.
Partial purification of the APase from SJL 46 cells by Nonidet P40 solubilization and butanol extraction yielded a 500-800-fold purification.

Mouse myeloma cells SP2/OAg14, were fused with the spleen cells of Spraque-Dawley rats immunized with the partially purified APase. Using enzyme-antigen-immuno-assay method, eight clones producing monoclonal antibodies against SJL 46 APase were isolated. The objective of the investigation was met by isolating 8 clones secreting anti-APase monoclonal antibody.
ACKNOWLEDGEMENTS

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<td></td>
</tr>
<tr>
<td>APase</td>
<td>Alkaline phosphatase</td>
<td></td>
</tr>
<tr>
<td>But Sup</td>
<td>Butanol supernatant</td>
<td></td>
</tr>
<tr>
<td>DEA</td>
<td>Diethanolamine</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
<td></td>
</tr>
<tr>
<td>EAIA</td>
<td>Enzyme-antigen-immunoassay</td>
<td></td>
</tr>
<tr>
<td>ELIZA</td>
<td>Enzyme linked immunosorben assay</td>
<td></td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine aminopterin thymidine</td>
<td></td>
</tr>
<tr>
<td>HGPRT</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
<td></td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>NP40 Sup</td>
<td>Nonidet P40 supernatant</td>
<td></td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
<td></td>
</tr>
<tr>
<td>PNP</td>
<td>p-nitrophenol</td>
<td></td>
</tr>
<tr>
<td>pNPP</td>
<td>p-nitrophenyl phosphate</td>
<td></td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
<td></td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
<td></td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund's adjuvant</td>
<td></td>
</tr>
</tbody>
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CHAPTER I
INTRODUCTION

Several immunological techniques have been developed within the last ten years, most important of which is the production of monoclonal antibodies. By fusing normal antibody-forming cells with those of their malignant counterparts, Kohler and Milstein (1975) were able to select and clone the hybrid cell which secreted specific immunoglobulin, known as monoclonal antibody. Their monumental achievement culminated from discoveries by previous workers. Such discoveries that have helped to broaden the understanding of the underlying principles of the production of hybridomas are:

1) the clonal selection theory (Nossal and Lederberg, 1958),
2) the artificial induction of plasmacytomas (Potter and Boyce, 1962), and
3) the development of hybrid selection techniques after fusion (Littlefield, 1964).

The principle of the monoclonal antibody production technique involves the cloning of a single antibody-secreting B-lymphocyte so that homogenous antibody molecules with identical antigen-binding sites, can be produced in large quantities. Since B-lymphocytes have limited life spans in culture, the antibody-producing B-cells from immunized mouse or rat are fused with myeloma cells. The hybrids, having the ability to make the desired antibody and the ability to multiply indefinitely in tissue culture, are selected from the heterogenous mixture of hybrid cells resulting from the fusion of the B-lymphocytes and the myeloma cells. Such hybridomas are then propagated as individual clones which provide a stable source of monoclonal antibody.
The application of monoclonal antibody technology immunology, generally, is enormous. One area where monoclonal antibody makes an immense contribution is in the functional distinction of T-cell subsets. In man the identification and characterization of antigens like T4 and T8 were discovered by using monoclonal antibodies (Thomas et al, 1982). Similarly, the specificity of localization and distribution of the Class I and Class II major histocompatibility molecules has been improved by the use of monoclonal antibodies against these molecules (Lemke et al, 1978).

Cell surface markers like, T4, T8, Lyt1, Lyt2, and Lyt 3 are associated with mature T-cells in man and mouse. In T-cell differentiation, however, certain markers such as terminal deoxynucleotide transferase (TdT) (Chang, 1971) and FT-1 (Pizmino et al, 1978) have been associated with pre-T cells. Alkaline phosphatase (APase) is a cell surface enzyme found on the cell membrane of normal lymphoid tissues and some lymphoma cell lines (Smith, 1961, 1962; Lagerlof and Kaplan, 1967; Lumb and Doell, 1970). Pre-T cell lines (SJL-46) and pre-B cell lines (18-81, 18-48) are all rich in APase (Lumb and Silverstone, in preparation). It has been hypothesized that APase acts as an early differentiation marker for pre-T cells and its appearance precedes that of TdT (Lumb and Silverstone, in preparation). The goal of this research is to produce monoclonal antibodies against APase using one of the pre-T cell lines, SJL-46, as the source of the enzyme. The monoclonal antibody will then be used for the isolation of APase+ cells, and identification of inactive cross-reacting material.

The specific research objectives are:

1) to grow SJL-46 cells (the source of APase) in mice to obtain them in large quantities,
2) to partial
3) to immunize
   APase, ially purified
4) to fuse the
   myeloma cell
5) to select
6) to screen
   bridomas, and
7) to clone
   limiting dilution.
Alkaline Phosphatase

Alkaline phosphatase (APase) represents one of the most widely studied enzymes. It is found in living organisms from bacteria to higher plants and animals including humans. Malani and Horecker (1964) showed this enzyme to be located in the region lying between the proplasmic membrane and the cell wall of Escherichia coli. APase is also abundant in fishes (Bodansky et al, 1931).

APase has been widely studied in mammalian tissues such as intestine, placenta, kidney, liver, and bone (McComb, et al, 1979). Using histochemical techniques, Clark (1961) and Ito (1969) showed that APase was located on the membrane of the epithelial cells of intestinal microvilli.

The study of the localization of APase on the membrane of lymphoblastic cells, fetal thymus, thymic lymphoma, and other lymphoid tissues has mainly been based on the results from histochemical and ultrastructural studies. Smith was the first to observe that the normal adult thymus was negative for APase during a histochemical study (Smith 1961, 1962). Similar studies showed that the enzyme was located on the fetal thymus cells up to 16 days gestation, on thymic lymphoma cells and on some cells in normal spleen in areas surrounding the germinal center (Lagerlof and Kaplan, 1967). Ruuskanan and Kouvalainen (1974) showed by ultrastructural techniques that APase is localized on the
Hybridoma Technology

Hybridoma technology was an outgrowth of the early work on somatic cell hybridization. Littlefield (1964) developed a method for the selection of somatic cell hybrids using two drug resistant sublines of mouse cells, one to 8-azaguanine and the other to 5-bromodeoxyuridine. The former was deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) while the latter was deficient for the enzyme thymidine kinase (TK). Mammalian cells have two pathways to synthesize nucleotides for DNA synthesis, the "de novo" pathway, which synthesizes nucleotides from sugars and amino acids and the "salvage" pathway which synthesizes nucleotides from preformed hypoxanthine and thymidine. When the "de novo" pathway was blocked by aminopterin, the only optional pathway left for the cell in nucleotide synthesis, was the "salvage" pathway, which was dependent on the presence of the enzymes, HGPRT and TK. The two drug resistant cell lines, one TK⁻ and the other HGPRT⁻, were therefore incapable of surviving in a medium containing hypoxanthine, aminopterin and thymidine (HAT). The hybrid on the other hand, which contained TK⁺ and HGPRT⁺ genes, one from each parent, made both HGPRT and TK enzymes and hence had the capability to grow in HAT. The gene coding for the HGPRT was located on the X chromosome and this made the production of the mutant myeloma cells through single mutation of the X chromosome in male cells fairly easy.

Cotton and Milstein (1973) showed that when two antibody-producing cells were fused, four immunoglobulins (Ig) were produced, the two original molecules, plus two molecules obtained by combining the H chain of
one with the L chain from the other cell. Immunology witnessed a new era when it was reported that specific antibodies can be manufactured by means of continuous cultures of antibody secreting fused cells (Kohler and Milstein, 1975). They fused mouse myeloma cells and spleen cells from mouse immunized to sheep red blood cells (SRBC). They selected and expanded the hybridoma cell line secreting anti-SRBC antibody. The myeloma cell line used for this hybridoma also secreted immunoglobulin (Ig). Later other myeloma cell lines not secreting Ig were developed. Table 1 shows some of the myeloma tissue culture lines that have been developed and found useful for hybridoma production.

Fusing Agent

Inactivated Sendai Virus was first used by Harris and Watkins (1965) to promote fusion. Cotton and Milstein (1973); Kohler and Milstein (1975) also used Sendai Virus as a fusing agent. Although this technique is satisfactory, the system tends to be biased towards IgM producing hybrids (Fazekas de St Groth and Scheidegger, 1980). Davidson and Gerald (1976) used polyethylene glycol (PEG) to improve induction of mammaline cell hybridization. The fusion efficiency of PEG was reported to have improved in the presence of 5% dimethyl sulfoxide (DMSO) (Norwood et al, 1976). Comparing different glycols, Fazekas de St Groth and Scheidegger (1980) reported that PEG of lower molecular weight was less effective while PEG of molecular weight 4000 (PEG 4000) was most effective. Many investigators have recommended concentrations of 30-50% PEG for fusion. Induction of cell fusion is not limited to Sendai Virus and PEG. Recently electric field-induced cell to cell fusion was carried out and reported to be a highly efficient technique (Zimmermann and Yienken, 1982).
Table 1. Myeloma cell lines used in hybridoma production

<table>
<thead>
<tr>
<th>Myeloma cell line</th>
<th>Origin</th>
<th>Derived from</th>
<th>Ig Secretion</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>P3 X63 Ag8</td>
<td>BALB/c mouse</td>
<td>P3.6.2.8.1</td>
<td>+</td>
<td>Kohler and Milstein, 1975</td>
</tr>
<tr>
<td>NS-1</td>
<td>BALB/c mouse</td>
<td>P3 X63 Ag8</td>
<td>-</td>
<td>Kohler and Milstein, 1976</td>
</tr>
<tr>
<td>SP2/O Ag14</td>
<td>BALB/c mouse</td>
<td>P3 X63 Ag8 X BALB/c spleen</td>
<td>-</td>
<td>Shulman et al, 1978</td>
</tr>
<tr>
<td>P3 X63 Ag8.653</td>
<td>BALB/c mouse</td>
<td>P3 X63 Ag8</td>
<td>-</td>
<td>Kearney et al, 1979</td>
</tr>
<tr>
<td>Y3-Ag 1.2.3</td>
<td>Lou rat</td>
<td>210 Lou plasmacytoma rat</td>
<td>-</td>
<td>Galfre et al, 1979</td>
</tr>
<tr>
<td>U-266R^-</td>
<td>Human</td>
<td>U-266</td>
<td>+</td>
<td>Olson and Kaplan, 1980</td>
</tr>
<tr>
<td>82266 AR/NIP4-1</td>
<td>Human</td>
<td>82266AR</td>
<td>-</td>
<td>Pickering and Gelder, 1982</td>
</tr>
</tbody>
</table>

1 All myeloma cell lines listed are HGPRT^-  
2 Ig = Immunoglobulin  
+ = Produce and secrete Ig  
- = Neither produce nor secrete Ig
Screening Assays

The choice of an appropriate screening assay is one of the most vital aspects of hybridoma production. Basically, any method which enables one to detect the desired antibody with specificity and efficiency may be used. The guiding principles in the selection of a particular screening assay are reliability, speed, cost, and reduction of labor. Unless the assay can be performed with specificity, in a reasonable time, at low cost, and little effort, the production of hybridomas is likely to fail. Catt and Tregear (1967) developed the solid-phase radioimmunoassay technique and the method was later used to identify monoclonal antibodies. The assay is based on the fact that polyvinyl surfaces tightly adsorb most proteins. Soluble protein antigen was first attached to the tube, then its corresponding immunoglobulin (Ig) was added. This resulted in antigen-antibody reaction. To reveal this reaction $^{125}I$-labelled anti-immunoglobulin or $^{125}I$-labelled staphylococcal protein A, which binds specifically with the Ig, was added (Goding, 1978). Specific antigen-antibody reaction was revealed by positive count using a scintillation counter. This method was later adapted to detect monoclonal antibodies to viral antigens (Oi et al, 1978; Nowinski et al, 1979).

Engvall and Pesce (1978) and Voller et al (1979) adapted the solid-phase radio-immunoassay to enzyme-linked immunosorbent assay (ELISA) to detect monoclonal antibodies. Here the anti-immunoglobulin or protein A was conjugated to an enzyme such as peroxidase, alkaline phosphatase or β-galactosidase. Addition of the corresponding enzyme substrate revealed the specificity of the antigen-antibody reaction. The avoidance of isotopes and the visual reading provided by this method are some of the advantages of ELISA over the solid-phase radioimmunoassay.
Using the catalytic activity of placental alkaline phosphatase, Jemmerson and Fishman (1982) developed a convenient assay for the detection of monoclonal antibodies which bind the enzyme by reversing the reagents of the ELISA test. Goat anti-mouse IgG was first attached to the solid-phase. Culture fluid from anti-APase secreting hybridomas was then added. This was followed by the addition of alkaline phosphatase. The specificity of the monoclonal antibody to the enzyme, was revealed by using p-nitrophenyl phosphate as the substrate for the enzyme. The sensitivity of the assay was 10 ng antibody per milliliter and the assay was applicable to detection of monoclonal antibodies of other isozymes of alkaline phosphatase (Jemmerson and Fishman, 1982).

Monoclonal Antibodies Raised Against Enzymes

Monoclonal antibodies have been produced to several enzymes. The majority of the antibodies was raised by the mouse-mouse hybridoma method (Kohler and Milstein, 1975), while in few cases rat-rat hybridoma (choo et al, 1980) or rat-mouse hybridoma (Levy et al, 1981) methods were used. In each of these hybridomas the enzymes were either from different species of animals or were polymorphic enzymes. In this hybridoma the SJL 46 cell line which is the source of the APase (antigen) is a mouse cell line. Mice will not be used for immunization because the enzyme is not polymorphic and therefore, it is not likely to stimulate an adequate immune response in mice. A different species of animal, Sprague-Dawley rat, is a better choice of animal for immunization. Spleen cells from such immune rat will be fused with SP2/0 Ag 14 myeloma cell line (Shulman et al, 1978) to obtain the enzyme-specific hybridomas. The hybridoma cell line, which is foreign to both the rat and the normal mouse must be grown in serum-free
culture medium or in nude mice for the production of monoclonal antibodies.

Highly purified enzymes have been used as antigen in some cases, while in others, enzyme-specific monoclonal antibodies were still obtained by using partially purified enzymes. Fambrough et al (1982) and Choo et al (1980) obtained enzyme-specific hybridomas by using highly purified human acetylcholinesterase and monkey phenylalanine hydroxylase as antigens respectively. Kramer et al (1980) and Siddle et al (1981) on the other hand obtained enzyme-specific hybridomas by using only partially purified RNA polymerase II and 5'-nucleotidase as antigens. Also, Arklie et al (1981) and Wray and Harris, (1982) used partially purified human alkaline phosphatase, as the immunogen for the hybridoma production.
CHAPTER III
MATERIALS AND METHODS

Preparation of Lymphoid Cell Line SJL 46

The source of the antigen (APase) was the SJL 46 lymphoma cell line which was obtained from Dr. Ed Siden, University of Florida, Gainesville, Florida. The cells were first cultured in RPMI 1640 medium (Appendix A) and incubated at 37\(^\circ\)C with a constant supply of 5\% CO\(_2\). Large quantities of SJL 46 cells were obtained by injecting 300 mice intraperitoneally with 10\(^5\) cells per mouse in 0.5 ml of RPMI 1640 medium. Six days after injection, each mouse was killed by cervical dislocation and disinfected with 70\% alcohol. The abdominal skin was removed, leaving the peritoneum. Ten milliliters of 0.1 M Tris buffer containing 0.15 M NaCl was then injected into the peritoneal cavity with a 18 gauge, 1 1/2-inch needle. After gently massaging the abdomen, the fluid was withdrawn. The cells were collected into 50 ml centrifuge tubes and washed thrice with 0.1 M Tris buffer containing 0.15 M NaCl. The cells were counted in a hemocytometer, pooled and frozen at -20\(^\circ\)C deep freezer ready for enzyme purification.

Alkaline Phosphatase Assay

The volume of the reaction mixture was 0.5 ml and consisted of 0.8 M diethanolamine (DEA) at pH 10.38 mM p-nitrophenyl phosphate (pNPP), and the enzyme extract (Njoku, et al, 1982). Dephosphorylation of the substrate, pNPP catalyzed by the APase released p-nitrophenol (PNP). The absorption of the released PNP was read at a wavelength of 400 nm in Zeiss PMQII Spectrophotometer. The spectrophotometer was interfaced to a Hewlett-Packard 9825 computer which calculated the change in product per
unit time. Enzyme activity is expressed as the amount of PNP in μmole,
liberated per milliliter per minute in the presence of 1.0 M DEA pH 10.0.
The specific activity was calculated by dividing the enzyme activity by
the protein concentration.

**Measurement of Protein Concentration**

Protein concentration was determined by the Bradford method (Bradford,
1976). The method is based on the fact that Coomassie blue dye when re-
acted with protein, forms protein-dye complex which has an absorbance of
595 nm. The absorbance, which is directly proportional to the concentration
of the protein, was measured using the Nissei Hitachi Model 1023 spectro-
photometer. A standard curve of absorbance versus concentration of bovine
serum albumin (BSA) was constructed and the concentration of the unknown
protein was determined by interpolation.

**Alkaline Phosphatase Purification**

**Purification Scheme.** The primary aim was to purify the enzyme at least
300-500 times. At this level of purification the enzyme would then be used
as an immunogen containing 200 mg protein per dose. The flow chart of the
purification scheme is shown in Figure 1.

The membrane fraction was pelleted, solubilized with a detergent, NP40,
and extracted with cold butanol.

**Pelleting of the SJL 46 cell Membranes.** The cell suspension (crude
sample) was first thawed on ice and vortexed. At this stage all the cells
were lyzed. The APase activity and the protein concentration of the crude
sample were estimated. The crude sample was centrifuged at 150,000 x g. The
Crude Sample

Lysed by freezing whole cells
centrifuge 150,000 x g

1st Pellet

1st Sup

Homogenize in 1% NP40
centrifuge 190,000 x g for 1 hour

1st NP40 Pellet

1st NP40 Sup

Extract with cold 30%
butanol. Centrifuge
190,000 x g for 1 hour

2nd NP40 Pellet

2nd NP40 Sup

Pellet (Discard)

But Sup-1

But Sup-2

3rd NP40 Pellet (Discard)

3rd NP40 Sup

Pellet (Discard)

But Sup-3
residue (1st Pellet) and the supernatant (1st Sup) were then estimated for enzyme activity and protein concentration.

**Solubilization with NP40.** The 1st Pellet was solubilized in 1% NP40, incubated at 45°C for 30 minutes and centrifuged at 190,000 x g 1 hour. The residue (1st NP40 Pellet) and the supernatant (1st NP40 Sup) were estimated for APase activity and protein concentration. The procedure was repeated twice to obtain 2nd NP40 pellet, 2nd NP40 Sup, 3rd NP40 pellet, and 3rd NP40 Sup. The three NP40 supernatant samples were stored frozen for extraction in butanol.

**Butanol extraction.** Each NP40 supernatant was extracted in the cold with 30% butanol. The sample was placed in a vial which was in turn placed in an insulated metal container. The whole assembly was placed on a cold plate, for which the temperature was adjusted to about 3-4°C. The butanol was brought to 4°C before use. The butanol was gradually added to the NP40 supernatant with continuous stirring until the required concentration was reached. The samples were left stirring for 30 minutes after which they were centrifuged at 190,000 x g for 1 hour at 4°C. The clear aqueous layer which contained the enzyme was removed. The enzyme activity and protein concentration of the extracts were estimated. The extracts were accordingly referred to as ButSup-1, ButSup-2, and ButSup-3, respectively.

**Removal of butanol by dialysis.** The enzyme used for immunization was the ButSup-2 from Crude Samples II and III. The butanol was removed by dialysis before the APase was injected in Spraque-Dawley rats. The dialysis bag was soaked in distilled water overnight. The ButSup-2 was placed in the bag and both ends were tied. The samples were dialyzed in 0.1 M Tris buffer
containing 0.15 NaCl, for 10 hours with continuous stirring in the cold.
The enzyme activity and the protein concentration of the samples were
determined after dialysis.

Electrophoresis

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed on the partially purified APase. The
electrophoresis was done in a slab gel using the Laemmli gradient gel
electrophoresis method (Laemmli, 1970). The components of the gels are in
Appendix B. The gradient gel was mixed using the gradient mixer. The
stacking gel was layered on the gradient gel and allowed to set. Electrophoresis was run in 1.0 M Tris HCl at pH 8.8.

The APase sample containing 30 μg protein, enzyme activity of 311
nmoles PNP liberated per ml per minute, and the tracking dye in 25 μl
were placed in 6 wells. An additional well contained standard membrane
glyco proteins. Electrophoresis was run at 20 mA per gel for 6 hours.

The gel was stained with silver stain to indicate the protein bands.
The components of the silver stain are in Appendix C. The gel was fixed
in solution I for 30 minutes, in solution II for 10 minutes, and in solution III for 30 minutes. It was then rinsed in deionized water for 30
seconds and transferred into solutions IV and V for 30 minutes each. The
gel was then rinsed in deionized water for 30 seconds and developed in
solution VI for about 10 minutes. Finally it was destained in solutions
VII and VIII for 5 minutes and 45 seconds respectively.

The APase band was identified by staining the gel with the APase
stain. The stain contained 25 mg Fast blue RR and 25 mg naphthyl phos-
phate ASMX dissolved in 25 ml 0.2 M Tris HCl pH 9.0. The gel was stained
in the dark at 4°C for about 2 hours.
Immunization of Sprague-Dawley Rats

Four Sprague-Dawley rats were obtained from the Center for Laboratory Animal Resources, Atlanta University Center. The partially purified APase was emulsiified in Complete Friend's Adjuvant (CFA) at the ratio of 2 parts APase to 1 part CFA. The rats were bled prior to injection. Each rat was injected intraperitoneally with 1.0 ml of the APase-CFA emulsion containing 200 µg protein. Two additional injections of the same dosage were given to each rat at two week intervals in CFA. The rats were bled prior to each injection.

The antibody titer of the pre-injection, and post-injection sera samples were determined by the enzyme antigen immunoassay (EAIA) (Jemmerson and Fishman, 1982). A booster injection was given three days before fusion. The booster dose was given intravenously with 0.1 ml APase containing 10 µg protein per ml and at the same time intraperitoneally with 0.5 ml APase containing 10 µg per ml. Since this was determined to be inadequate, subsequent booster injections were given at 50 µg/ml intravenously and intraperitoneally.

Standardization of the Screening Assay

To ensure that the EAIA method would work, the test was standardized using dog anti-goat IgG as the secondary antibody, goat anti-<i>Escherichia coli</i> APase as the primary antibody, <i>E. coli</i> APase as the antigen, and pNPP as the enzyme substrate. The secondary antibody was diluted to 10 µg/ml in 0.1 M Na₂CO₃ pH 9.0 and was plated into microtiter wells at 100 µl per well. The plate was incubated at 4°C overnight. The wells were washed twice with 25 mM Tris HCl pH 7.5 containing 0.1% Tween 20 and 2 mM MgCl₂ (wash buffer). Each well was blocked with 200 µl of 25 mM Tris HCl pH 7.5
containing 5% FCS and 2 mM MgCl₂ (working buffer) for 4 hours at 4°C. The wells were then washed twice. Two fold serial dilutions of the primary antibody beginning with 1:20 dilution, were performed in the working buffer. Two hundred μl of each dilution was added to the corresponding well and the test was incubated at 4°C for 4 hours. The wells were washed twice. One hundred μl of E.coli APase with enzyme activity of 50 nmoles PNP liberated per ml per minute was added to each well and incubated at 4°C for 4 hours. One hundred μl of 33.8 mM pNPP prepared in 1.0 M DEA pH 8.6 was added to each well and incubated at 37°C for 1 hour. Normal goat serum was used as the negative control while the working buffer was used as the buffer control.

The test was repeated with the following variations:

1) the test was incubated at room temperature for 1 hour instead of at 4°C for 4 hours,
2) enzyme activities of 20 nmoles/ml/minute and 30 nmoles/ml/minute were used instead of 50 nmoles/ml/minute.

Table 2 shows the results of the EAIA experiments. With enzyme activity of 50 nmoles/ml/minute and other factors constant, non specific positive reactions were obtained (Experiment I). With enzyme activity of 30 nmoles/ml/minute and other factors constant specific reactions were obtained (Experiment II). Further decrease of enzyme activity to 20 nmoles/ml/minute gave the same result as Experiment II except the time for color development was much longer (1½ - 2 hours after substrate addition). Incubation temperature of 4°C for 4 hours and room temperature for 1 hour gave the same result as Experiment II.
Table 2. Enzyme–antigen–immunoassay

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Primary Antibody</th>
<th>Dilution 20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
<th>640</th>
<th>1280</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Anti-\textit{E. coli} APase</td>
<td>+² + + + + + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat serum</td>
<td>+ + + + + + + + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Buffer control</td>
<td>+ + + + + + + + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Anti-\textit{E. coli} APase</td>
<td>+ + + + + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goat serum</td>
<td>- - - - - - - - - - - - - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Buffer control</td>
<td>- - - - - - - - - - - - - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

² Experiment I included 50 nmoles PNP/ml/minute of \textit{E. coli} alkaline phosphatase activity.

Experiment II included 30 nmoles PNP/ml/minute and 20 nmoles PNP/ml/minute of \textit{E. coli} alkaline phosphatase activity.
The following conclusions were drawn:

1) for hybridoma screening assays, the EAIA steps should be incubated at room temperature for 1 hour, but incubation after substrate addition should be at 37°C for 1 hour,

2) the standard activity of the APase in the test should be 30 nmoles/ml/minute,

3) the concentrations of the secondary antibody and the buffers should be as recommended by Jemmerson and Fishman, 1982.

**Determination of Antibody Titers of Rat Sera**

The experiment was carried out as described above. The secondary antibody was the goat anti-IgG, purchased from Flow Laboratories. The secondary antibody concentration, the buffers used and their concentrations were as described in the standardization procedure above. The incubation steps were carried out at room temperature for 1 hour and after substrate addition the incubation was at 37°C for 1 hour. The substrate was prepared in 1.0 M DEA pH 10 instead of in 1.0 M DEA pH 8.6 used for *E. coli* APase above. The APase activity used was 30 n mole PNP/ml/minute.

**Preparation of Mouse Myeloma Cells**

Figure 2 shows the flow-chart of the processes of cell fusion and cloning. The SP2/0 cells were obtained from Dr. Alison Mawle, Immunology Laboratory, Center for Disease Control, Atlanta, Georgia. The cells were subcultured in Dulbecco's Modified Eagles Medium (DMEM) (Appendix D) with 10% Calf serum. Incubation was at 37°C in 6% CO₂. Subculturing of the cells was done twice a week. For fusion, the cells were transferred from 25 cm²
SEED SP2/0 CELLS

IMMUNIZE SPRAQUE-DAWLEY RATS

PREPARE SINGLE CELL SUSPENSION OF SPLEEN CELLS

FUSE SPLEEN AND SP2/0 CELLS WITH PEG 4000 AND DISTRIBUTE INTO 96 WELL PLATES

SCREEN BY EAIA METHOD

CLONE (96 WELL PLATE)

SCREEN BY EAIA METHOD

EXPAND POSITIVE CLONES
growth area (T-25 flask) to 75 cm² growth area (T-75 flask) and grown for 4 days. Twenty four hours before fusion, the cells were split 1:1 in fresh medium. On the day of fusion, each flask was shaken gently to dislodge the cells from the flask wall. The cell suspension was then centrifuged at 700 x g for 5 minutes. The supernatant was filtered and stored frozen to be used as conditioned medium for cloning. The pellet was resuspended in 50 ml serum-free DMEM and centrifuged at 700 x g for 5 minutes. The centrifugation was repeated twice and the pellet was finally suspended in 5.0 ml serum-free DMEM. The cells were then counted in a hemocytometer.

Preparation of Spleen Cells for Fusion

The rat, which was boosted 3 days before, was anesthesized with ether, then slaughtered at the neck to collect blood. The animal was wiped with 70% alcohol and then placed face up under the hood and a cut was made through the skin with scissors just below the ribs. The skin was separated along the incision and pulled firmly towards the head and tail to expose the peritoneum. The peritoneum was disinfected with 70% alcohol and the spleen was removed with sterile scissors and forceps into a sterile petri dish containing 10.0 ml serum-free DMEM. With two sterile, bent, 21 gauge needles, the spleen cells were teased into the medium. The medium was withdrawn and expelled several times with the pipette to separate compact cells and get them into suspension. The cell suspension was transferred into a sterile 12.0 ml centrifuge tube and left for 2-3 minutes. While the large particles settled at the bottom of the centrifuge tube, the
cell suspension was transferred to a 50 ml centrifuge tube that was filled to 50 ml mark with serum-free DMEM. The cells were pelleted by centrifugation at 700 x g for 5 minutes. The process was repeated twice and the final pellet was suspended in 10.0 ml serum-free DMEM. The cells were then counted in a hemocytometer.

**Preparation of Polyethylene glycol**

Polyethylene glycol molecular weight 4,000 was weighed into a clean screw cap bottle. The bottle and the PEG were then autoclaved for 20 minutes. The autoclaved polyethylene glycol (PEG) was allowed to cool and then added to the DMEM containing 5% DMSO. The final concentration of PEG was 50% (w/v). The solution was dispensed in 1.0 ml aliquots and stored frozen at -20°C until used.

**Hybridization of SP2/0 and Rat Spleen Cells**

The SP2/0 and the spleen cells were first mixed in a 50 ml centrifuge tube in the ratio of 1:4 (2.4 X 10^7 SP2/0 cells to 9.8 X 10^7 spleen cells). The mixed cells were pelleted at 700 x g for 5 minutes. The pellet was broken up by tapping the bottom of the tube. The 50% PEG was gradually added to the pellet. One ml PEG was added to 1 X 10^8 cells. The mixture was left for 45 seconds with continuous shaking. Ten ml of serum-free DMEM was added to the mixture over a period of 3 minutes, shaking the tube continuously. The volume was then brought to 50 ml with serum-free DMEM. The cells were centrifuged at 700 x g for 5 minutes to remove the PEG. The supernatant was decanted. Mouse spleen cells (1.2 X 10^8 cells in 4 ml DMEM) were added to the tube as feeder cells. The suspension was divided evenly into two tubes and centrifuged. Cells in one tube were resuspended in DMEM-HAT medium (Appendix F) with 10% FCS to contain 1 X 10^6 cells per ml. The
cell suspension was distributed into 96-well microtiter plates at 200 μl per well. Cells in the other tube were resuspended in DMEM medium with 10% FCS to contain $1 \times 10^6$ cells per ml. The suspension was similarly distributed into 5 microtiter plates at 200 μl per well. Twenty four hours later the DMEM medium was changed to DMEM-HAT medium. All the plates were incubated at 37°C in 6% CO₂ for at least 10 days.

**Screening of Culture Supernatants**

After 14 days of incubation at 37°C, the culture supernatants of the fused cells were screened for anti-APase antibody production. The positive wells were cloned while the negative wells were kept and rescreened at 21 days. The screening assay method was the EAIA described earlier. In this case the tissue culture supernatants were not diluted serially. The controls used with the test were:

1) a positive control containing the polyclonal antibody from the immunized rat,

2) a negative control containing the SP2/0 myeloma supernatant fluid, and

3) a buffer control containing the 25 mM Tris buffer containing 5% CFS and 2 mM MgCl₂.

**Preparation of Medium for Cloning**

The supernatant fluid of SP2/0 myeloma cells fed for 24 hours, was used as the conditioned medium (Appendix F). The tissue culture supernatant was transferred into 50 ml centrifuge tubes and spun at 208 x g for 15 minutes to remove dead cells. The fluid was filtered through a 22 micron membrane filter. Its sterility was checked by incubation at 37°C for 24 hours. It was then stored frozen at -20°C. For use, the conditioned
medium was thawed and mixed with equal volume of fresh feeding medium (DMEM). Ten percent calf serum, 50 I.U. penicillin/50 μg streptomycin, and 0.3 mg glutamine were added to replace the spent additives in the conditioned medium.

Preparation of Macrophage Feeder Cells

Three mice were injected intraperitoneally with 0.3 ml CFA 4 days before cloning. Twenty four hours before cloning, each mouse was killed by cervical dislocation and disinfected with 70% alcohol. A cut was made through the skin just below the ribs. The skin was separated along the incision and pulled firmly towards the head and tail to expose the peritoneum. With 18 gauge needle 10.0 ml of DMEM was injected into the peritoneal cavity of each mouse. After gently massaging the abdomen, the ascites fluid was collected in a sterile 50.0 ml centrifuge tube. The macrophages were counted in a hemocytometer. They were then plated into microtiter plates at 100 μl containing 1 X 10^4 cells per well. The plates were incubated overnight at 37°C.

Cloning of Hybrids

Wells giving strong positive EAIA test were selected for cloning. With a sterile pipette, the medium of the well was withdrawn and expelled several times to dislodge the cells. The cells were resuspended in 1.0 ml of DMEM and counted in a hemocytometer. The cells were diluted to give 80 cells per ml in a 20 ml amount. A doubling dilution was made in 5 tubes with a total volume of 10.0 ml per tube. The final cell concentrations were:
8 cells per well,  
4 cells per well,  
2 cells per well,  
1 cell per well, and  
0.5 cell per well.

Each dilution was plated into 96 wells of a microtiter plate, containing the mouse peritoneal macrophage feeder layers at 100 ml per well. Each well was observed with inverted microscope as from the 7th day for the appearance of single ball of cells which later form clusters of cells. After satisfactory cell growth, each monoclonal well was again screened by EAIA method for the presence of anti-APase monoclonal antibody.
CHAPTER IV
EXPERIMENTAL RESULTS

Preparation of Lymphoid Cell Line SJL-46

To obtain enough APase for purification, the SJL-46 cells were grown in mice. From the $2.38 \times 10^7$ SJL-46 cells injected into mice $6.19 \times 10^{10}$ cells were recovered after 6 days.

Alkaline Phosphatase Purification

The cells were split into Samples I and II. Each sample was solubilized with NP40 and extracted with butanol. Tables 3 and 4 show the results of these treatments. In each experiment the butanol extract of the 2nd NP40 Sup gave the best results. The fold purification was 524 and 838 for the two experiments with 41% and 69% overall yield respectively. Thus, the level of purity of the two samples met the expected objective of 300-500, and the percent yield was good.

Electrophoresis

The partially purified APase was electrophoresed. Figure 3 shows the result of the electrophoresis. The protein bands were stained yellow with silver stain. The corresponding APase band was identified by the APase stain which stained blue. From the standard protein bands, the molecular weight of the APase was $135,000 \pm 8,000$ (Figure 4).

Immunization of Sprague-Dawley Rats

Table 5 shows the anti-APase titer of pre-injection and post-injection sera. After two weeks of injection, the anti-APase antibody titer increased 8-fold, and after four weeks it was increased in all four rats 64-fold.
Table 3. Partial purification of APase from Sample I

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total APase Activity¹</th>
<th>Total Protein</th>
<th>Specific Activity²</th>
<th>Percent Yield</th>
<th>Fold Purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>182.26</td>
<td>3,645.60</td>
<td>0.050</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>1st Pellet</td>
<td>146.30</td>
<td>175.84</td>
<td>0.836</td>
<td>80.27</td>
<td>16.64</td>
</tr>
<tr>
<td>1st Sup</td>
<td>14.52</td>
<td>1,860.51</td>
<td>0.008</td>
<td>7.97</td>
<td>0.16</td>
</tr>
<tr>
<td>1st NP40 Pellet</td>
<td>95.82</td>
<td>81.01</td>
<td>1.182</td>
<td>52.57</td>
<td>23.66</td>
</tr>
<tr>
<td>1st NP40 Sup</td>
<td>16.50</td>
<td>55.59</td>
<td>0.296</td>
<td>9.05</td>
<td>5.94</td>
</tr>
<tr>
<td>2nd NP40 Pellet</td>
<td>4.83</td>
<td>43.32</td>
<td>0.111</td>
<td>2.65</td>
<td>2.22</td>
</tr>
<tr>
<td>2nd NP40 Sup</td>
<td>88.12</td>
<td>18.45</td>
<td>4.771</td>
<td>48.35</td>
<td>95.52</td>
</tr>
<tr>
<td>3rd NP40 Pellet</td>
<td>0.08</td>
<td>32.49</td>
<td>0.002</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>3rd NP40 Sup</td>
<td>8.07</td>
<td>3.70</td>
<td>2.181</td>
<td>4.43</td>
<td>43.62</td>
</tr>
<tr>
<td>But Sup-1</td>
<td>11.34</td>
<td>6.93</td>
<td>1.636</td>
<td>6.22</td>
<td>32.72</td>
</tr>
<tr>
<td>But Sup-2</td>
<td>74.97</td>
<td>2.86</td>
<td>26.210</td>
<td>41.13</td>
<td>524.26</td>
</tr>
<tr>
<td>But Sup-3</td>
<td>4.80</td>
<td>0.58</td>
<td>8.286</td>
<td>2.63</td>
<td>165.50</td>
</tr>
</tbody>
</table>

¹Total APase activity is expressed in µmole PNP liberated/minute

²Specific activity is expressed in µmole PNP liberated/mg protein/minute
Table 4. Partial purification of APase from Sample II

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Apase Activity(^1)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity(^2)</th>
<th>Percent Yield</th>
<th>Fold Purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>255.7</td>
<td>4942.00</td>
<td>0.052</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>1st Pellet</td>
<td>199.5</td>
<td>336.10</td>
<td>0.594</td>
<td>78.03</td>
<td>11.42</td>
</tr>
<tr>
<td>1st Sup</td>
<td>28.01</td>
<td>3261.00</td>
<td>0.009</td>
<td>10.96</td>
<td>0.02</td>
</tr>
<tr>
<td>1st NP40 Pellet</td>
<td>150.64</td>
<td>81.61</td>
<td>1.850</td>
<td>58.92</td>
<td>35.58</td>
</tr>
<tr>
<td>1st NP40 Sup</td>
<td>40.80</td>
<td>201.80</td>
<td>0.202</td>
<td>15.96</td>
<td>3.88</td>
</tr>
<tr>
<td>2nd NP40 Pellet</td>
<td>21.16</td>
<td>65.92</td>
<td>0.320</td>
<td>8.28</td>
<td>6.17</td>
</tr>
<tr>
<td>2nd NP40 Sup</td>
<td>127.00</td>
<td>20.05</td>
<td>6.330</td>
<td>49.66</td>
<td>121.79</td>
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<td>3rd NP40 Pellet</td>
<td>3.38</td>
<td>36.71</td>
<td>0.092</td>
<td>1.32</td>
<td>1.77</td>
</tr>
<tr>
<td>3rd NP40 Sup</td>
<td>17.27</td>
<td>6.40</td>
<td>2.700</td>
<td>6.75</td>
<td>51.88</td>
</tr>
<tr>
<td>But Sup-1</td>
<td>29.40</td>
<td>20.95</td>
<td>1.460</td>
<td>11.50</td>
<td>26.98</td>
</tr>
<tr>
<td>But Sup-2</td>
<td>177.00</td>
<td>4.06</td>
<td>43.590</td>
<td>69.22</td>
<td>838.33</td>
</tr>
<tr>
<td>But Sup-3</td>
<td>13.42</td>
<td>1.34</td>
<td>10.010</td>
<td>5.25</td>
<td>192.50</td>
</tr>
</tbody>
</table>

\(^1\)Total APase activity is expressed in \(\mu\)mole PNP liberated/minute

\(^2\)Specific activity is expressed in \(\mu\)mole PNP liberated/mg protein/minute
Table 5. Immunization of Sprague-Dawley rats

<table>
<thead>
<tr>
<th></th>
<th>Rat 1</th>
<th>Titer</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preinjection</td>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40</td>
<td>40</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>2 weeks</td>
<td>320</td>
<td>320</td>
<td>320</td>
<td>160</td>
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<tr>
<td>4 weeks</td>
<td>2560</td>
<td>2560</td>
<td>2560</td>
<td>2560</td>
<td></td>
</tr>
<tr>
<td>Positive control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>320</td>
<td>320</td>
<td>320</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>Negative control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
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</tr>
<tr>
<td>Buffer&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Titer is the inverse of the last dilution which gave a positive result.

<sup>b</sup>Positive control was *E. coli* APase and Goat anti *E. coli* APase.

<sup>c</sup>Negative control was SJL 46 APase and normal rat serum.

<sup>d</sup>Buffer control was working buffer without APase.
Screening of Culture Supernatant

After 14 days of incubation of the plates, clusters of large cells started to appear in many wells while the tiny spleen cells disappeared from almost all the wells. All the 960 wells plated were screened by EAIA method. Eight wells were positive by showing strong yellow color after 1 hour incubation of the test at 37°C. Figure 5 shows an example of one EAIA plate with 4 positive wells.

Cloning of Hybrids

Six of the eight positive wells were cloned. The positive clones obtained from the six wells are shown in Tables 9 and 10.

Eight positive clones from cells suspended in DMEM-HAT immediately after fusion were obtained. These clones appeared earlier. Five positive wells obtained from hybrids, suspended in DMEM after fusion and 24 hours later changed to DMEM-HAT became negative after the first cloning (Table 7).

Estimation of Fusion Frequency

Figure 5 shows the estimation of the fusion frequency of the hybridoma. The overall fusion frequency was 1 in $2.8 \times 10^7$. 
Table 6. Cloning results of the positive well from DMEM-HAT

<table>
<thead>
<tr>
<th>Positive well after fusion</th>
<th>Number of cells plated per well</th>
<th>Number of positive wells obtained from each plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Positive wells after fusion</td>
<td>Cell count per ml</td>
<td>Number of cells plated per well</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>1B</td>
<td>$7.5 \times 10^4$</td>
<td>$8,4,2,1,0.5$</td>
</tr>
<tr>
<td>2B</td>
<td>$4.5 \times 10^4$</td>
<td>$8,4,2,1,0.5$</td>
</tr>
<tr>
<td>3B</td>
<td>$4.5 \times 10^4$</td>
<td>$8,4,2,1,0.5$</td>
</tr>
<tr>
<td>4B</td>
<td>$5.0 \times 10^4$</td>
<td>$8,4,2,1,0.5$</td>
</tr>
<tr>
<td>5B</td>
<td>$1.9 \times 10^5$</td>
<td>$8,4,2,1,0.5$</td>
</tr>
</tbody>
</table>
**Estimation of fusion frequency**

Spleen cells used

9.8 $\times 10^7$

HAT at time 0

= 4.9 $\times 10^7$

HAT after 24 hours

= 4.9 $\times 10^6$

Number of positive wells

1 7

Fusion frequency

1 in 4.9 $\times 10^7$ 1 in 7.0 $\times 10^6$

Overall fusion frequency

1 in 2.8 $\times 10^7$
This investigation proposed to produce monoclonal antibody against alkaline phosphatase (APase) isolated from a lymphoid cell line, SJL 46. A high degree of purification was achieved by solubilizing the enzyme with NP40 detergent and extracting it with butanol. Purification on the order of 20-30 fold was achieved by Davis (1977) who used this method to purify APase from mouse placenta tissue. The improved purification achieved with Samples I and II could be due to a better recovery of APase from pellets. Unlike the procedures of Davis in which the supernatants were pooled before butanol extraction, the individual NP40 supernatants were extracted separately with butanol. While Davis obtained a purification fold of 16.6 after butanol extraction of the pooled NP40 supernatant a much higher purification fold of 838 was obtained in this investigation when the individual NP40 supernatants were extracted separately with butanol. The higher fold purification achieved could be due to the fact that the APase was purified from a homogeneous cell line instead of from tissue which contained more extraneous materials.

Electrophoresis of the partially purified APase shows three protein bands including that of the APase. The proteins used as standards are the red cell membrane proteins (Fairbanks et al, 1971) instead of the commercially available standard proteins. This is because the APase is also a membrane protein. The molecular weight of the partially purified APase was 135,000 ± 8000. Davis (1977) obtained the molecular weight of 170,000 for placental alkaline phosphatase. The difference could be due to the fact that Davis purified the APase from a tissue while this investigation purified the APase from a cell line.

Our immunization protocol gave a very satisfactory immune response in rats. Although 2-4 volumes of complete adjuvant to 1 volume of antigen has been
suggested to give a very good immune response (Hurn and Chantler, 1980) our investigation demonstrated that 1 volume of complete Friend's adjuvant to 2 volumes of APase could still give an equally adequate immune response.

The proportion of the myeloma cells to the spleen cells (1:4) used in the fusion yielded satisfactory results. This agrees with the suggestion that higher proportion of the spleen cells to myeloma cells should be used to increase the chance of myeloma-spleen cell fusion and decrease that of myeloma-myeloma cell fusion (Kohler and Milstein, 1975, 1976).

The fusion frequency of the hybridoma is very low. This agrees with Stahli et al (1980) who said that soluble antigens induce low fusion frequencies. In general, low fusion frequency is due to a low number of specific B cells in the heterogenous mixture of spleen cell population at the time of fusion. The specific B cell population could be increased by giving 3-4 booster dose injections prior to fusion (Stahli et al, 1980) or by culturing the immune spleen cells in vitro in the presence of the antigen, for 4 days, prior to fusion (Fox et al, 1981).

One of the most striking features in the cloning of hybrids in this investigation, was that out of the six strongly positive fusion wells cloned, only one yielded 8 positive clones, while the rest gave negative clones after the first cloning. Also, it was observed that the positive clones appeared earlier and came from the hybrids suspended directly in DMEM-HAT medium, while the unstable clones appeared later and came from hybrids suspended in DMEM but changed to DMEM-HAT after 24 hours. While the issue of frequent loss of chromosomes by hybrid cells, generally, could be attributed to the instability of the positive clones, the observation by Fazekas de St Groth and Scheidegger (1980) that SP2/0-hybrids were more prone to
chromosome loss than the X63-hybrids could be more applicable here. They explained that many clones positive for antibody production in the first incorporation test (usually between day 14 and 21) registered later as non producers. Interspecies hybridization is another contributive factor to chromosome loss. Most interspecies hybrids are unstable, and it is not surprising if this is the case. The enhancement of positive clones by direct suspension of hybrids in DMEM-HAT in the experiment agrees with Fazekas de St Groth and Scheidegger (1980), when they observed that clones of hybrid cells resuspended directly in HAT medium appeared earlier and had a higher frequency of survival than those suspended directly in the growth medium and changed to HAT after 24 hours.

The screening assay employed in the experiment was the enzyme-antigen-immunoassay (EAIA) by Jemmerson and Fishman (1982). It specific and reliable, if the test is properly standardized for the desired experiment before being employed. The positive and negative controls gave their expected results throughout the experiments and reproducible results were obtained. This agrees with the observation of Jemmerson and Fishman (1982) that EAIA technique could be applicable to the detection of antibodies to isozymes of human placental APase.

Finally, the objective of the investigation to produce monoclonal antibody against SJL46-APase was accomplished by obtaining eight clones secreting anti-APase monoclonal antibody.

To fully utilize the anti-APase monoclonal antibody in the study of T cell differentiation pathway the following additional work on the monoclonal antibody is recommended:

1) to perform Ouchterlony test on the antibody using anti-rat IgM, IgG1-4, IgA to determine its isotype,
2) to perform EAIA test using the neuramimidase-treated APase antigen to determine whether the monoclonal antibody binds to the sugar moiety or the protein moiety of the APase,

3) to perform Western blot of the APase to further confirm its specificity, and

4) to perform Ouchterlony test on the monoclonal antibody using extracts from other APase lymphoid cell lines, human placenta, E. coli, calf intestine, beef liver, mouse placenta, and SJL 46 antigens so as to determine the immunological relationship between the SJL 46 APase and these isozymes.

In spite of the success of this investigation, there are problems unsolved in the hybridoma technology. The specific role of the macrophage feeder cell is still not quite understood. The issue of chromosome loss by hybrid cells can still not be completely remedied.

While the problems remain, a lot can be achieved in APase-hybridoma technology of certain factors are kept in mind. Before starting APase-hybridoma, every step must be clearly understood. It is necessary to partially purify the enzyme to enhance its specific immune response. It is very important to decide on the screening assay and to standardize it to the desired specificity before starting the hybridoma experiments. Contamination of culture cells is a major problem in hybridoma technology, and it can be reduced if aseptic techniques are always observed.
CHAPTER VI

SUMMARY

1. Five hundred to eight hundred fold of APase purification was obtained from SJL-46 cells by solubilization with NP40 detergent and extracted with butanol.

2. One part of complete adjuvant to two parts of the partially purified SJL 46 APase gave adequate immune response in rats for hybridization.

3. Mouse myeloma cells (SP2/0) and spleen cells from a rat immunized with the APase were fused in the ratio of 1:4.

4. The anti-APase monoclonal antibody was detected by enzyme–antigen–immunoassay (EAIA), a reversed ELISA technique, using the catalytic activity of the SJL 46 APase.

5. Eight clones secreting anti-APase monoclonal antibody were isolated.

6. Clones from cultures resuspended immediately after fusion in DMEM-HAT appeared earlier and had a higher number of surviving hybrids than those resuspended in DMEM after fusion and changed to DMEM-HAT after 24 hours.
LITERATURE CITED


APPENDIX A

RPMI 1640 Medium

RPMI 1640 (Gibco Laboratories) 98.0 ml
Fetal Calf Serum 10.0 ml
2-Mercaptoethanol (5 X 10⁻³ M) 0.01 ml
Penicillin/Streptomycin (5000 I.U./ml, 5000 mcg/ml) 1.0 ml
Glutamine (29.2 mg/ml) (Gibco Laboratories) 1.0 ml

Filter sterilize and incubate at 37°C for 24 hours to check sterility. Store in the refrigerator.
APPENDIX B

Components of SDS PAGE

1. Stacking gel

4.5% Acrylamide

1.2% N,N'-methylene bis acrylamide

0.077% Sodium dodecyl sulfate

2. Gradient gel (5-15% (16.0 ml)

<table>
<thead>
<tr>
<th>Low (5%)</th>
<th>Components</th>
<th>High (15%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.83 ml</td>
<td>30% Acrylamide</td>
<td>8.5 ml</td>
</tr>
<tr>
<td>1.7 ml</td>
<td>1.0 M Tris HCl pH 8.8</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>12.47 ml</td>
<td>Deionized water</td>
<td>6.88 ml</td>
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<tr>
<td>17.0 μl</td>
<td>100% N,N,N',N'-tetramethylene diamine</td>
<td>17.0 μl</td>
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<tr>
<td>32.0 μl</td>
<td>10% Ammonium persulfate</td>
<td>17.0 μl</td>
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**APPENDIX C**

**Components of Silver Stain**

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>45% Methanol, 12% Acetic acid</td>
</tr>
<tr>
<td>II</td>
<td>10% Ethanol, 5% Acetic acid</td>
</tr>
<tr>
<td>III</td>
<td>10% Glutaraldehyde</td>
</tr>
<tr>
<td>IV</td>
<td>10 μg/ml Dithiothreitol (DTT)</td>
</tr>
<tr>
<td>V</td>
<td>0.012 M AgNO₃</td>
</tr>
<tr>
<td>VI</td>
<td>0.28 M Na₂CO₃ with formalin (3 drops)</td>
</tr>
<tr>
<td>VII</td>
<td>1% Acetic acid</td>
</tr>
<tr>
<td>VIII</td>
<td>Farmer's reducer for reduction of surface stains</td>
</tr>
</tbody>
</table>

   Solution A = 7.5% Potassium ferricyanide
   Solution B = 24% Sodiumthiosulfate

Working solution: 2.5 ml A + 10 ml B + 250 ml deionized water.
APPENDIX D

Dulbecco's Modified Eagle's Medium (DMEM)

Reinforced Minimal Essential Medium 816 ml

1M HEPES (N-2-Hydroxyethyl piperazine-N2-ethan-sulfonic acid) 12.0 ml
Glutamine (100 X) (Gibco Laboratories) 12.01 ml
Insulin-transferrin-selinium (ITS) 24.01 ml
Sodium bicarbonate (7.5%) 12.0 ml
5 X 10^-3 M 2-Mercaptoethanol 12.0 ml
Nonessential amino acids (100 X) 12.0 ml
Fetal calf serum 100 ml

Filter sterilize; incubate at 37°C for 24 hours to check sterility. Store in refrigerator.
APPENDIX E

Preparation of DMEM-HAT Medium

DMEM (Appendix C) 49.0 ml
HAT (Hypoxanthine, Aminopterin, Thymidine)-media supplement (50 X) 1.0 ml
Store frozen
Thaw at 37°C water bath for use
APPENDIX F

Conditioned Medium

SP2/0 Culture supernatant 100.0 ml
Spin at 1000 rpm for 15 minutes
Filter sterilize
Store frozen

For use:
Conditioned medium (thawed at 37°C water bath) 50 ml
Glutamine (29.2 mg/ml) (Gibco) 0.5 ml
Calf serum 5.0 ml
DMEM (Appendix D) 50 ml