RAT X MOUSE HYBRIDOMA SECRETING MONOCLONAL ANTIBODY TO MOUSE ALPHA-1-PROTEASE INHIBITOR:

DEVELOPMENT AND CHARACTERIZATION

By

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I am indebted to Miss Nancy Lyons for typing this manuscript.
In our investigations concerning the control of synthesis of mouse alpha-1-protease inhibitor (α1Pi), also called alpha-1-antitrypsin (α1AT), a major inhibitor of serum proteases, we had reason to attempt to develop a monoclonal antibody to this molecule to clarify some apparent molecular heterogeneity, as conventional approaches to separate the isoproteins had met with limited success. We immunized Lewis rats with purified mouse α1Pi and fused the immune spleen cells with mouse SP2 plasmacytoma cells after optimizing some of the fusion parameters such as the source of polyethylene glycol (PEG), its concentration and exposure time to cells. In addition, we examined two different fusion protocols for their ability to produce xenogenic rat × mouse hybrid cells. Several hybridomas were produced, one in particular secreting rat IgM specific for mouse α1Pi. Screening of the positive clones was carried out by modified ELISA and radioimmunoassays. Characterization of the cell product was completed by SDS-PAGE and radioimmunoelectrophoresis. Specificity of the secreted immunoglobulin (D7-IgM) was determined using crossed radioimmunoelectrophoresis with 35S-methionine labelled monoclonal antibody. The establishment of a mouse ascitic form of the hybridoma was not successful even after prolonged subcutaneous adaptation of the hybridoma line in ALS treated, bone marrow reconstituted, X-irradiated Balb/c recipients. Mice bearing the solid tumor exhibited
pathology in the liver in the form of discrete necrotic lesions
suggesting metastatic localization of the hybridoma in the liver and
local production and interaction of the monoclonal antibody with the
hepatocyte via one of its secreted molecules, alpha-1-protease inhibitor.
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1.0 INTRODUCTION

Inflammation is a series of complex processes mediated by cellular and humoral mechanisms. Central to humoral involvement are the major biochemical pathways of inflammation, consisting of the Complement system, the Clotting system including Coagulation and Fibrinolysis, and the Kallikrein-kinin system. These pathways are not isolated but interact with each other with the products and fragments of one system turning on and/or accelerating the others.

Interactions between these systems must be controlled to avoid undue activation and subsequent non-specific tissue destruction. This regulation is supplied by a group of proteins termed serum protease inhibitors which include alpha-1-protease inhibitor (α1Pi), otherwise known as alpha-1-antitrypsin (α1AT), which inhibits elastase, trypsin, chymotrypsin, plasmin and other leukocyte neutral proteases; antithrombin III (AT3) which regulates coagulation by inhibiting thrombin; Cl-esterase inhibitor (ClNH) which inhibits Cl, plasmin, kallikrein and activated Hageman factor (factor XII); and alpha-2-macroglobulin (α2M) a broad spectrum protease inhibitor capable of interacting with most endopeptidases tested. In addition, there is α1-antichymotrypsin and α1-antiplasmin which have major inhibitory activities on chymotrypsin and plasmin respectively (1,2,3,4,5).
α1-Protease inhibitor (α1Pi) also belongs to a group of serum proteins termed acute phase reactants (APR) which are characterized by showing an increase in serum levels within hours in response to acute tissue damage (6). Other acute phase reactants are fibrinogen, C-reactive protein (CRP), serum amyloid A protein (SAA), ceruloplasmin, α1-acid-glycoprotein, haptoglobin and C3 (7). Recently, serum amyloid protein P (SAP) of the mouse (8) and the acute phase α2M of the rat (9) have also been recognized as acute phase reactants. APR stimulation has been suggested to occur as the result of serum factors derived from the site of inflammation, inducing increasing synthesis of the reactants by an increased number of hepatocytes (10).

Although the identity of the messenger is not confirmed, possible candidates are cortisol (11) or a polypeptide termed interleukin 1 (12) having properties similar, if not identical to leukocyte endogenous mediator (LEM) (13), lymphocyte activating factor (LAF) (14), endogenous pyrogen (EP) (15), leukocyte pyrogen (LP) (16) and crude leukocyte extract (CLE) (17). Whether each of the above activities can be ascribed to a single messenger is not established, although the LAF and EP activities have been claimed to be derived from the same molecule (18).

We chose to examine α1Pi as a model to investigate how this inhibitor's regulatory capabilities affects inflammation. Investigation of the sites of synthesis, storage and degradation of this molecule provide further insight into mechanisms governing α1Pi levels and subsequent interaction with the proteases involved in inflammation.
1.1. Inherited Deficiency of Alpha-1-Protease Inhibitor

Inherited deficiencies in α1Pi levels in the human correlates with a markedly increased incidence of juvenile cirrhosis (19), early onset of emphysema (20), and respiratory distress syndrome (21,22). It is believed that the decreased control of inflammation, in particular that caused by neutrophil derived elastase, predisposes those with little or absent circulating α1Pi to develop these diseases. These people can be phenotyped according to their Pi protease inhibitor genotype and have either greatly decreased (PiZZ) or totally absent (Pi--). Persons who are homozygous or heterozygous for the PiZ allele can be shown to have deposits in their hepatocytes which contain periodic acid-Schiff (PAS) positive material identified as α1Pi by immuno-histochemistry (23,24,25). PiZZ deficiency is most likely caused by a defect in the α1Pi transport mechanism in the hepatocyte rather than lack of synthesis. As a possible consequence of accumulation in the hepatocyte, PiZZ, PiMZ and PiSZ phenotypic individuals have increased susceptibility to juvenile hepatitis, cirrhosis and primary liver cancer (26).

Detailed biochemical studies have not exposed significant structural differences between the various α1Pi molecules except in the case of the type Z inhibitor, which shows 20-25% less carbohydrate (27,28). However, subtle amino acid substitutions have been detected in PiZZ and PiSS α1Pi variants (27,29,30) and these single point mutations may alter the molecules' solubility leading to decreased circulating levels.

Laurell proposed a model to represent the development of emphysema (31). Infiltrating leukocytes responding to lung inflammation release
neutral proteases and elastase, destroying elastin fibres of the lung in response to low or non-existent levels of α1PI. Loss of lung tissue elasticity ensues with subsequent wasting. Individuals with normal α1PI levels but who are exposed to oxidizing agents such as those found in cigarette smoke, air pollutants or leukocyte myeloperoxidase may be prone to the development of emphysema through inactivation of α1PI and an effective decrease in inhibitory activity (32). Chemical oxidants themselves may also indirectly inactivate α1PI as they are chemotactic for neutrophils and monocytes which may subsequently release myeloperoxidase and oxidize α1PI in situ.

1.2. Alpha-1-Protease Inhibitor: Characterization

The inhibitor has a defined electrophoretic mobility in the α1 region on serum electrophoresis. Human α1PI is a glycoprotein containing 12-15% carbohydrate, present as oligosaccharide (33). Purified human α1PI has a molecular weight of 50,000 (34) while that of mouse has been described as 53,500 (35). Turnover studies have indicated mouse serum α1PI to have a half life of 15.5 hrs, while that of human is 5-6 days.

α1PI has a major inhibitory activity against elastase and neutral proteases of polymorphonuclear leukocyte origin. In addition, it demonstrates activity against trypsin and to some extent plasmin, plasminogen proactivator and prevents thrombin from interacting with factors V, VIII, XIII of the clotting system (36-40).

α1PI has a methionine residue at its active site and most likely interacts with serine proteases via an acylation reaction involving the
hydroxyl group of serine at its active site with an acyl group on α1Pi (41).

1.3. Alpha-1-Protease Inhibitor: Synthesis and Degradation

In the mouse the majority of α1Pi is synthesized by hepatocytes (42). Human studies of lung (43), alveolar macrophage (44) and gut (45) have demonstrated the presence of α1Pi in these tissues but have not, as yet, shown synthesis of the inhibitor. Recent reports have shown α1Pi to be found associated with platelets (46,47), polymorphonuclear leukocytes (48) and on the surface of Con A stimulated lymphocytes (49). Thus, α1Pi may, in some way, be responsible for taking part in modulating the immune response at a cytotoxic and/or humoral level (50).

In the mouse, alveolar macrophages have been shown, by immunohistochemistry, to contain significant amounts of α1Pi during acute inflammation of the lung probably as a result of uptake of a complex with undetermined proteases (Gauldie, unpublished). This evidence may suggest a possible role in the degradative pathway of α1Pi by the reticuloendothelial system.

1.4. Immunological Analysis of Alpha-1-Protease Inhibitor

During initial investigation of the sites of synthesis and degradation of α1Pi in the mouse, a sheep antiserum was used extensively for serologic and immunohistologic studies. Antisera used in crossed immunoelectrophoresis (XIEP) of α1Pi revealed two immunologically distinct forms of α1Pi in purified preparations of the molecule, as well as in serum, plasma,
ascitic fluid, liver extracts or supernatants of cultured hepatocytes which did not appear to be a result of a purification artifact (35). Both forms bound $^{125}$I-trypsin, increased identically in response to inflammation and demonstrated the same antigenic characteristics (52). Lee and Janoff have reported similar findings in the rat with respect to amounts, capabilities of binding $^{125}$I-trypsin and XIEP pattern (53). $\alpha$1Pi and its associated electrophoretic and immunologic non-identity in both mouse and rat suggest there are two distinct, separate molecules each possibly playing a different function in controlling the mediators of inflammation. Specific $\alpha$1Pi antisera capable of identifying the two variants by XIEP precipitated only a single labelled precursor polypeptide from mRNA extracts of hepatocytes in a rabbit reticulocyte system (data unpublished). Since the hepatocyte synthesizes both serum forms, the electrophoretic heterogeneity may arise from post-translational events such as glycosylation, while the immunological non-identity may be explained by the occurrence of false spur formation in two-dimensional immunoelectrophoresis (54).

To clarify the apparent molecular heterogeneity we chose to develop monoclonal antibodies to one or both forms of the inhibitor since attempts at conventional purification procedures did not yield any purified molecules of a single type. Monoclonal specificity provided by the development of such a reagent could not be duplicated by absorptions of antisera. Additionally the monoclonal reagents would allow for rapid purification of $\alpha$1Pi by affinity chromatography while providing ultra pure reagents for further immunohistochemical studies of the sites of synthesis and degradation of $\alpha$1Pi.
1.5. Monoclonal Antibodies/Hybridomas

Conventional antisera raised to a specific molecule involves isolation of the antigen by immunological and/or biochemical techniques but these approaches can rarely result in homogeneous, pure preparations. Subsequent injection of the antigen preparation into animals induces antibody responses to wanted as well as unwanted molecules. In addition, the induced response causes stimulation of many different antibody-producing clones resulting in a heterogeneous polyclonal antiserum having a mixture of specificities and binding affinities. It would be ideal if one could stimulate only a single specific antibody-producing clone resulting in a homogeneous response with a defined/restricted activity.

Plasma cells derived from B lymphocytes are responsible for antibody synthesis but are themselves unable to survive long term culture. Plasmacytomas are a malignant form of plasma cells with a capacity to proliferate rapidly and survive for exceptionally long periods of time, under tissue culture conditions. Plasmacytomas while maintaining this extraordinary ability also continue to synthesize and secrete immunoglobulin though it is usually of undetermined specificity.

Fusion between appropriate spleen cells and plasmacytomas can give rise to hybrid cells or hybridomas which have the properties of both cell types, in particular, retaining the ability to secrete specific homogeneous antibody while growing in culture for extremely long periods of time. The supernatant of these cultures contain monoclonal antibody which can be used as an immune reagent.
1.6. Selection of Hybridomas

Spleen cells maintain DNA synthesis through both the purine/pyrimidine biosynthetic pathway and through the salvage pathway which itself requires the presence of both enzymes thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT). Treatment with the folic acid antagonist aminopterin, blocks the main pathway for DNA synthesis. However, if these cells are given hypoxanthine and thymidine, DNA synthesis may continue through the salvage pathway (Figure 1).

Plasmacytoma cells used in fusions have been selected for the deficiency of the enzyme HGPRT (HGPRT\(^{-}\)). Treatment with aminopterin blocks the major DNA synthetic pathway and, because these cells lack HGPRT, no DNA synthesis can occur via the alternate or salvage pathway, resulting in cell death.

Fusion of spleen cells with plasmacytomas results in the formation of hybridomas which contain the HGPRT enzyme. Addition of hypoxanthine, aminopterin and thymidine (HAT) allows continued selective growth of hybridomas (HGPRT\(^{+}\)) while killing plasmacytomas (HGPRT\(^{-}\)). Unfused spleen cells die in culture within a few days (55) (Figure 1).

1.7. Production of Monoclonal Reagents

Köhler and Milstein in 1975 fused mouse spleen cells sensitized to sheep red blood cells (SRBC) with the plasmacytoma cell line x63-Ag-8 (HGPRT\(^{-}\)) and selected for a hybridoma secreting anti-SRBC (56). Most of the mouse plasmacytoma cell lines subsequently used for hybridoma fusion are derived from the original x63-Ag-8 line and include two subtypes:
Figure 1

HAT selection. Cell DNA synthesis requires appropriate nucleotide precursors from either the main purine/pyrimidine biosynthetic pathway or, via the salvage pathway. Folic acid, a coenzyme participates in the methylation reactions involved in the biosynthesis of nucleotides and is blocked by the presence of aminopterin. DNA synthesis however, may still continue via the salvage pathway if both thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzymes are present. Cells lacking HGPRT and supplied with aminopterin are killed.
P3NS1/1-Ag 4-I (P3NS1) which synthesizes only a K light chain but does not secrete it; Sp2/O-Ag 14 (SP2) which has completely lost the ability to synthesize its own immunoglobulin heavy and light chain.

Sendai virus was initially used as the fusing agent, however, polyethylene glycol (PEG) has been shown to give better results and is the fusing agent of choice (57,58).

Hybridoma cells are screened for the production of specific antibody by appropriate assays. When cells are shown positive for specific antibody they are quickly cloned by either limiting dilution cultures or isolated in semi-solid agarose. This subcloning prevents overgrowth by irrelevant hybridomas which may replicate faster resulting in the apparent loss of a positive clone. Cells which survive this cloning appear to be more stable and resistant to chromosome loss and disengagement of immunoglobulin synthesis (59).

Hybridomas grown under normal tissue culture conditions produce average antibody yields ranging from 10 to 100 μg/ml of media. If hybridomas are introduced into recipient animals of an appropriate genetic background ascites result and monoclonal antibody may be collected in quantities of milligram/ml of ascitic fluid.

1.8. Detection of Monoclonal Antibodies

Assay systems chosen must be reliable, fast and flexible enough to allow examination of large numbers of individual wells containing cells. Examples of a number of assay systems include enzyme linked immunosorbent
assay (ELISA) (60), Chromium release cytotoxicity (61), immunofluorescence (62), hemagglutination (63), and both solid and fluid phase radioimmunoassay (RIA) (68). We used both fluid phase RIA and ELISA methodology to screen for positive hybridomas and to monitor production of specific anti-mouse αIPI antibody by the hybridoma lines.

1.9. Xenogenic Fusion - Rat X Mouse

Immunization increases the number of proliferating B cells in the spleen specific for the eliciting antigen and, increases the probability of generating relevant hybridomas, as the proliferating B cell appears to be the cell favouring hybridization (65). Immunization schedules vary according to the antigen, the method of presentation to the immune system and the degree of antigenicity. Generally, soluble as well as cellular antigens are first presented to the immune system with an adjuvant such as complete Freund's adjuvant (CFA) by an intraperitoneal (IP) or intramuscular (IM) route. This is followed by a boost administered intravenously (IV) three days prior to fusion. IV presentation of the antigen is critical since it induces B cell proliferation and localization within the spleen, which is the organ taken as the cell source for fusions.

In our case, immunization of an appropriate animal species was governed by the ability of that species to mount an immune response against mouse αIPI while also forming stable hybridomas with mouse plasmacytomas. Rabbits, when immunized against mouse αIPI, develop good circulating levels of specific antibody but when fused to mouse plasmacytomas, in the majority of cases, form unstable hybridomas as a result of chromosome
loss. Allotypic immunization in mice was expected to generate a response due to polymorphic differences in strain specific α1PI, but, this response would be weak at best because of the marginal antigenic differences. These expected polymorphic forms however, have not yet been demonstrated. Mouse spleen cells, however, were the best choice in fusions with mouse plasmacytomas, because they form stable secreting hybridomas. Xenogenic fusions between rat and mouse have been reported to produce stable hybridomas capable of long term culture while maintaining their ability to secrete specific immunoglobulins (66). Although rat and mouse α1PI were shown to cross react (35), enough antigenic differences were anticipated to induce sufficient immune responses.

This thesis reports the successful application of hybridoma technology resulting in the production, isolation and characterization of rat X mouse monoclonal antibodies to the mouse protein alpha-1-protease inhibitor (α1PI).
2.0 MATERIALS AND METHODS

2.1. Media

Media and their supplements were purchased from Grand Island Biological Company (GIBCO).

2.1.1. Complete medium for cultures

Roswell Park Memorial Institute (RPMI) media, and Dulbecco's modified Eagles media (DMEM) were used as base medium and were supplemented with 1% v/v 10^{-7} M Hepes, 8.9 \times 10^{-3} M \text{NaHCO}_3, 1-glutamine 0.03% w/v, 100 units/ml penicillin, 100 \mu g/ml streptomycin and were referred to as RPMI complete and DMEM complete, respectively. Each of the complete media were supplemented with 10% v/v fetal calf serum (FCS).

2.1.2. Hybridoma medium (Hy)

Complete media were supplemented with 10^{-3} M oxaloacetate, 4.5 \times 10^{-4} M \text{pyruvate}, 10% v/v NCTC 109 medium (Microbiological Associates) and 20% v/v FCS.

2.1.3. Hy-HAT

Hy medium was supplemented with 10^{-4} M hypoxanthine, 8.0 \times 10^{-4} M aminopterin and 1.6 \times 10^{-5} M thymidine (HAT) (67).

2.1.4. Freezing media

10% v/v dimethylsulfoxide was added to DMEM complete or neat FCS
and were referred to as freezing media. Cells were suspended at 1.5 - 2.0 X 10^7/ml in freezing medium, cooled 2 hrs at 4°C, placed in a -70°C freezer overnight and subsequently stored cryogenically in liquid nitrogen.

2.1.5. Serum for culture

Heat inactivated FCS (56°C, 30 mins) was tested in serum lots for their capability to support plasmacytoma and hybridoma cell growth. Cells were suspended in either DMEM or RPMI complete media supplemented with 10% v/v FCS from different lot numbers. Approximately 5-10 cells/200 μl were distributed into wells of a 96 well microtiter plate for each lot tested. Plates demonstrating cell growth in greater than 50% of the wells were considered to contain appropriate FCS for use in cultures.

2.2. Buffers

All chemicals were purchased from Fischer Scientific Company.

2.2.1. Barbital buffer

2.07 gms of barbital and 13 gms of sodium barbital were dissolved in 1 liter of deionized distilled water. Glycine (56.2 gms) and 45.2 gms of Tris were dissolved in a second liter of deionized distilled water and mixed 1:1 with the barbital solution (pH 8.8).

2.2.2. Bicarbonate/carbonate buffer

Na₂CO₃ (1.59 gms), 2.93 gms of NaHCO₃, 0.2 gms of NaN₃ were added to 1 liter of deionized distilled water and adjusted with HCl (1N) to pH 9.6.
2.2.3. Dulbecco's B buffer

\[
\text{CaCl}_2 \ (0.1 \text{ gm}) \text{ plus } 0.1 \text{ gm MgCl}_2 \text{ per liter of PBS (pH 7.3).}
\]

2.2.4. Phosphate buffered saline (PBS)

Twenty-three mls of a 0.2M solution of monobasic sodium phosphate and 77 mls of 0.2M dibasic sodium phosphate were diluted with 0.15M sodium chloride to a final volume of 1 liter (pH 7.3).

2.3. Plasmacytomás

P3NS1/1-Ag 4-1 (P3NS1) and SP2/0-Ag 14 (SP2), subclones of P3-x63-Ag-8, both being HPRT deficient were a generous gift from Dr. R.H. Kennett, Wistar Institute, Philadelphia, USA.

Plasmacytomás were maintained in stationary cultures at cell densities of 2-8 \( \times 10^5/\text{ml} \), in either RPMI complete or DMEM complete, both supplemented with 10\% v/v FCS.

Fused cells were first grown in Hy-HAT plus 20\% v/v FCS and once hybridomas were established, cells were maintained in Hy-media, with 20\% v/v FCS.

2.4. Cell Viability

2.4.1. Ethidium bromide

Stock solutions of ethidium bromide (EB) (Calbiochem), 10 mg/ml PBS were stored in the dark at 4\°C and fluorescein diacetate (FDA) (Sigma), 5 mg/ml acetone was stored in darkness at -20\°C. Immediately prior to use, EB and FDA were thawed and 400 \( \mu \text{l} \) EB stock plus 15 \( \mu \text{l} \) FDA stock were added to 4,000 \( \mu \text{l} \) of DMEM. Stain (EB/FDA) was stored on ice until used.
Cells to be tested were suspended in DMEM complete at densities of $5 \times 10^6$/ml. Five hundred $\mu$l of cells ($2.5 \times 10^6$) at room temperature were added to test tubes followed by 25 $\mu$l of EB/FDA stain. Cells were vortexed for 10 secs and 20 $\mu$l counted in a hemocytometer. After settling cells were viewed under fluorescent light, live cells appeared green while dead cells appeared red.

2.4.2. Trypan blue

Stock solution of 0.4% trypan blue was diluted 1:4 in PBS. Cells were mixed 1:200 with trypan blue and allowed to stand for 1 min. Cell viability was then determined using a hemocytometer. Cells taking up trypan dye were considered dead, while cells excluding the dye were viable.

2.5. Purification of Alpha-1-Protease Inhibitor

Purified $\alpha$1Pi was provided by Dr. J. Gauldie and has been described in detail elsewhere (35). In short, adult Balb/c mice were bled out and their sera pooled. Plasma was collected and precipitated with $(\text{NH}_4)_2\text{SO}_4$ at a final concentration of 40%. To remove antithrombin III, the resulting supernatant was passed through a Sepharose-heparin column. The protein solution was concentrated by ammonium sulfate precipitation, chromatographed on a DEAE-Sephadex A-50 column ($0.05\text{M Tris}$, $0.05\text{M NaCl}$, pH 8.8), and eluted with a linear gradient ($0.05$-$0.2\text{M NaCl}$). Fractions demonstrating antitryptic activity were pooled and chromatographed on a Sepharose-Concanavalin A column ($0.1\text{M Tris/HCl}$, pH 7.5 containing $0.5\text{M NaCl}$, and $1\text{mM}$ each of $\text{MnCl}_2$, $\text{MgCl}_2$ and $\text{CaCl}_2$), $\alpha$1Pi being eluted by
1-O-methyl-α-D-glucopyranoside (0.1M) in the starting buffer. The eluate was passed through a Cibacron blue-Sepharose column to remove albumin. The effluent was further chromatographed on a DEAE-Sephadex A-50 column (5 mM phosphate buffer, 0.05M NaCl, pH 6.5) and eluted with a linear gradient (0.05M - 0.2M NaCl). Preparative acrylamide gel electrophoresis was employed to separate a minor contaminant. The final preparation was homogeneous in analytical polyacrylamide gel electrophoresis and contained both forms of α1Pi as shown by two-dimensional immunoelectrophoresis against sheep anti-mouse α1Pi reagent.

2.6. Alpha-1-Protease Inhibitor Iodination

Fifty μl of purified α1Pi (2 mg/ml) was added to 1 mCi Na$_{125}^+$I (New England Nuclear) in a volume of 25 μl PBS in a glass test tube. Chloramine T (7.7 μl; 6.5 mg/ml of distilled water) was added and allowed to react for 60-90 sec. Immediately thereafter, 100 μl of potassium iodide (13.5 mg/ml PBS) was added, followed by the addition of 25 μl of sodium thiosulfate (10.5 mg/ml PBS). This was chromatographed on a 10 cm anion-exchange resin column (Baker Chemicals) and 10 drop fractions collected. Radioactivity was assessed for each fraction using an automated Beckman 8000 gamma counter. $^{125}$I-α1Pi was pooled and stored at 4°C.

2.7. Immunization

Male Lewis rats (Trudeau Institute) weighing 150-175 gms were used for the production of antisera and the generation of immune splenocytes for fusion.
Initial sensitization was accomplished by injecting 50 μg of purified mouse αλPi, emulsified 1:1 in complete Freund's adjuvant (CFA) in a volume of 1 cc, IP. Subsequent serial boosts of 50 μg of purified αλPi in saline were administered biweekly in a volume of 0.5 cc, IP. Animals were bled via the tail vein 10 days following each boost and the sera were assayed for antibody activity against αλPi by radioimmunoassay (see section 3.3.1.). Animals demonstrating good immunological responsiveness to αλPi were rested for 2 weeks, given a final boost (50 μg) IV and sacrificed 3 days later.

2.8. Reagents

Rabbit IgG anti-rat IgG (heavy and light chain specific), rabbit IgG anti-rat IgM (heavy chain specific), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were obtained from Cappel Laboratories.

Goat anti-rat IgM was a gift from Dr. H. Bazin. Purified rabbit IgG, sheep anti-mouse αλPi and anti-mouse α serum were routinely available in our laboratory.

2.9. Assays

2.9.1. Radioimmunoassay RIA

Fluid phase RIA was adapted from a technique described by Nisonoff et al. (68).

Wells of 96-well flat bottom microtiter plates were soaked with 200 μl of normal non-immune human sera, FCS, agamma horse sera or 1% bovine serum albumin (BSA) for 2 hrs at 4°C to block non-specific binding
of mouse α1Pi or antibody reagents to the plastic surfaces. Ten μl of
125I-mouse α1Pi, having an activity of 5,000 CPM (50 ng) were added to
each well followed by 100 μl of hybridoma supernatant, dilutions of
positive control sera, dilutions of negative control sera or media alone.
Plates were incubated for 1 hr at 37°C. Antibody binding 125I-α1Pi was
precipitated with saturated (NH₄)₂SO₄ at a final concentration of 40%.
Plates were incubated for 30 min at 4°C and then spun at 3,000 x g for
15 min at 4°C in a refrigerated MSE centrifuge. Supernatants were collected
and held separately while precipitates were washed twice with 100 μl of
PBS.

Wells were of the snap off type and were subsequently broken off
and counted along with their respective supernatants in an automated
Beckman 8000 gamma counter.

2.9.2. Enzyme linked immunosorbent assay (ELISA)

The ELISA protocol outlined was adapted from the technique
reported by Ruitenber et al.(69).

One μg of purified mouse α1Pi in 100 μl of 0.05M bicarbonate
buffer (pH 9.6) was added to each "break off" well of 96 well flat-
bottomed microtitre plates and incubated for 2 hrs at 37°C to allow
adsorption to the plastic. Excess α1Pi was decanted and the wells washed
2X with 100 μl of 0.85% saline with 0.05% Tween-20, a wetting agent.
Non-specific binding of reagents was prevented by blocking with 200 μl
of 1/40 dilutions (2.5%) normal human serum, incubated for 2 hr at 4°C.

Plates were washed 2X with PBS-Tween-20. One hundred μl of
hybridoma supernatants, dilutions of positive and negative control sera,
or media alone was added to wells and incubated for 2 hr at room temperature. Wells were washed as above and rat anti-mouse α1Pi immunoglobulin detected by the addition of 100 μl of rabbit anti-rat IgG or rabbit anti-rat IgM diluted 1/100 with PBS. Wells were washed and 100 μl of 1/200 HRP conjugated goat anti-rabbit IgG added and incubated 1 hr at 22°C after which the plates were washed free of unbound conjugate.

Bound HRP-conjugate was detected by the addition of 50 μl of substrate consisting of 5-aminosalicylic acid (5-AS) (9 ml of 0.08% 5-AS, pH 6, and 1 ml of 0.05% H₂O₂). Incubation was carried out for 25 min at 22°C and stopped by the addition of 50 μl of 1N NaOH. Colour optical densities were measured using a spectrophotometric analyser (Dynatech) reading at 490 nm.

2.10. Cell Fusion

Rats injected with antigen IV 3 days prior to fusion were sacrificed by ether anesthesia and their spleens removed aseptically. The spleen was cut into small (0.5 x 0.5 cm) pieces which were passed through a size 60 stainless steel mesh screen to make a single cell suspension. Cells were washed once in DMEM complete and resuspended in 9 ml of serum-free Hy-medium. The suspension was divided into three equal parts and each 3 ml was layered onto 4 ml of a Ficoll-hypaque-azide solution (FHA). Briefly, FHA was made by mixing 12 volumes of Ficoll 400 (14% w/v stock) plus 5 volumes of Na-hypaque (32.8% w/v H₂O), 0.1% Na-azide (25%w/v H₂O), and 1.72 volumes of H₂O. The cell suspension was spun at 1800 x g for 10 min at 22°C. Cells were aspirated from the Ficoll interface using
sterile pasteur pipettes and were washed twice in serum free Hy-medium counted and adjusted to 50 x 10⁶/ml.

Plasmacytoma cells harvested at midlog phase and displaying viabilities ≥ 95% were washed twice in serum free Hy-medium and adjusted to 5 x 10⁶/ml.

2.10.1. Petri Plate fusion

The protocol reported below was adapted from the method developed by T.J. McKearn(70). One ml each of washed spleen cells and plasmacytoma cells were added to four 60 mm petri plates, after which an additional 3 ml of serum free Hy-medium was added to each plate. Petri plates were then centrifuged using microtiter plate carriers at 250 x g for 3 min. The supernatant was gently aspirated from the resultant layer of cells and the dish flooded with 1 ml of a 50% PEG solution which had been made earlier by mixing equal volumes of liquified (56°C) BDH 1000 PEG and Hy-media additionally buffered with 2% Hepes (10⁻²M) and 2% sodium bicarbonate (8.9 x 10⁻³M).

PEG remained in contact with the cells for 30 sec at room temperature, after which the PEG was diluted with 5 ml of serum free Hy-medium which was immediately aspirated. The cell layer was washed twice more, followed by the addition of 5 ml of Hy-medium containing 20% v/v FCS. The petri plates were incubated overnight at 37°C in 5% CO₂. Following incubation cells were washed from the plate and pooled into a 50 ml tube and washed twice in Hy-medium. Cells were resuspended in 50 cc of Hy-HAT medium containing 20% v/v FCS and 40 µl were plated into each well.
of 12 96-well microtiter plates. Each well was supplemented with 100 µl of Hy-HAT 3 days post fusion to remove unfused spleen cell immunoglobulin. Medium was aspirated and each well supplemented with 200 µl of fresh Hy-HAT 6 days post fusion. Hybridoma colonies became visible 14-21 days post fusion.

2.10.2. Cell suspension fusion

This technique was a modified version adapted from the method reported by R.H. Kennett (71). Spleen cells isolated by Ficoll-hypaque azide and plasmacytoma cells in midlog growth phase were washed and adjusted to cell densities as reported above. One ml each of spleen cells and plasmacytoma cells were mixed in a 15 cc round bottomed plastic test tube (Falcon) and pelleted by centrifugation for 10 min at 250 x g. Medium was aspirated and the pellet loosened by gentle tapping. 0.2 ml 35% BDH 1000 PEG made earlier by mixing 350 µl of liquified PEG (56°C) with 650 µl of additionally buffered Hy-medium (2% Hepes 10^{-2}M, 2% NaHCO_3, 8.9 x 10^{-3}M) was added to each pellet of cells. PEG was kept in contact with the cells for 8 min during which time the cells were pelleted for 3-6 min at 250 x g at room temperature. PEG was quickly diluted with 10 ml of serum free Hy-medium and the supernatant aspirated. Cells were washed once more and resuspended in 15 cc of Hy-HAT medium containing 20% v/v FCS and plated (50 µl) into each well of 3-96 well microtiter plated. Wells were further supplemented with 100 µl of Hy-HAT 3 days post fusion and on day 6 supernatants were aspirated to remove any unfused spleen cell immunoglobulin and supplemented with 200 µl of fresh Hy-HAT.
medium. Hybridoma colonies appeared 14-21 days post fusion.

2.11. **Cloning by Limiting Dilution**

Hybridoma lines to be cloned were suspended in Hy-medium containing 20% v/v FCS and adjusted to concentrations of 10 and 20 cells/ml. One hundred μl of each hybridoma cell suspension was distributed into the wells of 96-well microtiter plates, one plate for each dilution of hybridoma cells. Plates were incubated at 37°C in 5% CO₂. Macroscopic colonies appeared 10-14 days after seeding. Supernatants were assayed from plates which demonstrated less than 30% positive cell growth.

2.12. **Labelling of Monoclonal Antibody with ³⁵S-methionine**

Hybridomas in midlog growth phase were removed from tissue culture and washed once in methionine deficient RPMI 1640 medium (GIBCO). Cells (10⁶) were resuspended in 4 ml of RPMI 1640 (methionine deficient) complete, plus 10% v/v dialysed FCS. Twenty-five μCi of ³⁵S-methionine (New England Nuclear) was added to each culture and incubated for 18 hours in 5% CO₂ at 37°C. Cell suspensions were centrifuged (1000 RPM, 10 min) to remove cells and supernatants stored at either 4°C for immediate use or at -20°C.

2.13. **Characterization**

2.13.1. **Ouchterlony double diffusion**

Rat immunoglobulin isotype secreted by the various hybridoma lines were initially determined using Ouchterlony double diffusion analysis. Briefly, 1 g of agarose (Seakem) was heated (60°C) in 99 ml of PBS to
dissolve. Two ml of liquid agarose was spread evenly over a 1 x 3 inch microscope slide using a Pasteur pipette and allowed to gel at room temperature. Wells (2 mm) were cut 6 mm apart. Five μl of anti-rat reagent was added to one well and 5 μl of the neat hybridoma supernatant was added to the second well. Slides were placed in humid chambers and incubated overnight at 37°C. Slides were extensively washed with PBS, dried and stained with Coomassie Blue.

2.13.2. Immunoelectrophoresis (IEP)

Microscope slides were coated with 2 ml of warmed (60°C) 1% w/v agarose in barbital buffer, pH 8.2 and allowed to gel at room temperature. Two-2 mm wells were punched in the gel on either side of a cut trough. The wells were filled with serum, ascites or 5X concentrated hybrid supernatants and subjected to electrophoresis at 2 mA, 4-6 volts per slide for 45 minutes. Subsequently, the agarose was removed from the trough and appropriately diluted class specific antisera was added and the slides incubated in a humid chamber overnight at 37°C. Slides were then washed with PBS, dried and stained with Coomassie blue.

2.13.3. Crossed immunoelectrophoresis (XIEP)

XIEP was carried out as previously described by Laurell (72). Ten ml of heated (60°C) 1% w/v agarose in barbital buffer, pH 8.2 was poured onto 9 X 9 cm glass plates. When cooled, a single 2 mm well was punched 1 cm from each side at the cathodal end of the gel and 10 μl of sample added (1/15 dilution of normal mouse serum [source of α1Pi], or 2 μg of purified mouse α1Pi). Electrophoresis was carried out in the primary
direction at 70 mA, 18 volts/cm at 4°C for 3 hr and in the second dimension into a gel containing sheep anti-mouse α1Pi antisera (3.2% v/v in agarose gel) which was poured adjacent to one side of the first gel. Electrophoresis was carried out at 25 mA, 7 volts/cm, 4°C for 12 hours. Gels were washed with PBS, dried and stained with Coomassie blue.

2.13.4. Crossed radioimmunoelectrophoresis (XRIEP)

Preparation of mouse α1Pi precipitin arcs by two-dimensional electrophoresis against sheep anti-mouse α1Pi was the same as described above (see section 2.13.3).

Five thousand CPM of 35S-methionine labelled hybridoma monoclonal antibody (see section 2.12) was incorporated into 1 ml of warmed (60°C) 1% w/v agarose in PBS and poured into a 1.5 X 3.0 cm mold and allowed to gel at room temperature. The radioactive gel was placed over the mouse α1Pi precipitin arcs and incubated for 24 hr at 37°C in a humid chamber. Top gels were removed and the primary gel containing the α1Pi arcs were extensively washed to remove unbound labelled Ab. Cells were dried, stained and exposed to Kodak RP X-OMAT X-ray film for 1-6 wks and developed.

2.13.5. Immunoprecipitation-sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

One hundred μl of 10X concentrated (Amicon filter) hybridoma supernatants were mixed with 50 μl (5,000 CPM) of 35S-methionine labelled hepatocyte culture supernatant containing α1Pi (a gift from L. Lamontagne) for 2 hr under rotation at 4°C. One hundred μl of 1/100 rabbit IgG anti-rat IgM or IgG (heavy and light) reagent was added, and incubated under
rotation for a further 2 hr at 4°C. Subsequently, 100 μl of packed protein A Sepharose beads (Pharmacia) swollen in PBS was added under rotation for 1 hr at 4°C, after which 35S-μIPI-immunoglobulin complexed to PrA beads was isolated by high speed centrifugation (9,000 RPM). Beads were washed 3X in PBS and μIPI released from immunoglobulin by solubilization in 50 μl of PBS containing 10.2-mercaptoethanol and 10% w/v sodium dodecyl sulfate (SDS) followed by boiling for 2 min. Fifty μl of each sample, plus 50 μl of molecular weight markers, 50 μl of purified mouse μIPI (100 μg) and 50 μl of 35S-labelled culture supernatant (containing μIPI) alone were loaded into separate channels of a 12% polyacrylamide gel containing 0.1% SDS and run at a constant current of 8 mA for 4 hr. Gels were stained with Coomassie blue overnight, destained, saturated with Enhance (New England Nuclear) and dried. Gels were then exposed to Kodak RP X-Omat film for 1-3 wk and subsequently developed.

2.14. Monoclonal Antibody Isolation

2.14.1. Ammonium sulfate precipitation

Monoclonal antibodies (Mc Ab) were precipitated from culture supernatants by mixing 1:1 with a saturated solution of (NH₄)₂SO₄ (50% final solution) for 1 hr at 4°C. Precipitates were collected by centrifugation in a refrigerated Sorval centrifuge at 10,000 RPM for 30 min. Precipitates were redissolved in 0.1M PBS, pH 7.2 and dialyzed extensively against several changes of PBS (4L) for 3 days.

2.14.2. Eucoglobin precipitation

Fifty ml of hybridoma supernatant were dialyzed against deionized
distilled water for 3 days to precipitate euglobulin, in particular, IgM. Precipitates were pelleted in 50 cc conical test tubes in a refrigerated Sorval centrifuge at 10,000 RPM for 30 min. Precipitates were resolubilized in 0.1M PBS, pH 7.2.

2.14.3. Affinity chromatography

Goat anti-rat Ig was coupled to Sepharose 4B (Pharmacia) according to the method of Cuatrecasas and Anfinsen (73).

A 10 cc chromatography column was poured and equilibrated with 1 BSA in PBS. One hundred ml of hybridoma supernatant was allowed to percolate through the column at the rate of 15.5 cc/hr. The column was washed with 30 cc of PBS and hybridoma specific rat IgM eluted by HCl-glycine, pH 2.7 in 10-drop fractions which were neutralized immediately by the addition of 1N NaOH dropwise. Fractions were scanned at 280 nM with a spectrophotometer and protein fractions pooled for subsequent isotyping using Ouchterlony.

2.15. Tumor Establishment

Intraperitoneal (IP) injection into normal Balb/c mice of all rat x mouse hybridomas tested failed to demonstrate any tumor growth (see section 3.9), necessitating the development of a more elaborate preparation of recipient mice to avoid graft rejection and establish tumor growth in vivo.

To accustom hybridomas to grow as ascites, recipient Balb/c mice were first given 50 μl of 1/2 diluted rabbit anti-mouse lymphocyte serum
ALS) IP four days prior to hybridoma injection. On the day of injection, mice were given sub-lethal X-irradiation at a dose of 100 rads/min for a total of 600 rads (6 min). Mice were rested 4-6 hr and were bone marrow reconstituted by injecting (IV) 10^7 anti-theta treated cells.

Bone marrow cells were aspirated from the femur of syngenic Balb/c mice and resuspended in 10 ml of DMEM complete. Three ml of this suspension was layered onto 2 ml of heat-inactivated fetal calf serum and allowed to settle under 1x g for 10 min to get rid of dead and clumped cells. The upper 1.5 ml of FCS containing bone marrow cells were gently aspirated with a Pasteur pipette, pooled in 15 ml of DMEM and spun at 4°C in a refrigerated MSE centrifuge at 250 x g for 10 min, after which cells were resuspended at densities of 50 x 10^6/ml in DMEM complete.

Anti-theta serum, a gift from Dr. M. McDermott, was diluted 1/25 with DMEM complete. One ml of dilute anti-theta serum was mixed with 1 ml of bone marrow cells (50 x 10^6/ml) and incubated at 4°C for 45 min. Cells were pelleted at 250 x g for 10 min and resuspended in 1 ml of a 1/5 diluted (DMEM complete) rabbit low-tox complement (Cedarlane Laboratory) and incubated at 37°C for 45 min. Cells were pelleted 250 x g for 10 min, resuspended in 1 ml of DMEM complete and 250 ul injected IV into each recipient Balb/c mouse.

Hybridoma cells (2 x 10^7; ≥ 95% viable) in a volume of 0.5 ml DMEM were either injected subcutaneously behind the neck or IP 6 hr after bone marrow reconstitution.
2.15.1. **Ascites induction**

Hybridoma cells (2 x 10^6) taken either directly from tissue culture or as a cell resuspension of primary solid tumors removed from immune suppressed mice were injected IP into ALS, X-irradiated, bone marrow reconstituted Balb/c mice which had been primed with pristane (2,6,10,14 tetramethylpentadecane, 0.5 ml IP) 2 wk previously.
3.0 RESULTS

3.1. Characterization of Plasmacytoma Cell Lines

Culture conditions for optimal growth of the plasmacytoma cell lines used in fusion experiments were determined. Optimizing the various parameters ensured that cells taken for fusion were actively dividing and growing. The two lines used throughout this study were P3-NS1-Ag 4-1 (P3NS1) and SP2/O-Ag 14 (SP2), both derived from the original line P3X63-Ag-8 described by Milstein et al. (56) and were supplied as a gift from R.H. Kennett of the Pennsylvania School of Medicine.

P3NS1 grows well at low or high cell densities and synthesizes but does not secrete k light chains. Upon fusion, hybridomas secrete a mixture of original spleen cell immunoglobulin, plasmacytoma light chain, and combinations of these molecules. SP2 dislikes extremes in cell concentrations and media pH. It has lost the ability to synthesize heavy or light chains and when fused, secretes only spleen cell derived immunoglobulin.

Plasmacytoma cell growth can be divided into three phases. Phase I is a lag period found immediately after diluting cells in culture and represents minimal growth. Phase 2 is a logarithmic or exponential growth period which begins slowly, increases rapidly, and represents the period of most active growth and division. The mid log point represents a
population of cells, all virtually dividing, with viabilities in excess of 95%. Phase 3 is a period of slow growth or plateauing and is in response to a depletion of culture nutrients and a lowering of pH as a result of cell metabolism.

Growth curves were plotted for P3NS1 and SP2 over a period of several days to determine at what time the different lines passed into the three growth phases. From the graph (Figure 2) the midlog point was established and cells were taken at this point in time for all subsequent fusions. P3NS1 reached midlog 60-65 hr after reculture, SP2 reached midlog sooner, 51-56 hr after reculture. Viabilities for both lines were ≥95%.

Each cell line was cultured in Dulbecco's modified Eagles medium (DMEM) or Roswell Park Memorial Institute medium (RPMI) to detect any differences in growth characteristics. The only dissimilarity occurred at high cell concentrations with both cell lines maintaining higher viabilities in DMEM (82% RPMI vs ≥95% DMEM).

3.2. Fetal Calf Serum: Batch Characterization for Culture Supplementation

Media is normally supplemented with 10% FCS to facilitate cultured cell growth. However, not all batches of FCS are appropriate for tissue culture and require screening for suitability. Medium was supplemented with different batches of FCS and cells cultured over 7 days. Poor batches of FCS retarded the midlog point for P3NS1 and SP2 by as much as 1 or 2 days. Lots promoting rapid growth and shorter midlog points were frozen and stored at -20°C for future use.
Figure 2

Plasmacytoma growth curves. P3NS1 and SP2 Balb/c plasmacytoma lines were tissue cultured over several days to establish their growth curves. Cell viabilities and numbers were determined for each line. The midlog point, is the period of time after reculture where cells are in their most active stage of division and growth, displaying viabilities ≥95%. At this point, cells are selected and used in fusions. SP2 reached midlog 51-56 hrs after seeding in tissue culture while P3NS1 reached midlog 60-65 hrs after seeding.
3.3. Assay Systems for Antibody Producing Hybridomas

Assays for screening supernatants were chosen for their simplicity and reliability. Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) were capable of being performed quickly and in large numbers. Initially, RIA was used to establish positive controls and screen supernatants of hybridomas. Subsequently, all screening of hybrids and subclones were carried out by ELISA.

3.3.1. Radioimmunoassay

During the development of the screening assay, a specific sheep anti-mouse α1-antiprotease (Shαmα1Pi) reagent was used in our group for quantitation and immunohistochemical localization of α1Pi in mouse tissues (42). This reagent served as a positive heterologous control and rat α mouse α1Pi sera produced as described in section 3.4 was used as a homologous positive control in the immunoassays.

Fluid phase RIA utilized saturated (NH₄)₂SO₄ at a final strength of 40% to precipitate immunoglobulin-antigen complexes. At this concentration of (NH₄)₂SO₄, less than 5% of native α1Pi is precipitated. RIA was carried out in 96-well microtiter plates (Falcon Plastics). To determine the minimal amount of protein necessary to stop non-specific adsorption and/or precipitation, dilutions of normal, non-immune human serum, sheep serum, rat serum, or 1% bovine serum albumin (BSA) were used in a standard RIA protocol. Sera at dilutions of 1/10 and 1/20 caused 20% apparent precipitation while corresponding concentrations of 1% BSA had values less than 12% (Figure 3). Precipitation of label was
Figure 3

Non-specific precipitation of mouse α1P1. To reduce the occurrence of false positive reactions in radioimmunoassay screening procedures, serial dilutions of normal, non-immune human, sheep and rat sera, or 1% bovine serum albumin (BSA) were tested for their ability to non-specifically precipitate $^{125}$I-mouse α1P1. The dilution of serum giving the least precipitation was to be used in all future RIA screening procedures for hybridomas making antibody to mouse α1P1.
minimal for non-immune sera at dilutions of 1/40 while greater dilutions showed a steady increase in the amount of label associated with the plastic. Increasing amounts of $^{125}$I-αPi were added to a constant volume of 1/40 diluted sheep anti-mouse αPi to determine the saturation level for this assay (Figure 4). Five thousand CPM of αPi (approximately 50 ng) saturated available binding sites and was chosen for all future screening assays to ensure sufficient antigen was present.

Titrations were performed for both immune rat serum as well as immune sheep serum. Immune sera were added in increasing dilutions to fixed volumes of the corresponding normal sera and the resultant mixture diluted to 1/40 with PBS.

Three immune rat sera (Figure 5) showed titers of 1/544, 1/440 and 1/136. In contrast, sheep anti-mouse αPi had a titer of 1/1570.

3.3.2. Enzyme linked immunosorbent assay (ELISA)

As hybridoma technology has expanded the frequency of reports in the literature using ELISA as a means of detection has increased (74). Enzyme linked immunosorbent assay (ELISA) is comparable to RIA in sensitivity, reproducibility and applicability to microtiter systems (75). Screening of hybrid supernatants has been made faster by employing a spectrophotometric reader allowing quicker assessment of 96 well microtiter plates than possible with automated RIA counting equipment.

Initial attempts at implementing ELISA technology met with some difficulties. The major problem was the repeated occurrence of "false positive" reactions presumably caused by non-specific binding of enzyme-antibody conjugate to the surface of the plastic wells. One percent
\( \alpha lPi \), saturation point determination. To optimize the detection of positive hybridomas, the saturation point of \( ^{125}I \)-mouse \( \alpha lPi \) was determined for a 1/40 dilution of high titered sheep anti-mouse \( \alpha lPi \) antisera. \( ^{125}I \)-mouse \( \alpha lPi \) at its saturation point ensured antigen was not a limiting factor in the detection of positive hybrid cells.
Figure 5

Titration of immune rat sera. Binding capacity for mouse α1Pi is expressed as a percentage of the binding capacity of sheep antisera to mouse α1Pi. Titors reflect the dilution of serum binding 50% of the added 125I-α1Pi.

<table>
<thead>
<tr>
<th>Source of Sera</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune rat 1</td>
<td>1:544</td>
</tr>
<tr>
<td>Immune rat 2</td>
<td>1:440</td>
</tr>
<tr>
<td>Immune rat 3</td>
<td>1:136</td>
</tr>
<tr>
<td>Immune sheep</td>
<td>1:1570</td>
</tr>
</tbody>
</table>
BSA solution, FCS, normal human serum (NHS) and agamma-horse serum were used to block non-specific binding with the best results obtained using 2.5% NHS in a solution of PBS with 0.025% Tween-20 as a wetting agent. Blocking resulted in consistent optical density measurements (OD) between 0.01 and 0.02 units in negative control wells, and readings of up to 0.72 in positive control wells.

Antigen was adsorbed to the plastic wells of microtiter plates prior to protein blocking. Normal mouse serum containing αIPi was used as an antigen source but resulted in the non-specific adsorption of mouse immunoglobulins to the plastic. Anti-rat reagents used in the ELISA protocol cross-reacted with mouse immunoglobulins creating false positive reactions in all wells. Substitution of purified mouse αIPi containing no contaminating immunoglobulins eliminated the problem. The reaction of horseradish peroxidase (HRP) with the substrate 5-aminosalicylic acid (5-AS) continued to generate a coloured product with time, even after quenching the reaction with 1N NaOH. This necessitated attention to the timing of assay readings for good comparative results.

3.4. Immunizations

Allotypic immunization between strains of a given species have a tendency to generate weak humoral responses to polymorphic differences. Xenoergic immunization between species such as the rat and mouse, expose the responding immune system to a greater number of antigenic differences on closely related proteins (76).
Route of immunization, dose and spacing between successive boosts varies for each antigen. After immunization of rats with α1Pi as described in section 2.7, serial bleeds were taken via the tail vein 10 days after each injection of antigen and the sera were assayed for anti-α1Pi activity by RIA. Figure 6 shows the typical immune response of 3 rats with the binding capacity of the antisera increasing with subsequent boosts. Detection of antibody responses by double diffusion assays in agar demonstrated only a faint precipitin reaction after the fourth antigen boost.

Rats developing positive antibody responses were rested for at least 2 wk and given a final IV boost of 50 μg α1Pi to induce B cell proliferation and localization within the spleen. Spleens were removed for fusions 3 days later.

3.5. Fusions

3.5.1. Fusion protocol

Xenogenic fusions have been used successfully to investigate mouse differentiation antigens (77), and Rat major histocompatibility antigens (76).

Fusions between mouse plasmacytoma cells and rat, human and rabbit spleen cells have met with variable degrees of success due to the instability of chromosome mixing particularly with human X mouse hybrids (78). Rat X mouse hybridomas because of the species' genetic similarity, are less likely to subsequently lose chromosomes, become unstable and stop immunoglobulin secretion.
Figure 6

Response of Lewis rats to immunization with purified mouse α1P1.
ISS - immune sheep serum - positive control.
NSS - normal sheep sera - negative control.
NRS - normal rat sera - negative control.

Sera were tested at 1/40 dilution.
Values represent mean ± standard deviations of triplicate assays.
To determine which plasmacytoma cell line would preferentially fuse to form rat X mouse hybridomas, a series of comparative fusions were undertaken using P3NS1 and SP2 cells.

Two fusion protocols were chosen for comparison. One was a modified version of the original Köhler and Milstein method described by R.H. Kennett (71), the second was a protocol used for the generation of xenotypic hybrids developed by T.J. McKearn (70). The two methods were dissimilar in a number of ways.

Kennett's protocol mixed spleen to plasmacytoma cells in the ratio of 10:1 and subjected the suspension of cells to a 30% polyethylene (PEG) solution for periods of time up to 8 min. Cells were immediately washed and plated into 96-well microtiter plates containing HAT selective medium. McKearn's approach also used a spleen to plasmacytoma ratio of 10:1 but cells were then centrifuged in 60 mm petri plates to form a layer which was subjected to a 50% PEG solution for 30 sec. Cells were washed, overlaid with HAT supplemented medium and incubated at 37°C in 5% CO₂ overnight. Cells were then resuspended in additional HAT medium and dispensed into microtiter plates.

Wells demonstrating cell growth at 14-21 days post fusion were initially recorded as a "positive" well. P3NS1 formed the most positive wells using either fusion technique, 20% for Kennett's and 40% for McKearn's, with SP2 only forming 10% positives for the former and 30% for the latter technique. The experiments demonstrated that for our purposes, generation of xenogenic rat X mouse hybridomas was favoured using McKearn's fusion protocol with either plasmacytoma cell line.
3.5.2. **Choice of PEG**

Using the Petri plate (McKearn protocol) fusion technique we investigated the possibility of optimizing hybridoma formation by varying PEG. Fazekas de St. Groth *et al.* (79) has reported the source as well as the molecular weight of PEG is an important factor for the generation of hybridomas. In addition, the longer cells are held in contact with PEG, the greater the number of hybrids formed, which however must be balanced against the toxic effects of PEG.

Baker 1000, Fisher 1050 and BDH 1000 PEG's were added to micro-titer plates containing either P3NS1 or SP2 as 50% solutions for 10 min. Cell samples were removed at 1 min intervals and the viabilities determined. BDH 1000 produced viabilities >80% for exposures of 3 min or less, while Baker 1000 and Fisher 1050 gave viabilities <50% for the same period. BDH-1000 as a 50% aqueous solution had a pH of 5.9. Addition of PEG to PBS (0.1M, pH 7.3) with 2% Hepes and sodium bicarbonate, resulted in a more favourable pH of 6.9 and reduced the toxic effects of PEG, in particular for SP2 (viability >95%). In addition, using buffered PEG solutions of BDH 1000, SP2 gave similar numbers of positive growing hybrids compared to P3NS1, 20% positive growing hybrids using Kennett's protocol and 40% for McKearns. SP2 grew faster than P3NS1 in culture, and it was hoped that hybrids, when transferred to tissue culture conditions, would themselves grow quickly allowing faster accumulation of immunoglobulin.

The plasmacytoma cell line SP2 was finally chosen as the fusing partner for immunized Lewis rat spleen cells on the basis that hybridomas would only secrete spleen cell-derived rat immunoglobulin and not mixtures as may be found with P3NS1.
3.5.3. Production of hybridomas secreting antibody to mouse alpha-1-protease inhibitor

Prior to our attempts to examine various fusion protocols, we carried out 3 fusions using the technique described by Kennett (see section 2.10.2.). Three Lewis rats were immunized by the protocol described in section 2.7. These rats were used for fusion immediately (3 days after boost) or after resting and boosting at 2 wk or 4 wk. Although a number of hybrid cells were formed in each fusion (approximately 30% wells were positive), none were shown to bind α1PI.

Subsequently, using the petri plate technique (see section 2.10.1.), fusions were carried out with cells from similarly immunized rats. Only with the rats sensitized and rested 4 wk were there any positive wells found that bound α1PI.

In the fusion, cells were distributed into 12 96-well microtiter plates. Four hundred and twelve wells (36%) showed hybridoma growth by 21 days with an additional 72 wells (6%) showing a slower appearance of hybrids at 28-31 days post fusion. RIA indicated 64 (15.5%) of the 412 wells bound 125I-α1PI at a level greater than two standard deviations above the mean. Figure 7 shows a representative distribution of counts bound in this assay. The hybrid supernatants shown positive by RIA were tested by ELISA (Figure 8) and demonstrated a similar spectrum of activities.

Two positive hybridomas, D4 and D7, plus a non-binding hybrid, D11 (to act as an internal negative control), were selected for further characterization. D7 was selected for subcloning while the remaining
Figure 7

Initial RIA screening of hybridomas. The hybridoma supernatants in this figure represent a random sampling from the last successful fusion. Cell lines were considered positive if they bound αlπ equal to or greater than 2x the standard deviation above the mean. Indicated on the graph are 37 positive lines:
<table>
<thead>
<tr>
<th>SAMPLE NO.</th>
<th>CLONE</th>
<th>SAMPLE NO.</th>
<th>CLONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-A2</td>
<td>20</td>
<td>3-A9</td>
</tr>
<tr>
<td>2</td>
<td>1-B6</td>
<td>21</td>
<td>3-B5</td>
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<td>1-D6</td>
<td>22</td>
<td>3-C8</td>
</tr>
<tr>
<td>4</td>
<td>1-D10</td>
<td>23</td>
<td>3-D4*</td>
</tr>
<tr>
<td>5</td>
<td>1-G1</td>
<td>24</td>
<td>3-D7*</td>
</tr>
<tr>
<td>6</td>
<td>1-G8</td>
<td>25</td>
<td>3-E11</td>
</tr>
<tr>
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<td>1-G9</td>
<td>26</td>
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<tr>
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<td>1-G12</td>
<td>27</td>
<td>4-B3</td>
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<td>30</td>
<td>4-C12</td>
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<td>3-A2</td>
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Figures 7,8: Sample Key

IRS - Immune Rat Serum, 1/40
NRS - Normal Rat Serum, 1/40
ISS  Immune Sheep Serum, 1/40
NSS  Normal Sheep Serum, 1/40
Figure 8

ELISA testing of putative positive hybridomas. A survey of 37 hybrid lines previously shown positive by RIA demonstrated binding ability for mouse αRI.

positive hybrid lines were frozen in liquid nitrogen and stored cryogenically.

3.6. Cloning

Hybridoma growth in wells following fusion may represent more than a single population of cells. Cloning by limiting dilution or in soft agarose separates the various populations of cells into distinct groups. D7 was selected for cloning by limiting dilution at a density of 1 cell/well. Cell growth was faster than for the initial generation of the hybrids, macroscopic colonies of cells appearing within 7-10 days following cloning. Wells were examined daily using an inverted microscope and at 5 days after cloning, 124 wells (20.6%) showed only a single focus of cells, while 12 further wells (2%) showed multiple foci of cells, probably indicating multiple clones.

Wells containing a single focus of cells were expanded into 24 well plates (Linbro) and screened by ELISA for anti-α1Pi activity. Forty-one (33%) of the colonies assayed secreted a rat IgM which bound mouse α1Pi in significant amounts, in particular two clones D7.11 and D7.32 (Figure 9). These two clones were expanded into 25 cm² tissue culture flasks for characterization while all other clones were frozen and stored in liquid nitrogen.

3.7. Characterization

Characterization was performed on the original uncloned hybrid lines D4, D7, D11 and on the monoclones D7.11 and D7.32. Each was
Figure 9

Graph demonstrating the specificity of a number of D7 monoclones for purified mouse α1Pi detected by ELISA. Antibody isotype responsible for binding was a rat IgM. Vertical columns represent an average from three separate experiments. Bars indicate standard deviation.

NRS - normal rat serum, dilution 1/100.
IRS - immune rat serum, dilution 1/100.
M - fresh media plus 10% fetal calf serum.
D7* - polyclonal D7 prior to cloning.
isotyped to define secreted immunoglobulins. Of the two positive, uncloned lines, D4 and D7, only D7 and its monoclones (D7.11 and D7.32) were characterized for their binding specificity by crossed radioimmuno-electrophoresis.

3.7.1. Isotype secretion

Investigators have reported generating hybridomas secreting all forms of immunoglobulins. Fusion and plating of cells into microtiter plates (96-well) often result in the growth of multiple colonies in a single well. Not surprising is the occurrence of two or more immunoglobulin isotypes when cells are initially screened. The secreted products of the uncloned lines D4, D7 and D11 were first typed by Ouchterlony double diffusion analysis using class specific reagents. D4 secreted rat IgG while D7 and D11 secreted both rat IgG and IgM.

Analysis by immunoelectrophoresis confirmed the diffusion results with D4 showing a single long cathodal arc characteristic of IgG, and both D7 and D11 having a short and long arc identified as IgM and IgG, respectively.

Cloned lines D7.11 and D7.32 both bound αlPi and both secreted only rat IgM. IEP using a specific rabbit anti-rat IgG failed to show the presence of IgG in the cell supernatants (Figure 10).

3.7.2. Specificity

Assays such as RIA or ELISA demonstrate hybridoma specificity towards the eliciting antigen by binding activity. However, if the antigen is not purified or exists in more than one form, as does mouse αlPi,
Characterization of secreted products. Characterization by immunoelectrophoresis (IEP) utilizing a number of subclass specific anti-rat immunoglobulins.

Figure 10

- 1

D7- concentration of D7* supernatant

- 2

No lines were detected for

7.32 vs rabbit anti-rat IgG

D7- polyclonal D7 prior to cloning

Supernatant rabbit anti-rat IgG (heavy-light)
a more rigorous method is required to demonstrate which specific antigen
the hybridoma immunoglobulin is directed against.

3.7.2.i. Immunoprecipitation

Direct immunoprecipitation of the eliciting antigen by monoclonal
immunoglobulin in the majority of cases is not possible. If truly mono-
clonal, it can only recognize single antigenic determinants on the molecule.
Cross-linking can not occur to a significant degree, prohibiting the
formation of latticies necessary for the formation of a precipitate. To
circumvent this problem, hybridoma antibody binding antigen was indirectly
precipitated by the addition of Sepharose beads bearing specific anti-
immunoglobulin reagents. Once isolated, the complex is solubilized in
2-ME/SDS and electrophoresed on SDS-PAGE for analysis.

$^{35}$S-methionine labelled fast and slow electrophoretic forms of
mouse αlPi obtained from in vitro cultures of isolated mouse hepatocytes,
was a gift from L. Lamontagne. Neat culture supernatants of D7.11 and D7.32
were mixed with labelled hepatocyte culture supernatants. Subsequent
analysis revealed both clones bound a labelled protein having a molecular
weight of 53,000 corresponding to the position and MW of native, purified
mouse αlPi (Figure 11).

3.7.2.ii. Crossed radioimmunoelectrophoresis

Crossed immuno-electrophoresis (XIEP) using sheep anti-mouse αlPi
serum resolves mouse αlPi into two closely associated immunoprecipitin
arcs, the closest to the cathode representing the slow variant with the
more distant precipitin arc representing the fast variant (35).
Figure II

SDS-PAGE analysis of the material immunoprecipitated by monoclonal antibody to murine α1PI.

1. Labelled culture medium.
2. D7.32 precipitated α1PI.
Both precipitin arcs cross each other suggesting immunological non-identity, possibly the result of false spur formation (54).

The two clones, D7.11 and D7.32 plus the negative line, D11, were cultured separately in $^{35}$S-methionine supplemented media to internally label the secreted immunoglobulin products. $^{35}$S-immunoglobulins (5,000 CPM) were added to 1 ml of 1% agarose at 56°C and the mixture was poured into a 1.5 x 3.0 cm mold and cooled. These gels were then overlaid onto the $\alpha_1$Pi precipitin arcs of a crossed immunoelectrophoresis previously run against sheep anti-mouse $\alpha_1$Pi serum and incubated at 37°C for 24 hr. After subsequent washing and staining, plates were exposed to Kodak RP-X-Omat film for 7 days (Figure 12). Immunoglobulin products of both clones D7.11 and D7.32 bound only the fast electrophoretic variant of mouse $\alpha_1$Pi, while no activity was demonstrated for either $\alpha_1$Pi variant by D11. No binding was seen at the albumin arc.

3.8. Purification of Rat IgM anti-Mouse Alpha-1 Protease Inhibitor from D7.11 and D7.32 Culture Supernatants

Antibodies can be precipitated from supernatants by 50% saturated ammonium sulphate solutions (80).

Although all classes of immunoglobulins are initially precipitated, reconstitution using distilled water or weak salt solutions induces unstable euglobulins including IgM to reprecipitate resulting in their loss. Initially, supernatants from the uncloned lines D4, D7 and D11 were concentrated 10X using $(\text{NH}_4)_2\text{SO}_4$ for isotyping in Ouchterlony double diffusion. The resulting IgM lines were weaker than when neat supernatant was assayed.
Combined crossed immunoelectrophoresis and autoradiography. Mouse α1PI was subjected to electrophoresis in the first dimension (cathode, left) in 1% agarose and electrophoresed in the second dimension against 3.2% sheep anti-mouse α1PI. XJEPI arc patterns were overlaid with D7.32 35S-methionine in 1% agarose gel for 24 hr. XJEPI was washed extensively for 3 days and exposed to Kodak-RP X-Omat film.
To collect IgM from D7.11 and D7.32 media was dialyzed against distilled water to precipitate the euglobulin fraction. Precipitates were resolubilized in 0.1M PBS, pH 7.3 and fractionated on a 1 meter long Sephadex G300 column equilibrated with 0.01M PBS, pH 7.3. IgM fractions were detected by double diffusion immunoanalysis, pooled and concentrated by Amicon pressure filtration.

IgM was also purified by affinity chromatography using specific goat anti-rat IgM coupled to Sepharose. The bound IgM was eluted by 0.1M glycine-HCl, pH 2.5 and neutralized immediately with 1N NaOH. One hundred and eighty-three μg of pure rat IgM anti-mouse α1PI was isolated from 300 ml of D7.11 culture supernatant by affinity chromatography.

3.9. Tumor Induction

Culture supernatants may contain 1-100 μg of immunoglobulin/ml, in contrast to serum from animals supporting subcutaneous tumors or ascitic fluid from animals bearing an ascites which contain mg/ml quantities of antibody (82).

Generally, interspecies hybridoma cells do not readily adapt to in vivo growth as ascites (McKearn, personal communication). Problem lines are first used to induce solid subcutaneous tumors and cell suspensions from these tumors are then transferred to the peritoneal cavity of pristane-primed mice for the generation of ascites. Mice normally will produce 10-20 ml of ascitic fluid while rats generate 60-100 ml (82).

D7, D11 and clones D7.11 and D7.32 were injected at cell densities of 2 X 10⁷/ml IP into pristane-primed Lewis rats, nude mice and Balb/c
mouse hosts. There was no evidence of any successful development of tumor. It was necessary to carry out marked immunosuppression of Balb/c mice before successful tumor induction occurred. After pristane-priming, anti-lymphocyte serum treatment (ALS), sub-lethal X-irradiation and syngeneic bone marrow reconstitution, subcutaneous solid tumors were produced with $2 \times 10^7$ hybrid cells in Balb/c mice. These tumors became obvious and palpable approximately 10 days after injection. Attempts to induce ascites with these in vivo adapted hybrids were unsuccessful, resulting in only solid peritoneal tumors. The inability to generate ascitic fluid and the complexing of secreted hybrid immunoglobulin to free \( \alpha \)-Pi in circulation as detected by immunoelectrophoresis restricted isolation of anti-\( \alpha \)-Pi immunoglobulin to methods utilizing culture supernatants.

Examination of mice 18 days after induction of subcutaneous tumor growth by D7, D7.11 or D7.32 cells, revealed small white nodular areas on the surface of the liver which was not seen with D11 cells (Figure 13a). Histological studies showed these nodules to be small areas of necrosis adjacent to arteries and veins and intermittently dispersed throughout the liver (Figure 13b). Sera taken from mice showing liver involvement were assayed for their \( \alpha \)-Pi levels and in all cases, levels were 16-25% below normal suggesting either reduced liver capacity for \( \alpha \)-Pi production or that \( \alpha \)-Pi is complexed to circulating hybridoma immunoglobulin. In support of the latter possibility, IEP analysis of the sera was performed and showed free \( \alpha \)-Pi plus a small amount complexed to immunoglobulin, appearing as an extension of the \( \alpha \)-Pi arc back into the area normally associated with IgM immunoglobulin.
a. Gross demonstration of necrotic liver lesions. White nodules were seen on the liver in mice supporting solid subcutaneous tumors from D7, D7.11 and D7.32 hybridoma lines. No nodules were seen with the D11 negative hybrid line or Sham-treated mice.

b. Histological examination of necrotic liver lesions. Nodular areas were revealed to be necrotic foci adjacent to arteries and veins interspersed throughout the liver lobule. Section is stained with hematoxylin-eosin.
4.0 DISCUSSION

4.1. Rationale

Alpha-1-protease inhibitor (α1Pi) is one of a group of serum protease inhibitors which interact as a "group" controlling the interactions between the biochemical pathways of inflammation: the Complement, Coagulation/Fibrinolytic, and Kallikrein/Kinin systems. The actual mechanisms involved in the regulatory processes are not, as yet, known.

In human studies, agarose and acid starch gel electrophoresis have identified as many as 20 different α1Pi phenotypes displaying immunologic identity. Gauldie et al. have described, in mice, two variants or phenotypes with electrophoretic as well as immunologic heterogeneity (35).

The use of polyclonal antisera to resolve the molecular heterogeneity of α1Pi was not possible owing to the many different immunoglobulins comprising the antisera some of which recognized common antigenic determinants shared between the two variants, not allowing separation and/or purification of the two forms. Fusion and the production of hybridomas allowed the collection of monoclonal specific antibody to a single variant of α1Pi providing a means for its isolation and purification. Additionally, hybridomas acted as a continuous source of
homogeneous immunoglobulin eliminating complicated purification and absorption techniques.

This thesis deals, in part, with a comparison of different fusion techniques examining parameters allowing a more favourable production of xenogenic rat × mouse hybridomas. The work was not designed to test all of the possible variable parameters and their interactions. The main focus of the work involved the production of a rat monoclonal immunoglobulin against one or both of the forms of mouse α1Pi.

4.2. Detection Systems

Production of specific hybridomas are irrelevant if there are no ways of detecting cells producing specific antibody before positive lines are lost to overgrowth by non-producers or through chromosome loss due to instability. Prior to specific fusion we attempted to select an appropriate assay system that was simple, reliable and allowed rapid screening of a large number of cell supernatants.

Radioimmunoassay (RIA) as well as enzyme linked immunosorbent assay (ELISA) are two techniques which have been used to detect immunoglobulins to soluble as well as insoluble antigens.

4.2.1. Radioimmunoassay (RIA)

RIA has been used extensively to detect specific hybridomas to human chorionic gonadotropin (83), Concanavalin A (84) and insoluble antigens such as those found on leukocytes (85,86). Our initial attempts at using RIA as a means of detection met with several problems not the
least of which was the generation of "false-positive reactions". Elimination of cross-reactive proteins such as mouse immunoglobulin and blocking of non-specific binding sites through the use of pooled normal heterologous sera reduced non-specific binding (Figure 3).

Dilutions of normal, non-immune sheep serum, rat serum and human serum gave 20% non-specific precipitation at a dilution of 1/10. In comparison, a 5% precipitin value was obtained with BSA at comparable protein concentrations. This implied labelled antigen was trapped by immunoglobulin when induced to precipitate by the addition of ammonium sulfate. Increasing dilutions to 1/40 resulted in decreased, non-specific precipitation approaching background levels with normal non-immune human serum giving the lowest value (2.5%) and was chosen for subsequent use in all further RIA. Dilutions beyond 1/40 for all sera tested resulted in an increased ability to non-specifically remove labelled antigen from solution by allowing it to bind to the surface of the plastic wells, whose charged surfaces were previously blocked by higher concentrations of non-specific protein.

To ensure labelled αlPi was not a limiting factor for the detection of specific antibodies in RIA increasing amounts of labelled αlPi were added to fixed volumes of 1/40 ShāmalPi to determine the saturation point (Figure 4). All 125I-αlPi was bound within the range of 300 (3 ng) to 5000 CPM (50 ng) higher amounts showing no increased binding to Ig indicating the saturation point was reached. To ensure antigen was in saturating conditions, 5,000 CPM 125I-αlPi was used in all subsequent RIA.
Two positive controls, sheep anti-mouse α1Pi and immune rat sera were used throughout all RIA's. Titrations were carried out on three immune rat's sera giving titers of 1/544, 1/440, 1/136, while that for Shāma1Pi was 1/1570 (Figure 5). The large difference in titers seen between the rat and sheep sera suggested that mouse α1Pi was not as antigenic for rat, as for sheep, a possible consequence of the close similarity and cross reactivity of α1Pi in the rat and mouse species.

4.2.2. Enzyme linked immunosorbent assay (ELISA)

Midway into our studies, we developed an enzyme linked immunosorbent assay as a means of both detecting antigen specific immunoglobulin and its isotype. ELISA methodology has been shown to be comparable to RIA in sensitivity, reproducibility and applicability to the microtiter system while avoiding the biological hazards of radiation (74).

Reports in the literature cited increasing use of Staphylococcal Protein A for detection of secreted immunoglobulins (87). Conflicting reports on the specificity of Protein A for rat immunoglobulin (88) lead us to employ class specific anti-rat reagents which we used exclusively throughout these studies.

Initial implementation of ELISA gave "false-positive" reactions with all controls and hybrid supernatants tested.

The first layer of mouse α1Pi was initially laid down as dilutions of normal Balb/c mouse serum containing the antigen as well as immunoglobulins, both of which adsorbed to the surface of the plastic wells.
Subsequent use of specific anti-rat reagents to detect antibody bound to α1Pi cross-reacted with the adherent mouse Ig's causing false positive results. Substitution of purified mouse α1Pi devoid of all Ig's substantially reduced the background. The persistence however, of higher than expected optical densities (O.D.) in negative control wells suggested non-specific adsorption of reagents onto plastic surfaces. To reduce the non-specific adsorption, dilutions of 1% BSA and normal, non-immune agamma-horse serum, FCS and human serum were tested (data not shown). The use of 2.5% normal human serum gave O.D.'s in negative controls between 0.01 and 0.02 while similarly treated positive controls had readings as high as 0.72, permitting differentiation between positive and negative hybridoma supernatants.

Access to a spectrophotometric analyzer allowed faster reading of ELISA results than previous enumeration of RIA's using automatic gamma counting systems.

4.3. Generation of Immune Cells

Selection of a spleen cell population which preferentially fuses to plasmacytomas forming hybridomas is crucial. The cell type most likely is the B cell undergoing blastogenesis (blast cell) and/or plasma cells. These cells have been reported as the most likely candidates for fusion from mitogen studies involving lipopolysaccharide (LPS) and lipoprotein (LPP) (65). An immunization schedule that parallels the induced formation of B-blasts as seen in the mitogenic study also favours the formation of
specific hybridomas 1-2 orders of magnitude over other protocols (83).

4.4. Selection of Lewis Rats

Selection of an animal species for the generation of appropriate spleen cells for fusion was dictated by their ability to mount immune responses to αLPI while maintaining an ability to fuse to mouse plasmacytomas, forming stable antibody secreting hybridomas. The rabbit, when immunized against mouse αLPI, develops good circulating levels of specific antibody but spleen cells, when fused to mouse plasmacytoma lines, form very unstable hybrids as a result of chromosome loss. Mouse spleen cells in contrast, fuse to their plasmacytoma counterparts readily to form stable hybridomas capable of secreting immunoglobulin, however, we were unsure whether there were sufficient antigenic differences on αLPI molecules between mouse strains necessary to induce allogenic antibody responses and therefore appropriate B cell-proliferation for fusion. The rat has been reported by some to supply spleen cells capable of fusing to mouse plasmacytoma lines producing stable, xenogenic hybridomas (76,83). Rat and mouse were expected to demonstrate antigenic differences on αLPI molecules to allow rats to make sufficient antibody responses, thus providing proliferating B blasts for fusion.

Ouchterlony analysis of immune rat sera demonstrated weak reactions towards mouse αLPI, while RIA analysis confirmed weak responses by the demonstration of low titers in immune rat sera in contrast to high titers seen with sheep anti-mouse αLPI (Figure 5). This indicated antigenic differences between rat and mouse αLPI were not great, but were sufficient to induce immune responses. The choice of the rat was a compromise.
between rabbit and mouse, thus allowing the possibility of generating rat X mouse hybridomas secreting specific antibody.

4.5. Immunization Schedule

Our immunization methods employed a technique that favoured the induction of hyperimmunization, which is considered by some (90) counter-productive in producing favourable B cells for fusion. Our experiences do not support this view, as we were able to demonstrate both circulating levels of anti-αlPi Ig in immunized animals which when fused later allowed the formation of hybrid cells.

The state of immune responsiveness depends on the nature of the antigen, route of immunization, dosage and timing between successive injections (67). The antigen was presented (doses of 50 μg's) in complete Freund's adjuvant (CFA), and specific immunoglobulin could be detected by RIA with titers increasing with each subsequent boost (Figure 6).

The final boost prior to hybridization is given IV to ensure cellular localization and proliferation within the spleen. Spleen cells taken 3-4 days later represent an enriched source of stimulated cells for fusion.

4.6. Fusion

Comparative studies performed between P3NS1 and SP2 for selective formation of rat X mouse hybrids revealed P3NS1 to favour hybrid formation (section 3.5.1.). The efficiency could be increased for SP2 fusions by raising the pH of the fusing media to 6.9. The increase in hybrid number
formed (section 3.5.2.) was a reflection of the sensitivity of SP2 towards variable pH ranges. Our final decision to use the plasmacytoma line SP2 for production of specific hybrids was a result of increased fusion efficiencies and the knowledge that the hybrids formed would secrete immunoglobulins of spleen cell origin and specificity and not mixed or "scrambled" products as is seen with x63 or its derivative P3NS1 (91).

4.6.1. Polyethylene glycol (PEG)

A further variable examined in fusion was the source and molecular weight of polyethylene glycol as well as the length of exposure to cell suspensions. Two systems, one a modification of Köhler and Milstein's original method reported by R.H. Kennett (71) and a second, designed and reported by T.J. McKearn (70) incorporated different procedures for the presentation of PEG to cells as well as using different sources, strengths and exposure times. Our results (section 3.5.1.) concluded the McKearn technique to be superior for our purposes. McKearn used 50% PEG solutions as did Stahli (83), Oi and Herzenberg (90), all reporting substantial hybrid yields (10-40% of the seeded wells showing hybridoma growth). Fazekas de St. Groth (79) reported a comparison between sources of PEG and their MW's with their ability to induce the formation of hybrids. The report confirmed that the higher MW PEG's (1,000 - 4,000) favoured the formation of hybrids while lower MW PEG's (200) appeared to have a toxic effect. In our own comparison, we found BDH 1000 to promote the greatest formation of hybridomas owing to its minimal toxic effect (section 3.5.2.). Optimization of PEG conditions and exposure times to
cell populations increased hybridoma yields from 10-20% to an average of 40% (section 3.5.2.).

4.6.2. **Alpha-1-protease inhibitor binding hybridoma**

In our screening assay hybridomas were not selected on the basis of their Ig isotype, rather on their ability to bind α1PI.

Our failure to detect positive hybridomas in early fusions using the Kennett protocol with animals immunized in a similar manner to those used later in successful fusions, suggested we most likely were experiencing difficulties with our assay system as well as possibly forming fewer rat X mouse hybrids as a direct result of the specific fusion technique (section 3.5.1.).

In the successful fusion utilizing the McKearn protocol, 412 (36%) of the plated wells demonstrated growth of hybridomas in HAT selective media. Sixty-four of the supernatants from these wells bound mouse α1PI, representing a fusion efficiency of 15.5% specific hybrids. Stähli (83) has reported that hybrid products, in response to soluble antigens, occur at efficiencies <1%. It would appear that our immunization protocol and optimization of fusion parameters gave a substantially higher efficiency, approaching that reported with particulate antigens (83).

4.6.3. **Initial selection**

A random sample of hybridoma supernatants from the last fusion, examined by RIA are represented in Figure 7. Those wells binding α1PI in quantities greater than, or equal to, twice the standard deviation above the mean were considered positive. In this representation, 120
wells are shown, of which 37 are indicated as α1Pi binding. Also included are
the two hybridoma lines D4 and D7 which bound α1Pi in significant amounts
compared to the other positive wells. D4 bound 27.3% less counts compared
to D7 during initial screening. Over a period of time the ability of D4
to bind α1Pi decreased while that of D7 remained constant. Figure 8
represents an ELISA survey of 37 hybridoma lines previously shown positive
by RIA. Comparison between D4 and D7's ability to bind α1Pi indicated D4
bound less than 50% compared to D7, indicating D4 was either losing chromo-
somes and therefore decreasing its production of anti-α1Pi antibody or
it was slowly being overgrown by a second hybrid cell population within
the well. Neat D7 supernatants gave O.D.'s equal to those obtained with
1/160 immune rat serum.

Observations of other positive hybrid lines in Figure 8 and
comparison to their previous abilities to bind α1Pi by RIA (Figure 7)
indicated some lines increased their binding capacity while others decreased,
and may have shared the same fate as D4.

Two positive lines, D4 and D7, which showed optimum binding in both
RIA and ELISA (Figures 7 and 8), plus one negative line, D11, were
selected for further study. All three secreted rat IgG while only D7 and
D11 secreted, in addition, rat IgM indicating initially D7 and D11 were
at least biconal (Figure 10). The ability of D4 to bind α1Pi decreased
slowly over a period of weeks as stated above and prompted us to freeze
D4 and pursue analysis of D7.
4.7. Cloning

Cloning of D7 was accomplished by limiting dilution culture. Ninety-six-well microtiter plates were seeded at cell densities of 1 cell/well. Estimates by Poisson distribution (92) expect at least 37% of the wells seeded should have negative cell growth for the positive wells to have arisen from a single colony or clone. Cloning of D7 resulted in single colony growth in 124 wells while 472 (79.4%) were negative. These results reflect a high probability that all lines isolated would have resulted from a monoclonal cellular expansion.

ELISA survey of the 124 single clones indicated 41 (33%) bound 1Pi by a rat IgM immunoglobulin (Figure 9). The remaining clones were negative for 1Pi binding but secreted rat IgG of undetermined specificity (data not shown). Positive clone D7.11 gave O.D.'s equal to that for uncloned D7, while surpassing the 1Pi binding ability of immune rat serum by 20%. Other positive clones such as D7.27 and D7.32 bound 57.4% and 62.3%, respectively, compared to the immune rat serum control. Due to a lack of time, we did not carry out competition experiments to determine if all IgM antibodies were directed against the same antigenic site. The lower O.D.'s generated by positive clones in Figure 9 most likely represented differences in cell growth and immunoglobulin secreting characteristics. Since those wells that were negative for IgM were all positive for IgG it is probable that D7 uncloned represented the expansion of two clones of cells, one secreting IgM and the other IgG.
4.8. **Specificity**

Specificity of IgM secreted by the two clones D7.11 and D7.32 for mouse α1Pi was demonstrated by immunoprecipitation and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 11). \(^{35}\text{S}\text{-methionine} \) labelled α1Pi present in hepatocyte culture supernatants was precipitated with monoclonal immunoglobulin and specific anti-rat IgM linked to Sepharose beads. Subsequent solubilization and SDS-PAGE analysis demonstrated the labelled material bound by the rat IgM co-migrated with purified unlabelled α1Pi. The labelled antigen had a MW of 53K, indicative of mouse α1Pi (35).

Crossed immunoelectrophoresis (XIEP) resolves both serum and purified mouse α1Pi into two electrophoretically distinct forms, a fast and a slow variant. Two forms have also been reported for rat (Lee and Janoff, personal communication) and rabbit α1Pi (93).

Clones D7.11 and D7.32 and the negative line D11 were labelled internally with \(^{35}\text{S}\text{-methionine} \) and 5,000 CPM of each was overlaid onto the precipitin arcs formed by normal mouse serum and sheep anti-mouse α1Pi (35). We used an unabsorbed antiserum so that a third arc representing albumin was precipitated as a control for non-specific binding of label. On autoradiography, both D7.11 and D7.32 bound only the fast variant of α1Pi showing no affinity for either the slow α1Pi component or the albumin arc while D11 showed no activity for any arc regardless of the length of exposure to the x-ray plate. The results suggested that the specificity of the rat X mouse monoclonal IgM antibody was solely directed towards the fast α1Pi variant.
4.9. Speculation

In the human, different electrophoretic migration patterns seen with deficiency associated Pi types may be a result of amino acid point mutations and may become critical when they occur in sequences near carbohydrate linkage positions. These substitutions may impede growth and/or completion of oligosaccharide side chains. The latter possibility may be one explanation for the observed 20-25% reduction in carbohydrate in the inhibitor of the PiZZ phenotype. In the majority of cases, the absolute carbohydrate and amino acid compositions vary only slightly (94). Owen (30) and Yoshida (29) have demonstrated a substitution from glutamic acid in the normal PiMM to a valine in the PiSS variant. Additional changes may have taken place with concealed amino acids but these have not, as yet, been found. Yoshida (57) and Miller (28) have found a negatively charged glutamic acid in PiMM changed to a positively charged lysine in PiZZ.

Amino acid point mutations may effect the tertiary structure of the α1Pi protein altering its solubility, which may result in a decrease in circulating levels of α1Pi in some individuals.

The mouse has not yet been examined for phenotypic variation in α1Pi as occurs in the human. A survey of the majority of mouse strains by isoelectric focussing may reveal species polymorphism. It is probable that α1Pi in the mouse as in human is a codominant expression of two alleles (95), one from each of the available paternal/maternal Pi alleles. If molecular polymorphism exists in the mouse, amino acid variation, with
its associated alteration of post translational glycosylation, may account for the anticipated migratory differences demonstrated by fast and slow α1PI. If this were the case, we would expect to see (a) partial or total identity of the two forms on two-dimensional XIEP, (b) a single polypeptide precursor molecule in the mRNA translation experiment and (c) highly probable occurrence of a monoclonal antibody that binds to both forms. As we did not see identity on XIEP and our monoclonal antibody binds only one form, we must consider that the two forms represent distinct different proteins, each inhibiting trypsin, each behaving as an acute phase reactant and each being synthesized by the same cell. Further, there were two polypeptide precursor molecules precipitated in the mRNA experiment but the technology was unable to separate them. That only the rodent species should contain two proteins of such similar behaviour and identity with different polypeptide structures is peculiar and awaits confirmation by studies using purified and isolated inhibitors.

A second explanation of the electrophoretic differences between fast and slow mouse variants may involve their association with serum proteases, this however, is not supported by experiments where both variants bind trypsin and express different electrophoretic behaviour in the complexed form compared to their normal state (data not shown).

Trypsin has been shown to cleave off a 6-8,000 MW peptide (96) from human α1PI which would alter the electrophoretic migration. However, reduction in MW by 6-8,000 should be detected by SDS-PAGE and no such differences in MW between the two variants was seen. In addition, since both variants are secreted by a cloned hepatoma line and by normal mouse
hepatocytes in culture, these findings argue against serum protease interaction to produce variant α1Pi. These observations indicate that the two separate forms are distinct and may possess different anti-protease specificities such as reported by Lee and Janoff for the rat inhibitors (53).

The monoclonal immunoglobulin from lines D7.11 and D7.32 present a means of separating large quantities of fast and slow α1Pi variants. Separation and isolation of the two forms would allow initiation of studies such as amino acid sequencing to determine structural differences as well as variations in their biological activities. Establishment of different specificities would give further insight into the role played by α1Pi in regulating inflammation.

4.10. Tumor Induction

Direct induction of ascitic forms of D7 or its two clones, D7.11 or D7.32 was not possible even after extensive immunosuppressive measures. McKearn (personal communication) has reported similar resistance of some rat X mouse hybridoma lines to adapt to ascites. Although it was possible, with extensive immunosuppressive measures, to establish D7, D7.11, D7.32 and D11 (negative line) as solid subcutaneous tumors, it was still not possible to use these in vivo generated cells for induction of ascites as had been suggested by McKearn (97). Instead, only solid peritoneal tumors resulted from this approach. It may be that the selection of the Lewis rat strain may produce hybridomas that have a tendency for solid tumor induction and that no amount of immunosuppression would allow
ascitic growth. This explanation is consistent with the fact neither D7 subclones nor the D11 cell line produced ascites, but in each instance established only solid tumors.

The generation of solid subcutaneous tumors by D7 or its clones demonstrated, upon gross examination, small white nodules (Figure 13a) on the surface of the liver, which was not seen with sham-treated mice or mice implanted with the negative hybrid D11. On further histological examination, the nodular areas were revealed to be necrotic foci adjacent to arteries and veins interspersed throughout the liver lobule (Figure 13b). It is possible that hybrid cells from the parent solid subcutaneous or peritoneal tumor undergo metastasis to the liver where they lodge and secrete rat IgM anti-α1Pi. The local secretion of the hybridoma immunoglobulin to a specific liver protein may have been directly responsible for the destruction of the surrounding hepatocytes. Analysis of serum samples from mice displaying liver nodules indicated α1Pi levels were reduced 16-25% compared to mice supporting D11 tumors. The D11 tumor mice demonstrated normal to slightly elevated (<5%) levels of α1Pi. We postulate in D7, D7.11 and D7.32 tumor bearing mice, secretion of a specific antibody to circulating α1Pi induces a reduction of normal levels. This reduction may be a result of a decreased liver capacity to synthesis α1Pi as a result of hepatocyte destruction. Conversely, Ag-Ab complexes may be formed and subsequently removed, but this was not investigated. However, IEP analysis of serum taken from the animals indicated a portion of the circulating α1Pi was complexed to rat IgM immunoglobulin and may account for the reduced α1Pi levels detected. The slightly elevated levels seen
in Dll animals may be a result of inflammation caused by the tumor itself resulting in an acute phase response.

4.11. Animal Model of Alpha-1-Protease Inhibitor Deficiency

To date, the only animal model available for the study of human α1Pi deficiencies is the turkey round heart disease model (98). These birds reduced α1Pi levels and PAS positive material in their hepatocytes. With the development of monoclonal antibodies to mouse α1Pi, a second animal model of α1Pi deficiency may be possible.

4.12. Conclusion

In conclusion, our work has reported on the production of a rat × mouse hybridoma cell line secreting a rat IgM capable of binding mouse α1Pi. Two clones, D7.11 and D7.32 demonstrated specificity towards the fast electrophoretic variant of α1Pi and will be useful in elucidating structural differences between the two variants as well as allowing possible investigation into the different roles each variant may play during interactions with serum proteases. Monoclonal antibody to α1Pi may also provide a second animal model for the study of α1Pi deficiencies and the possible association with liver and lung diseases.
5.0 APPENDIX

The work reported in this section is an attempt to describe some of our early investigations on the production of a monoclonal mouse immunoglobulin directed against rat mast cell (RMC) surface markers. This reagent was to be used for mast cell identification and for use in studies on the differentiation pathway of mast cell precursors.

The inability to demonstrate an immune response in mice to rat mast cell surface components was possibly a result of the suppressive effects of histamine released from injected mast cells. This problem forced us to abandon these investigations and pursue instead, our studies on mouse alPi.

This appendix describes the development of a method for the isolation in high purity and yield of mast cells from the peritoneal cavity of Sprague Dawley rats. In addition, this report briefly discusses the apparent suppressive effect of injected whole rat mast cells on the humoral immune response of recipient mice.

5.1. Isolation of Rat Mast Cells.

Methods in the literature have reported several different approaches for the isolation of specific cells from suspensions of mixed cell populations. In particular, velocity sedimentation (99, 100) and differential centrifugation (101), have been employed successfully to
isolate various cell types. These techniques have been used to purify rat mast cells, but have reflected inherent difficulties with high purities (97%) and low yields (<50%).

We have used a modified technique reported by Cooper and Stanworth (102) to attempt to overcome these problems. During our investigation, we improved the reported mast cell yields of Cooper and Stanworth from 72% to greater than 85% while maintaining and/or improving mast cell purities to 98%.

Outbred Sprague Dawley rats (Biobreeding), weighing between 175 and 300 gms were sacrificed by ether euthanasia. This method reduced the degree of blood contamination in the peritoneal cavity normally associated with other techniques of euthanasia.

Twenty-five ml of phosphate buffered saline (PBS) was infused into the peritoneal cavity of rats with a 30 cc syringe and 18 gauge needle and the peritoneum palpated for 2 min. A midline incision was made in the abdominal wall to facilitate the removal of peritoneal exudate cells (PEC) using a 20 cc syringe affixed with a 6 inch tygon catheter. Polypropylene plastic tubes (Falcon) were used for collection and isolation of cells to reduce non-specific adsorption to glass and other types of plastic surfaces. All preparations were kept at 4°C on ice. Cells were spun in a MSE refrigerated centrifuge at 750 x g, 4°C for 1 min including acceleration. Pellets were tapped lose, resuspended at 2 x 10^6 cells/ml in PBS supplemented with Dulbecco's B buffer (500 ml PBS with 2.5 ml Dulbecco's B, pH 7.3), supplemented with 0.1M dextrose. Three ml were layered onto 2 ml of a 30% w/v Ficoll
(Pharmacia) solution in PBS/Dulbecco's B buffer (500 ml PBS with 2.5 ml Dulbecco's B), supplemented with 1.0% BSA. Cells were spun in an MSE centrifuge at 150 x g, at 4°C for 5 min. Cells at the interface and in the upper buffer were mainly lymphocytes, mononuclear cells, red blood cells and a small number of mast cells. The majority of mast cells were found throughout the Ficoll cushion. The upper contaminating phase was aspirated and the Ficoll diluted with 10 ml of PBS/Dulbecco's B buffer supplemented with 1.0% BSA. Cells were pelleted by centrifugation at 750 x g, 4°C for 1 min and resuspended in PBS/Dulbecco's B buffer (no BSA) and their viability checked by trypan blue exclusion. Table 1 shows the results of a typical mast cell isolation after Ficoll separation, with average purities ≥95% and yields in excess of 85%. This represents 1-1.5 X 10⁶ RMC's isolated/animal.

The results of Table 1 were confirmed using cytocentrifuge smears and differential counting using May-Grünwald Giemsa staining. In all smears, the purity approached 100% by this evaluation. Figure 14 is a representative section of a cytocentrifuge smear of the final preparation of purified rat mast cells.

5.2. **Immune Response Alterations by Histamine and Whole Rat Mast Cells**

Intact, isolated mast cells were resuspended in 0.01M PBS, pH 7.3, at a density of 1.5 X 10⁶ cells/250 µl and injected IV via the tail vein into recipient Balb/c mice.

Serum samples were taken from immunized mice 6, 10 and 14 days after injection and assayed for anti-mast cell activity by RIA. In each
Table 1

Rat Mast Cell Purity and Yield Before and After Ficoll Separation;
(Individual Adult Sprague Dawley Rats)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Pre-Isolation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Post-Isolation&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEC</td>
<td>RMC</td>
<td>Purity&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>512</td>
<td>20</td>
<td>3.9%</td>
</tr>
<tr>
<td>2</td>
<td>560</td>
<td>32</td>
<td>5.7%</td>
</tr>
<tr>
<td>3</td>
<td>484</td>
<td>36</td>
<td>7.4%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results enumerated using a hemocytometer.

<sup>b</sup> RMC Purity: Expressed as a fraction of the total cell population.

<sup>c</sup> RMC Yield: Expressed as a fraction of the starting mast cell population.

Abbreviations: PEC - peritoneal exudate cells; RMC - rat mast cells.
Figure 14

Purified mast cell preparation following Ficoll separation. Cytocentrifuge smears of purified rat mast cells were stained with May-Grünwald Giemsa. Virtually all cells were RMC.
treated animal, no immune response could be detected, even following a schedule of multiple boosts (1 to 6 given at bi-weekly intervals) no immune response was detectable.

Histamine content of mast cells range from 7 to 32 pg/cell (103). Each mouse injected with $1.5 \times 10^6$ mast cells, received between 10 and 50 μg of histamine. Wang et al. (104) have reported a suppressive effect of histamine on the in vitro proliferative responses of lymphocytes to lectins PHA and Con A, suggesting inhibition of lymphocyte function. In addition, Shearer et al. (105) have demonstrated that histamine inhibits the production and/or release of antibodies from lymphocytes. We examined the possibility that histamine liberated from injected mast cells had a suppressive effect on the immune responsiveness of recipient mice leading to the lack of antibody production.

Five-hundred μg of TNP-BGG, a T cell dependent antigen, was injected IP into recipient mice 1 day following sensitization with either 10 μg of reagent grade histamine, or $1.5 \times 10^6$ intact rat mast cells, or membrane fragments (MF) from $1.5 \times 10^6$ rat mast cells or PBS. The immune response was monitored for specific IgM and IgG to TNP-BGG antigen by a modified Jerne-Nordin plaque assay (106). Figures 15 and 16 indicate both IgM and IgG antibody responses to TNP-BGG were inhibited by intact rat mast cells but not appreciably by their membranes.

Our observations suggested the inability to demonstrate an immune response in mice, to rat mast cell surface antigens was attributable to the suppressive effects of administered histamine from the mast cells. Future investigations may make use of histamine free membrane preparations
Mouse IgM Response to TNP-BGG

Treatment
- 10μg Reagent grade histamine
- Rat mast cell membrane fragments (RMC MF) $1.5 \times 10^6$ intact cells
- Rat mast cells (RMC) intact $1.5 \times 10^6$
- PBS control

Figure 15

Inhibition of mouse IgM responses to TNP-BGG following histamine challenge. Both reagent grade histamine and $1.5 \times 10^6$ intact rat mast cells induced a suppression of mouse IgM to TNP-BGG, while membrane preparations of mast cells had no apparent effect. Detection of mouse IgM was by the use of a Jerne-Nordin plaque assay.
Inhibition of mouse IgG responses to TNP-BGG following histamine challenge. Mouse IgG as detected by a Jerne-Nordin plaque assay was suppressed by pre-treatment with either reagent grade histamine or $1.5 \times 10^6$ intact rat mast cells but not with membrane preparations of mast cells.
to generate specific immunological reagents, thereby expanding the capabilities for recognizing and studying mast cells and their related biochemistry and biology.
6.0 BIBLIOGRAPHY


