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Generation of Cross-Reactive Monoclonal Anti-Streptococcal Cell Membrane Antibodies: The Evolution of a Murine Model for Goodpasture's Syndrome

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GENERATION OF CROSS-REACTIVE MONOCLONAL ANTI-STREPTOCOCCAL CELL MEMBRANE ANTIBODIES: THE EVOLUTION OF A MURINE MODEL FOR GOODPASTURE'S SYNDROME

by

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LIST OF ABBREVIATIONS AND SYMBOLS

ABM	Alveolar Basement Membrane
Ab	Antibody
AGN	Acute Glomerulonephritis
APSGN	Acute Post-Streptococcal Glomerulonephritis
ARF	Acute Rheumatic Fever
BSA	Bovine Serum Albumin
CBM	Capillary Basement Membrane
CFA	Complete Freunds Adjuvant
CIC	Circulation Immune complexes
CNBr	Cyanogen Bromide
Con A	Concanavalin A
CPM	Counts Per Minute
EHS	Engelbreth-Holm-Swarm
ELISA	Enzyme Linked Immunosorbant Assay
FITC	Fluorescein Isothiocyanate
GBM	Glomerular Basement membrane
GP	Goodpasture's
H and E	Hematoxylin and Eosin
HAT	Hypoxanthine, Aminopterin, Thymidine
HBSS	Hank's Balanced Salt Solution
HLA	Human Leukocyte Antigen
HPLC	High Pressure Liquid Chromatography
HRA	Heart Reactive Antibodies
HSA	Human Serum Albumin

HSPG	Heparin Sulfate Proteoglycan
Ig	Immunoglobulin
IL-1	Interleukin-1
IP	Intraperitoneal
Kd	Kilodalton
LBM	Lung Basement Membrane
L/B ratio	Lung/Body Weight Ratio
mAb	Monoclonal Antibody
M Alpha + H	MEM Alpha Medium with L-Glutamine, and Hybri-Sure™ (Sterile Fetal Bovine Serum)
MEM	Minimum Essential Medium (Eagles)
MHC	Major Histocompatibility Complex
MOR	Multiple Organ-Reactive
MW	Molecular Weight
NC	Noncollagenous
NDRI	National Diabetes Research Interchange
nm	Nanometers
NSAP	Nephritis Strain-Associated Proteins
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PEG	Polyethyleneglycol
PHA	Phytohemagglutinin
pI	Isoelectric Point
RIA	Radioimmunoassay
RPM	Rotations per minute

SCM	Streptococcal Cell Membrane
SDS	Sodium Dodecyl Sulfate
SI	Stimulation Index
SLE	Systemic Lupus Erythematosus
S. mutans	Streptococcus mutans
TNF	Tumor Necrosis Factor
ul	Microliter
Wt	Weight
X-63	P3-X63-Ag8 Plasmacytoma Cell

CHAPTER I

INTRODUCTION

A. DEVELOPMENT OF MONOCLONAL TECHNOLOGY

When the immune system responds to a foreign antigen, the antibodies which are generated, bind to a variety of epitopes in that antigen. Each B cell recruited by the immune system produces a single antibody which is directed towards only one of a whole group of epitopes which as a whole make up the antigen. Monoclonal antibody production is a powerful tool which enables the formation of large amounts of a single antibody. This technique allows the selection of a single B cell, its expansion by fusion with an immortal cell line, followed by isolation of the desired clone, and thus the generation of large quantities of the B cell specific antibody.

Much of the early work on cloning, was carried out by Kohler and Milstein (1975). These investigators fused the myeloma cell P3-X63-Ag8 (a cell line which was derived from Balb/c mice) with spleen cells from Balb/c mice immunized with sheep red blood cells (SRBC). The fusion of these cells was accomplished through the use of an inactivated Sendi virus. This was an important step since the single antibody that was produced was directed against a preselected antigen (ie. SRBC). In addition different cell lines could be made against the various epitopes that were part of the antigen. In later

experiments designed to determine the rate of spontaneous loss of immunoglobulin secretion, Kohle et al.,(1976) isolated nonsecreting plasmacytomas. These immortal cells could then be used for hybrid production.

By putting hybrids back into a mouse of the same haplotype (Balb/c) solid tumors could be generated. The tumor would continue to produce the monoclonal antibody as long as the mouse survived the tumor. In addition, not only were all nutrient requirements of the cell line met by the host animal, but also the tumor was maintained in an environment free from the cells own waist products and from contamination by bacteria,molds and fungi.

B. STREPTOCOCCAL SEQUELAE

Since the early 1940's an association between Group A, streptococcal infection and acute glomerulonephritis (AGN) was suggested (Futcher,1940). Despite many attempts to elucidate the connection between these two disease states, the pathogenic mechanisms which lead to post streptococcal sequelae are still unclear. One reason for this obscurity is that streptococcal infections only occasionally result in detectable kidney damage. A possible explanation for the lack of consistent kidney pathology may involve the existence of nephritis strain-associated proteins (NSAP). Villarreal et al. (1979) have compared a number of strains of Streptococci, and determined that only a select few produce NSAP. These antigens are secreted by the organism and form immune complexes with circulating antibodies. The immune complexes then lodge in the kidney resulting in tissue necrosis. The theory that bacterial strain specific factors are

responsible for post-streptococcal sequelae is supported by Van de Rijn et al. (1978). These investigators demonstrated that equivalent levels of circulating immune complexes were found in patient populations suffering from two different post-streptococcal diseases, acute rheumatic fever (ARF) and acute post-streptococcal glomerulonephritis (APSGN). Villareal et al. (1979) have identified a 46 Kd protein from bacterial strains isolated from patients diagnosed with post streptococcal acute glomerulonephritis. NSAP contains structural and biochemical properties identical to streptokinase. Both molecules share the first 21 amino-terminal amino acids (Johnston and Zabriskie, 1986).

A second reason for sporadic glomerulonephritis following streptococcal infection may be genetic susceptibility to the disorder. Pre-liminary studies examining the HLA type of Japanese patients suffering from APSGN implicate the HLA antigen DR.1 as a possible prerequisite for the disease. APSGN as well as other glomerulopathies in the study seem to be associated with MHC class II antigens rather than MHC class I antigens (Naito et al.,1987).

The class and subclass of anti-GBM antibody may be a third factor in determining those individuals who are susceptible to glomeruloneph-ritis (Lockwood et al., 1987). By examining the sera from 20 patients with anti-GBM nephritis a correlation was drawn between IgG subclasses and the occurrence of kidney pathology. Levels of IgG_2 were exceedingly low, while IgG_3 was not even detectable. IgG_1 and IgG_4 on the other hand, were detectable in the sera and eluted from the kidneys of these patients. Lockwood et al. (1987)

were presented with a case history of an individual whose kidney biopsy showed a linear deposition along the basement membrane but failed to follow the normal course of anti-GBM nephritis. Subclass analysis showed that the individual's serum anti-GBM antibody was exclusively IgG_4. In addition to the clinical data, IgG_4 antibodies are univalent, do not fix complement, and do not have the facility for binding to macrophages through the Fc receptor. IgG_1 has, therefor, been implicated as the subclass of anti-GBM antibody responsible for the damage directed towards the kidney.

APSGN is by definition associated with previous infection by group A, Beta hemolytic streptococci. Yoshimoto et al. (1987) developed 15 monoclonal antibodies to SCM, and isolated the antigen which bound to each monoclonal antibody. They then screened human serum for antibodies that would bind to these isolated antigens. Sera of children with APSGN were compared with sera from children with uncomplicated streptococcal pharyngitis, and from normal controls. The children with APSGN had significantly higher levels of antibodies to the isolated SCM (Yoshimoto et al.,1987).

The presence of granular immune deposits in the GBM, as detected by fluorescent staining, seems to indicate that it is immune complexes which induce the glomerulonephritis. At least three theories have been proposed to explain the occurrence of APSGN: 1) Immune complex deposition in the GBM; 2) In situ formation of immune complexes and 3) Production of anti-streptococcal cytoplasmic membrane (SCM) antibodies cross-reactive with GBM epitopes. Overall the etiology of APSGN is unclear.

In the theory involving immune complex deposition, processing of the streptococci by the immune system produced a pool of soluble antigens which readily complex with their respective antibodies. Friedman et al. (1984) extracted immune complexes from sera of patients with post streptococcal sequelae they demonstrated that the complexes had streptococcal substituents as their antigenic component. Sera from rabbits receiving the immune complexes contained antibodies which reacted with streptococcal strains associated with either ARF or APSGN (Friedman et al., 1984). The circulating immune complexes (CIC) are trapped by the GBM while being filtered through the kidney. Once trapped the immune complexes initiate the activation of the complement cascade resulting in destruction of the GBM (Zabriskie et al., 1973; Rodriguez-Iturbe et al.,1980). The correlation between CIC and tissue pathology however, is tenuous (Yoshizawa et al., 1983).

The more elaborate theory of in situ formation of immune complexes proposes that streptococcal antigens circulating within the vasculature are trapped by the GBM. Subsequent to this the antigens are complexed with anti-GBM antibodies and a complement mediated reaction occurs (Michael et al., 1966; Yoshizawa et al.,1973). The interaction between the GBM and the macromolecules (antigens, antibodies or immune complexes) it comes in contact with occurs because of an over-all negative charge on the GBM. This phenomena results in an electrostatic repulsion of anionic macromolecules and an attraction of cationic macromolecules. While neutral or negative charged molecules, ~68Kd in size (about the size of albumin), enter the GBM in very small numbers (Farquhar, 1978), highly cationic macro-

molecules can penetrate into all three layers of the GBM (Rennke et al., 1975). Rather than an immune complex being deposited into the GBM, Vogt (1984) suggested that cationic macromolecules (57Kd or greater) enter the GBM, and the immune complex arises in situ. Support of in situ immune complex formation was provided by Stinson et al. (1984) who absorbed discreet antigens from extracts of S. mutans MT703 with rabbit and monkey kidney homogenates. Intact cells of S. mutans were extracted sequentially with a combination of organic solvents and ammonium hydroxide. After incubation, the bacterial extracts which bound to the homogenated kidneys were eluted and loaded on to acrylamide gels. S. mutans antigens of 65Kd, 35Kd, and 24Kd were isolated by SDS-PAGE. Immune complexes with a net positive (cationic) charge also have the capacity to permeate the various layers of the GBM (Caulin-Glaser et al., 1983).

While the above mentioned theories propose the interaction of antibody and foreign antigen, the remaining theory states that antibodies produced in response to a challenge by streptococci cross-react with antigens in mammalian basement membranes. This theory implies an epitope(s) common to mammalian basement membrane and SCM. If this is true an animal model of kidney pathology could be induced simply by injection of the appropriate anti-SCM antibody. Markowitz et al. (1960) demonstrated a direct relationship between the Group A streptococci and AGN by injection of a virulent strain of Group A, beta hemolytic streptococci into the peritoneal cavity of rats. After four weeks, rat kidneys showed extensive lesions throughout the entire organ. Antibodies produced in response to the bacterial antigens seem

to deposit in the kidney initiating the resulting tissue damage. Subsequently Markowitz and Lange (1964) established that the immunologic foci for the induction of a cross-reactive antibody was the SCM. Crude extracts of streptococcal cell wall, as well as human lung and kidney digests, formed a common precipitation pattern when immuno-electrophoresed with anti-human kidney antibodies (Holm, 1967). A common precipitation pattern indicates that the anti-human kidney antibodies are reacting with epitopes in both human tissue and bacterial cell wall. Why do these antibodies appear in the kidney in the first place? Perhaps they are produced in response to foreign antigens (eg. SCM). The presentation of SCM to the mammalian immune system induces the formation of antibodies that may cross react with mammalian basement membrane. The array of immune responses is, therefore, directed at host tissue, resulting in autoimmune phenomena with subsequent tissue pathology. Serological cross-reactivity existing between group A streptococci and antigens in the GBM appear not to be the result of antigen deposition but rather due to deposition of immunoglobulins (anti-SCM antibodies) in the kidney which are capable of cross-reacting with the GBM.

In support of this theory, Blue and Lange (1975) using indirect immunofluorescent staining procedures, demonstrated that rabbit anti-SCM antibodies were capable of binding to human GBM. Fluorescein isothio-cyanate (FITC) conjugated goat anti-rabbit gamma-globulin revealed a granular deposition of anti-SCM antibodies on normal human kidney sections. This data supports speculation regarding the cross-reactive nature of the anti-SCM elicited antibodies. Deposition was

inhibited by pre-absorption of antisera with either SCM or GBM; homogenized liver and spleen were unable to remove the fluorescent pattern. Taken together, these data suggest a unique cross-reactivity between SCM and GBM.

Blue and Lange (1976) went on to show that the age of the host played a significant role in the severity of the cross reactive response. Since major human GBM antigens have been shown to be glycoproteins, the effect of carbohydrase treatment on GBM was evaluated in terms of it's role in the extent of anti-SCM binding to GBM. Increased cross reactivity between anti-SCM antibodies and GBM of carbohydrase treated kidney tissue pointed to a glycoprotein as the cross-reactive epitope. This data was supported by previous work by Quish and Lange (1973) who demonstrated a greater precipitant reaction between SCM antiserum and GBM after treatment with a carbohydrase. Blue et al. (1980) confirmed, by an indirect fluorescent assay using rabbit anti-human GBM and FITC conjugated sheep anti-rabbit IgG, the role that carbohydrates play in masking GBM epitopes. Comparisons were made between the reactivity of anti-GBM antibodies to GBM before and after carbohydrase treatment of the kidney sections. Carbohydrase treated GBM showed greater antibody binding as indicated by greater fluorescence of tissue sections. Further, preabsoption of the anti-GBM antisera with carbohydrase treated GBM reduced the ability of the antisera to react with the kidney sections. These studies indicated that a correlation existed between host age, as it is reflected in the level of glycosylation, and tissue antigenicity.

The increase in the number of carbohydrate moieties that become

associated with proteins with increased age may mask the cross reactive epitope making it less reactive with SCM elicited antibodies. This supposition is reflected in the higher incidence of APSGN seen in children as opposed to adults. The relationship between glycosylation versus antigenicity was pursued further by Lange et al.(1981). Their research elucidated the role that the carbohydrate and protein moieties play in the immunologic cross-reactivity of the SCM and GBM. While the intensity of the cross-reactive response (anti-SCM antibodies binding to GBM) was determined by the extent of protection afforded by the carbohydrate, the cross-reactive moiety is in fact not the carbohydrate but rather it's associated protein structure. Antibodies directed towards the carbohydrates were only slightly cross-reactive with SCM while antibodies against the relevant proteins were extremely cross-reactive. Further, identification of the specific protein associated carbohydrate was achieved through the use of carbohydrases. Neuraminidase and Beta-Galactosidase treatment of normal human kidney increased the reactivity of the anti-SCM antibodies to the GBM. Lange et al., (1984) determined that these two sugars, at least in part, make up the overlying oligosaccharide side chain at or near the protein epitope. Anti-Heparin sulfate proteoglycan (HSPG) antibodies have induced subepithelial basement membrane thickening in the GBM (Mietinen et al., 1986). The presence of HSPG in the GBM, as well as the appearance of autoantibodies against HSPG in the sera of patients with PSAGN (Fillit et al., 1985), make this proteoglycan clinically significant.

It has been suggested that specific cross-reactive antibodies

directed towards epitopes on both the SCM and GBM account for the sub-epithelial humps seen in APSGN. Lange et al. (1985) developed an animal model showing that rabbit anti-SCM could stimulate synthesis of murine GBM. The results of this antibody treatment was a pathology which mim-icked PSAGN in humans (Nayyar et al. 1985a, Nayyar et al. 1985b,Lange et al. 1985). The fact that anti-SCM antibodies raised in rabbit could bind to human GBM, as indicated by indirect immunofluorescence (Blue and Lange, 1975), supported the concept of an SCM elicited antibody which was cross-reactive with GBM. Further investigations into the cross-reactive nature of anti-streptococcal antibodies involved the use of hybridoma technology. Cunningham et al. (1984) produced hybridomas against M type 5 Streptococcus pyogenes, and then cloned (by limiting dilution) cells which synthesized anti-SCM antibodies which were cross-reactive with human heart antigen. ELISA (Enzyme Linked Immunosorbant Assay) and Western immunoblotting techniques were used by these investigators to compare the anti-SCM monoclonal antibodies for their reactivity to triton X-100-extracted heart antigen. Some monoclonal antibodies cross-reacted primarily with heart extract while other clones reacted with similar epitopes ex-tracted from heart, kidney or skeletal muscle.

Lange and Weber (1985) produced hybridomas from SCM immunized mice. Antibody production specific for SCM and GBM was detected by ELISA, using either trypsin or collagenase digested membrane coated plates. Of the fusion products tested, one polyclone (III 3C) produced ascitic fluid which was highly reactive with both SCM and GBM. From this poly-clone sixteen monoclonal antibodies have been

prepared which are strongly reactive with both SCM and GBM (Lange and Weber,1986). Gross examination of animals bearing these clones intraperitoneally reveals hemorrhagic lungs ranging from multiple patches to totally hemorrhagic lungs. Necrotic lungs appeared intermittently in these mice, with fe-males being more predisposed to tissue damage. These observations suggest that the SCM induced antibodies are generally reactive towards basement membrane and not just to GBM. Thus, the presence of anti-SCM antibody in the murine model appears at least on gross observation, to mimic the pathology of Goodpasture's syndrome (ie. lung and kidney involvement).

However, the concept of a cross-reactive monoclonal antibody resulting from "antigenic mimicry" has recently been called into question. Swartzwelder et al. (1988) compared reactivity of anti-heart antibodies and anti-Streptococcal antibodies isolated from rabbits sensitized with Streptococcus mutans whole cell homogenate. These investigators first demonstrated that low levels of autoantibodies to heart antigens were endogenous to normal serum. Titers for heart reactive antibodies (HRA) were increased after immunization with S. mutans but the bacterial homogenate did not seem to induce the formation of new HRA. Affinity purified anti-S. mutans antibodies from immunized rabbits were tested for reactivity to solubilized heart components. Additionally, affinity purified anti-myosin antibodies, from the same rabbits were tested for reactivity to S. mutans. Neither experiment showed antibodies reactive with the reciprocal antigen. Swartzwelder et al. (1988), therefore, contend that immunizing an animal with S. mutans stimulate an increase in the production

of naturally occurring, non-cross-reactive autoantibodies, not the formation of new cross-reactive antibodies.

In disease states such as Goodpasture's syndrome and APSGN, autoantibodies directed against the various components of the glomerular basement membrane (including type IV collagen) arise and contribute to the pathology of these diseases. Glomerulonephritis in Goodpasture's syndrome may be distinguished from the nephritic state of APSGN in that the former disease is associated with a single linear deposition of antibodies along the endothelial aspect of the basement membrane (Schiffer et al., 1981). The latter condition includes granular deposits of immunoglobulins at this site. Case histories have been reported, however, of patients diagnosed with Goodpasture's having both linear deposits (antiglomerular basement membrane antibodies) and granular deposits (immune complexes). In addition to its characteristics subepithelial humps, APSGN appears to differ from Goodpasture's syndrome in terms of the variety of autoantibodies which arise in these diseases. Destruction of the kidney in APSGN may be traced to one or more autoantibodies. Serum from APSGN patients have been found to contain antibodies reactive against a number of basement membrane constituents, including heparin sulfate proteoglycan and galactose-containing proteoglycan (Fillit et al.,1984), as well as laminin and the 7-S fragment of type IV collagen (Kefalides et al.,1986). On the other hand, there seems to be a general agreement that serum from Goodpasture's patients contain autoantibodies primarily directed at the NC1 domain (Kefalides et al., 1984; Weislander et al., 1984; Butkowski et al., 1985; Weck et al., 1986).

Although these two disease states both result in glomerular damage due to autoantibody production, the antibodies seem to be directed toward different epitopes in the GBM.

C. GOODPASTURE'S SYNDROME

Goodpasture's syndrome has been defined classically as anti-GBM glomerulonephritis frequently preceded by lung hemorrhages (Wilson and Dixon, 1973). Circulating autoantibodies which can be detected by ELISA ultimately associate with the GBM and alveolar basement membrane (ABM). In both the GBM (Lerner et al., 1967) and ABM (Beechler et al., 1980; Wieslander and Heingerd, 1985) deposition of the autoantibodies occurs in a linear fashion. Goodpasture's syndrome presents with proteinuria resulting from glomerulonephritis which progresses rapidly to end stage renal failure. The characteristic linear deposition of immunoglobulins along the GBM is primarily composed of IgG, and may be accompanied by IgM and IgA (Savage et al.,1986). The severity of pulmonary hemorrhage ranges from mild hemoptysis to severe respiratory failure. In addition to antibody deposition on the basement membrane, lung pathology is characterized by intra-alveolar hemorrhaging and large numbers of hemosiderin-laden macrophages (Beechler et al.,1980; Donald et al., 1975). The autopsied lung may have relatively unaffected alveoli as well as some heavily damaged alveoli loaded with erythrocytes, neutrophils and macrophages (Rees and Lockwood, 1988). A possible cause for the patchiness of lung damage may be reflected in the histology of alveolar capillaries in that alveolar capillaries are lined with unfenestrated endothelium, which is relatively impermeable to IgG(Bill, 1977). Jennings et al.,

(1981) demonstrated that increased permeability of the alveolar capillary wall was necessary for the artificial induction of anti-ABM pneumonitis in rabbits. A number of environmental factors (i.e. bacterial infection and smoking) have been implicated as mediators of the severity of pulmonary hemorrhaging in Goodpasture's syndrome (Rees et al., 1977; Donaghy and Rees, 1983). Electron microscopy of lung biopsy show gaps between endothelial cells. These gaps contain platelet plugs, monocytes or processes from pneumocytes. The basement membranes were fragmented, irregular in outline and wider and less dense than normal (Donald et al.,1975).

Genetic predisposition to Goodpasture's syndrome is currently under investigation. Several investigators (Rees et al., 1978; Rees et al., 1984; Bernis et al., 1985) have reported a strong association between Goodpasture's syndrome and the histocompatibility antigen HLA-DR2. The etiology of Goodpasture's syndrome is unknown.

D. IMMUNOCHEMISTRY AND MOLECULAR ASPECTS

As early as 1919 associations have been drawn between glomerulonephritis and pulmonary hemorrhages (Goodpasture, 1919). Later Stanton and Tange (1958) used the term Goodpasture's syndrome to identify the pathologies associated with the lung and kidney. By injecting human anti-GBM antibodies into monkeys Lerner et al. (1967) were able to induce the state of glomerulonephritis, thereby implicating anti-GBM antibodies as the causative agent of nephritis. With the recognition that Goodpasture's syndrome tended to arise from an anti-glomerular basement membrane (anti-GBM) antibody, investigators began to restrict the use of the term Goodpasture's

syndrome to patients with evidence of anti-GBM antibody formation (Glassock, 1978). These anti-GBM antibodies react specifically with basement membrane antigens of the glomerulus (Wilson and Dixon, 1979), alveolus (Wilson and Dixon, 1973) and renal tubules (Briggs et al., 1979). Recently Cashman et al. (1988) were able to demonstrate the presence of Goodpasture's antigen at additional sites outside the kidney. Antibodies eluted from kidneys of Goodpasture's patients bound to basement membrane of the thyroid, eye, liver, adrenal gland, pituitary and breast tissue.

The putative autoantigen(s) involved in Goodpasture's syndrome may be different when compared with those antigens which play a role in other forms of nephritis (eg. SLE and periarteritis nodosa). Weislander et al. (1983) exposed human GBM to a variety of chemical modifications. Only the antigen(s) reacting with the IgG antibodies present in sera from patients with Goodpasture's syndrome is sensitive to a reducing environment. Additional support for the unique characteristics of the Goodpasture's antigen is the fact that collagenase treatment of the GBM left the reactive epitope intact, while pepsin treatment and exposure to a reducing environment inactivated the epitope. These data gives some insight into the structural elements and location of the Goodpasture's antigen.

The relationship between GBM and Goodpasture's syndrome has been reviewed by Kefalides (1987). The type IV collagen contains an alpha-1 and an alpha-2 chain which can: 1) form two distinct homotrimers $(alpha\text{-}1)_3$ and $(alpha\text{-}2)_3$ or 2) form one heterotrimer $[(alpha\text{-}1)_2 alpha\text{-}2]$ (Templ et al., 1981). Type IV collagen is comprised of

four distinct regions. They are; the collagenous domain, the noncollageous NC 1 and NC 2 domains, and the 7-S domain. The collagenous domain is a triple helix which occupies the center of the type IV collagen molecule, and may be isolated from basement membrane by pepsin or chymotrypsin digest. The collagenous domain is flanked on it's carboxy side by the NC 1 domain (a globular noncollagenous peptide), and on it's amino side by the NC 2 domain. These domains are left intact during collagenase treatment but are destroyed by pepsin. Sodium dodecyl sulfate (SDS)-gel electrophor-esis of the denatured NC 1 domain gives rise to monomeric and dimeric molecules of molecular weights 27Kd and 54Kd respectively. Although the 7-S domain is collagenous in nature, it is resistant to collagenase treatment. This is due to its location (the amino terminus of the molecule). Crosslinking of four separate type IV collagen molecules at this point protect the 7-S domain from collagenase digestion (Risteli et al., 1981).

The location of the Goodpasture's antigen appears to be the globular domain NC1, a structure associated with type IV collagen at its carboxyl terminal end (Weislander et al., 1984; Weislander and Heinegard, 1985). The NC1 domain is composed of a number of monomeric and dimeric peptides. Included among the monomers are three distinct peptides (M1, M2, M3) with slightly different molecular weights in the range of 25 Kd to 30 Kd. Of the three monomers only one, M2 was reactive with sera from Goodpasture patients (Butkowski et al., 1985) and therefore has come to be called the Goodpasture's antigen. M2 sequences comprise 2.5% of the mass of type IV collagen, being located

in the alpha one chain. The peptide dimers exist in two classes D1 and D2. Each of the peptide dimers in class D2 (D2a,b,c and d) contains the reactive epitope. Peptide mapping of these sequences reveals repeats of the M2 sequence (Butkowski et al., 1985). In an attempt to further characterize the autoantigen(s), basement membrane-producing tumor cells were adopted. Engelbreth-Holm-Swarm (EHS) sarcoma is a transplantable mouse tumor capable of producing extracellular basement membrane. Wick and Templ (1980) demonstrated the capacity of this tumor to remove the relevant antibodies from Goodpasture sera, as indicated by a complete loss of sera reactivity human and murine kidney sections. Laminin, the major non-collagenous component of the EHS tumor, however, was by itself unable to bind the anti-GBM antibodies of Goodpasture sera (Weck and Templ, 1980). Wick et al. (1986) also showed that intracutaneous immunization of mice with the NC1 domain of type IV collagen generated by the EHS tumor, would result in the generation of tissue reactive antibodies as demonstrated by ELISA and, caused kidney and lung pathology as indicated by immunofluorescence of tissues.

Anti-GBM antibody binding is essential for the characterization of the Goodpasture's antigen(s). Holdsworth et al. (1979) used collagenase solubilized antigens from human GBM to identify anti-GBM antibodies in sera from over 500 patients with anti-GBM diseases. With these anti- bodies and the antibodies eluted from 64 anti-GBM affected kidneys, Holdsworth et al. (1979) ran solubilized human GBM on 10% polyacrylamide gel electrophoresis (PAGE) and isolated 3 major reactive peaks (27 Kd; 53Kd and >150Kd). Human sera containing an-

tibodies to lung basement membrane also bound to the major GBM peaks (Holdworth et al. 1979). Fish et al. (1984) used PAGE separation of collagenase digested GBM followed by Western blotting, to show that sera from 13 Goodpasture's patients bound to a number of the membrane proteins. Anti-Goodpasture's (anti-GP) antibodies from the sera of these patients reacted with two protein bands in the range of 45-50 Kd and at least one and sometimes two or three bands in the 25-28 Kd range. These investigators (Fish et al. 1984) declined to speculate whether or not the larger molecular weight proteins represented repeating segments of the lower molecular weight proteins. Amino acid analysis of these proteins showed a low hydroxyproline, hydroxylysine and glycine content, suggesting that non-collagenous polar glycoproteins are present. The presence of cysteine indicates the presence of disulfide bonds (Fish et al. 1984).

Pressey et al. (1983) prepared a monoclonal antibody to the autoantigenic component of human GBM using BALB/c mice immunized with a soluble collagenase digest of human GBM. Competitive RIA studies between the monoclonal antibody and anti-GBM positive human sera identified the probable specificity of the monoclonal antibody. The finding that the monoclonal antibody bound to several protein fractions on nitrocellulose supported the contention (Pressey et al., 1983) that there is only one antigen which is present in multiple copies in the GBM.

More recently a clinical picture of Goodpasture's syndrome has developed, which includes confirmed anti-GBM antibody and intermittent involvement of the lung. The involvement of other organs (eg skin)

which contain type IV collagen is also variable. While Wieslander and Heinegard (1985) were able to show the presence of the presumptive autoantigen(s) (26Kd and 50Kd) in basement membranes of the glomerulus, placenta and lung, they were unable to demonstrate antibody binding to intact placenta basement membrane. The presence of the Goodpasture's antigen was determined by the binding of FITC-conjugated anti-IgG antibody to the tissue indirectly through the anti-GP antibody, when employing the method of indirect immunofluorescence. The lack of antibody binding was attributed to either a lack of accessibility of the antigen in certain tissues, or a difference in the distribution of any subtype of the type IV collagen (subtype being the result of epitope density) (Wieslander and Heingard, 1985). When the various solubilized basement membrane antigens were transferred from the polyacrylamide gels to nitrocellulose, immune staining with anti-GP antibodies showed variability of antibody binding. Only GBM was able to bind the antibodies with both its 26 Kd and 50 Kd proteins. Placenta and lung basement membrane showed antibody binding to the 50 Kd protein only, indicating a somewhat different organization of the type IV collagen from the three sources (Wieslander and Heingerd, 1985).

Pusey (et al., 1987) constructed a monoclonal antibody from mouse B cells stimulated with collagenase-solubilized human GBM. Immunoblotting techniques were used to show the binding of the monoclonal antibody to six bands of peptides from collagenase solubilized GBM. The two major bands had molecular weights of 26Kd and 58Kd respectively. The constructed antibody was not reactive with

types I or II collagen, laminin, elastin, fibronectin, proteoglycans, double stranded DNA or single stranded DNA. The six solubilized GBM bands were also recognized by anti-GP antibody from Goodpasture's syndrome patients.

The development of an animal model for Goodpasture's syndrome would have advantages in many aspects of the investigation of this disease. Using an immunologic agent which induces similar tissue pathology could be one means of generating an animal model. Autoantibodies elicited by Group A, beta hemolytic streptococcus may provide this pathology.

E. ANTI-GBM ANIMAL MODELS

In general the immune system is a highly regulated and specific set of responses to foreign antigens. Tolerance and/or suppression normally prevent the immune system from mounting a response against host tissue. Autoimmune diseases represent defects in the regulation of the immune system. Animal models are of great benefit since they provide insight into the break down of this highly regulated system. Anti-GBM animal models tend to be generated by one of two methods: 1) foreign antigens, primarily toxic chemicals or heterologous GBM injected into mammalian models for the generation of anti-GBM antibodies or 2) The direct *in vivo* addition of such anti-GBM antibodies.

Druet *et al.*, (1977) were able to induce the production of anti-GBM antibodies in Brown Norway rats by subcutaneous injecting Mercuric Chloride. Anti-GBM antibody production was detected by direct fluorescence of kidney sections using sheep anti-rat IgG antisera.

Heterologous GBM has been used in numerous studies to induce the formation of host re-active anti-GBM antibodies (Steblay, 1962; Steblay and Rudofsky, 1983a). Of greater significance is the fact that, when human LBM was injected into sheep, the animal developed autoantibodies directed not only to-wards their LBM but also GBM (Steblay and Rudofsky, 1983b). As early as 1956 rabbit antibodies directed against the rat kidney have been used as a tool in the formation of animal models for the study of nephritic states (Ortega and Mellor, 1956). Later Lerner et al., (1967) showed that anti-GBM antibodies were capable of inducing nephritis in an animal model.

To our knowledge no animal model exists for the study of Goodpasture's syndrome. An animal model would be of great benefit in addressing a number of questions regarding the nature of the disease. First, Goodpasture's syndrome by nature presents with sporadic lung hemorrhage with a wide range of severity. While smoking, bacterial or viral infection have been implicated (Bentoit et al., 1964; Rees et al., 1977; Rees, 1983) no definitive answer for the mediator of lung involvement is known at present. Second, this disease has been shown to effect both sexes (Benoit et al., 1964; Savage, 1986); although questions as to the role that the sex of the individual plays in the severity of tissue damage is still unknown. Third, the factor (s) which initiate Goodpasture's disease remain unknown. This is an especially difficult problem to address since it remains unclear whether the agent in question initiates the production of autoantibodies or simply elevate preexisting autoantibodies which are already present in very low levels

F. LUNG ASSOCIATED AUTOIMMUNE PHENOMINA

The glomerular damage induced during Goodpasture's syndrome is accompanied by lung hemorrhage. The hemorrhage is presumably the typical cascade of events resulting from the binding of antibody and compliment to alveolar basement membrane (Koffler et al., 1969; Mercole and Hagadorn, 1973). Antibody eluted by acid wash from lung and kidney of Goodpasture's patients bound equally well to normal GBM as judged by indirect immunofluorescence (Hagadorn et al., 1969,)

The fact that antibodies generated during Goodpasture's disease are found in linear deposition in the alveolar basement membrane implies a common antigen in the GBM and lung (Wieslander and Heingerd, 1985). This concept is supported by Hagadorn and Mercole (1971) who injected rats with rabbit anti-rat lung serum and showed lung and kidney lesions resembling those of Goodpasture's syndrome. Sado et al. (1984) employing soluble antigens, obtained from bovine glomerular basement membrane, demonstrated the induction of pulmonary hemorrhages in rabbits. The hemorrhages are thought to be due to binding of anti-GBM antibodies to the lung basement membrane. These investigators, however, were not able to demonstrate this phenomena by immunofluorescence. One feature which the GBM and lung have in common is the presence of type IV collagen as part of their structural organization (Konomi et al.,1981). Although both contain type IV collagen the basement membrane's gas exchange in the lungs has necessitated a more diverse arrangement of basement membranes in the lung. The wall between two alveoli contains alveolar cells, capillaries and their basement membranes. Each wall has a thin side and a thick side. The

thin side is made up of the alveolar epithelium, a basement membrane and the capillary endothelium. The thin side morphology allows gas exchange to occur. The thick side contains both an alveolar basement membrane (ABM) and a capillary basement membrane (CBM) which separates the epithelium from the endo- thelium. The (ABM) is dense and amorphous, containing 3 to 5 nm long filaments which run perpendicular from the ABM lamina densa to the cell plasma membrane of the epithelial and endothelial cells. The CBM is fibrillar and less compact than the ABM, containing only 1/5 the number of anionic binding sites. The ABM and CBM are in turn separated by interstitial type IV collagen and elastin. It is thought (Huang, 1978) that the thin side is in fact a unit membrane composed of both ABM and CBM in discreet but tightly packed layers. The alveolar wall also contains type I and Type II alveolar cells. Type I alveolar cells differ from type II cells in that type II cells have cytoplasmic processes (foot processes) which penetrate the ABM lamina densa and extend into the interstitium of the alveolar wall (Vaccaro and Brody, 1981).

The related features of the lung and kidney include a large area of basement membrane in intimate contact with circulating antibodies, and the presence of type IV collagen as a structural component of the mem-brane. With these similarities in mind, it is understandable how diseases involving the GBM may also affect the lung; especially those diseases which are thought to be collagen related. When human LBM was injected in to sheep, the animals developed autoantibodies directed towards their LBM and GBM. Conversely, human GBM produced only anti-GBM antibodies. Homologous antigen could not produce the disease in

sheep (Steblay and Rudofsky, 1983a and b). Apparently, there is a requirement for a heterologous or possibly exogenous antigen.

While Goodpasture's syndrome seems to imply pulmonary necrosis resulting from the presence of GBM directed antibodies, it must be kept in mind that lung hemorrhage and acute glomerulonephritis can occur in the absence of detectable levels of anti-GBM antibodies. Diseases which have pulmonary hemorrhages and glomerulonephritis as part of their symptoms yet lack the presence of anti-GBM antibodies include: Systemic Lupus Erythematosus (Byrd and Trunk, 1973; Kuhn,1972); Wegener's Granulomatoses (O'Donohue, 1974); and immune complex-mediated glomerulonephritis (Beirne et al., 1973; Agodoa et al., 1976; Loughlin et al., 1978). Ekholdt et al. (1985) have reported a case with pulmonary infiltrates, linear deposition of immunoglobulins in the alveolar basement membrane as well as kidney lesions without anti-glomerular basement membrane antibodies in the serum. These investigators (Ekholdt et al., 1985) believe this case to be a variant of Goodpasture's syndrome. The inability to detect circulating anti-GBM antibodies has been explained in relation with the observed pathology, that is, all the antibody is bound at the sites of injury. Only after specific sites are saturated can one find free circulating antibody.

Pulmonary hemorrhage in association with anti-GBM induced glomerulonephritis seems to be primarily restricted to young adults (Beirne et al., 1977, Briggs et al., 1979, Johnson et al., 1985), although it has been reported in men and women older than 50 years (Savage et al., 1986, Wilson and Dixon, 1973). Wilson and Dixon (1981) have even

noted GBM antibody-associated pulmonary hemorrhages in children less than 10 years old. Even though both sexes are susceptible to pulmonary damage, the studies reviewed seem to include a greater number of male subjects. The severity of the pulmonary hemorrhage can vary from mild hemoptysis without respiratory symptoms to severe respiratory failure with onset occurring very suddenly (Rees and Lockwood 1988). Support for antigenic similarities between the lung and the kidney comes from: 1) the observation that episodes of pulmonary hemorrhaging in patients with anti-GBM circulating antibodies have been halted by bilateral nephrectomy (Hal-grimson et al., 1971); 2) that antibodies eluted from homogenized human lung and isolated human glomeruli (both tissues from Goodpasture's patients) bind linearly to alveolar basement membrane (ABM) and GBM as demonstrated by indirect fluorescence (Koffler et al., 1969; Mc Phaul and Dixon, 1970); and 3) the similarity of western blots using anti-GP antibody and comparing collagenase digested human GBM and ABM (Yoshioka et al., 1988). Of equal interest is the observation that in these same patients, subsequent transplants of allogeneic kidneys appear to remain free of the nephritic state (Nowakowski et al., 1971). This observation can not be easily explained since the previous data indicate that anti-GBM antibodies from Goodpasture's patients reacts in vitro with GBM isolates.

Donaghy and Rees (1983), examined the relationship of inhaled toxins (cigarette smoke) to pulmonary hemorrhaging in patients presenting with glomerulonephritis due to anti-GBM antibodies. Of the starting patient population {51}, 43 {84%} had lung hemorrhaging as

detected either by hemoptysis {38} or by changes in chest X-rays and rise in single-breath carbon-monoxide constant{5}. All 37 smokers (smoked cigarettes daily at the onset of their clinical illness) had lung hemorrhage while pulmonary hemorrhage was found in only 2 of 10 non-smokers. The smoking history of 4 patients was unknown, while the remaining two cases of pulmonary hemorrhage were the result of fluid overload. Presumably, this difference in lung hemorrhage between smokers and non-smokers was not due to an increase in anti-GBM titer in the smoker since these titers were not significantly different between the two groups. In fact, no correlation seems to exist between the serum titer of anti-GBM antibodies and pulmonary hemorrhage (Lockwood et al., 1976; Rees et al., 1977). What then causes the difference in pulmonary pathology when comparing smokers and non-smokers?

It is speculated by Donaghy and Rees (1983), that lung capillaries which are normally relatively impermeable to IgG (Bill, 1977) become permeable due to the effects of cigarette smoke. The increased permeability makes the alveolar basement membrane (ABM) accessible to circulating antibodies. Alternatively, smoking may damage the ABM, leaving previously sequestered GBM cross reactive epitopes exposed to anti-GBM antibodies. Smoke altered antigenic determinants in the ABM, and smoke enhancement of local antibody induced inflammatory responses were also presented as possible mechanisms (Donaghy and Rees, 1983). Severity of lung hemorrhage may also be affected by bacterial infections. In 16 relapses of 7 patients being treated for Goodpasture's syndrome, bacterial infection preceded the relapse (Rees

et al., 1977).

Support for the role of increased permeability as an initiator of pulmonary damage was presented by Jennings et al. (1981). Goats immunized with rabbit ABM were bled and the gamma-globulin fraction of the antisera was injected into rabbits. While goat IgG was found in the GBM it was not found in the lungs. A linear deposit of goat IgG was, however, found in the ABM of 17 of 19 rabbits who had been injected with the anti-ABM antibody after a 62-66 hour exposure to 100% oxygen. In the latter experiment uptake of anti-ABM was greater in the lungs than in the kidney.

Autoimmune diseases represent a defect in the immune system's ability to distinguish self from non-self. The immune response tends to be directed towards specific organs (eg. rheumatic fever, APSGN) rather than a general systemic phenomena. The immune system appears to be responding to an epitope in the affected organ which is either not shared by other organs or is highly sequestered in unaffected organs. In autoimmune phenomena involving the kidney it is unclear whether the epitope is endogenous (eg NC1 domain of type IV collagen) or trapped in the GBM. SCM, whose presence would be the result of a previous infection, may be trapped by the filtering mechanism of the kidney. The immunologic response that follows would be directed towards the foreign antigen. On the other hand, the SCM may posses an epitope similar enough to epitopes in the kidney to induces a cross reactive response. An animal model which received anti-SCM antibodies without prior exposure to the bacteria would be useful in addressing this dilemma. The original purpose of this research problem was to

investigate the immunochemistry of the cross-reactivity of these mAb. The evolution and discovery of the potent anti-LBM activity of a select clone changed the thrust of this investigation toward an animal model for Goodpasture's syndrome.

Goodpasture's syndrome currently is described as an anti-GBM generated kidney pathology with lung hemorrhage. Is the severity or time of onset of lung damage precipitated by certain events (eg. smoking), making the reactive epitope more accessible to the reactive antigens, or is it simply a question of genetic disposition? Klasa et al. (1988) have speculated, based on a case study, that cigarette smoke could have played a role in the initial onset or reoccurrence of Goodpasture's syndrome. Two additional points also remain unclear in the investigation of Goodpasture's syndrome: 1) which epitope in the ABM or CBM is binding the anti-GBM antibody; and 2) what environmental stimuli is initiating the production of lung and kidney reactive antibodies? We have developed an animal model which mimics Goodpasture's syndrome. Proliferation of anti-SCM antibody producing clones in the peritoneum of mice has resulted in glomerulonephritis and pulmonary hemorrhages. These resultant pathologies would seem to indicate that a previous streptococcal infection could give rise to Goodpasture's event.

CHAPTER II

MATERIALS AND METHODS

Isolation of SCM:

Group A M-type 12 Streptococcus pyogenes Hektoen strain was grown in 10 L batches of Todd-Hewitt broth in a Biokulture Fermentor (Fermentation Design Allantown, Pa) at 37°C for 18 hrs. The bacteria were heat killed at 56°C for 1 hr. The bacteria were harvested in a Sharples centrifuge at full speed (a setting of 120 on the rheostat), washed 3 times in normal saline, weighed, and stored frozen. SCM was isolated by adding 1 liter of distilled water to 75g of streptococcal cells and placing the cell suspension in an Eppenbach Homo-Mixer (Gilford-Wood Co., Hudson NY) with Superbrite glass beads, type 120-5005 (Superbrite, 3M Co., St. Paul, Mn). The cells were disrupted for 35 min. with an aperture of 32 on the Homo-Mixer and a setting of 80 on the powerstat. The cell homogenate was then filtered by suction through a large coarse sintered glass filter.

The homogenate was passed through a Sharples centrifuge at top speed. Supernatant from the Sharples spin was passed at 2-3 ml/min. through a Sorvall KSB "Szent-Gyorgyi and Blum" Continuous Flow System (DuPont Co., Newton, CN), which was assembled on a Sorvall RC2-B centrifuge. The solution was centrifuged twice at 5,000 rpm and 6,000 rpm, respectively, to remove residual whole cells and cell

walls. The solution was then centrifuged a third time at 17,000 rpm to pellet cell membrane. The cell membrane pellet was the washed with distilled water in a continuous flow centrifugation at 17,000 rpm. The material was then lyophilized and checked for cell wall contamination by quantitative rhamnose determination by the method of Dische and Shettle (1948). Cell membrane preparations with a rhamnose content of less than 0.8% were considered free from cell wall contamination.

Isolation of GBM:

Renal cortices from human autopsy were obtained from the National Diabetes Research Interchange (NDRI), Philadelphia, PA. Human and mouse glomeruli were isolated by the method of Krakower and Greenspon (1978). Cortices were forced through a 64u mesh screen (F.P. Smith Wire Cloth Co., Franklin Park, Il) with a spatula into a container kept on ice. Cold phosphate buffer saline (PBS) with protease inhibitor, pH 7.4 was added to the filtrate, which was then spun at 2,000 RPM for 3 to 4 min. The pelleted glomeruli were resuspended in cold PBS. Glomeruli were then washed and purified by allowing them to sediment by gravity, 6 times in cold PBS which contained tannic acid at 1:20,000, at 30 min. per wash, removing the supernatant by suction each time. The purity of the resultant suspensions were evaluated by light microscopy. Purified GBM was obtained following the procedure of Carlson (1978) which employs the following sequential steps: Osmotic shock in distilled water containing 0.05% sodium azide (Mallinckrodat, St. Louis, Mo); a 3 hrs. wash with 3% Triton X100 (Sigma, St. Louis, Mo); incubation for 2 hrs. with DNAse (Sigma, St.

Louis, Mo) (1mg/100ml 1M NaCl); 4% deoxycholate (Sigma, St. Louis, Mo) treatment for 2hr; and final 6 washes with distilled water containing 0.05% sodium azide. The final product is essentially pure GBM, free of all serum components and cellular elements as judged by both light and electron microscopy.

Isolation of LBM:

Basement membrane was isolated from murine and human lung (National Diabetes Research Interchange, Philadelphia, Pa.). Lungs from DBA mice were pushed through a 64u mesh screen, washed once with PBS (pH 7.4) then washed 6 times in water containing 0.05% azide to isolate the basement membranes (LBM).

Solubilization of SCM, GBM, and SCM For ELISA:

Lyophilized SCM as well as freshly isolated GBM and LBM were weighed and 1mg trypsin (Sigma) in PBS, pH 6.0, was added per 100mg membrane preparation. The preparation was rotated at 37°C for 18 hrs., centrifuged at 13,000g for 30 min., and the supernatant was collected. The supernatant was dialyzed against distilled water overnight at 4°C with a membrane cut off of 3,000d (Spectrapor) (Spectrum Medical Ind, Los Angeles, CA) and concentrated to 5-10mg/ml (Speed-vac, Savant Instruments, Inc., Hickvill, NY). The concentrated digested membrane was stored at -20°C until used.

Preparation of SCM, GBM and LBM ELISA Plates:

Quantitation of the amount of digested protein was done on a spectrophotometer, using the Waddell equation $(OD_{215} - OD_{225}) \times 0.144$ = ug protein/ml Later this protein quantitation was confirmed by the use of a Beckman amino acid analyzer. The three digested membranes

were diluted to 4ug/ml with carbonate buffer (pH 9.6). Individual microtiter plates were sensitized with each of the digests. Each well of a 96 well plate received 100 ul of the digest and was incubated over night at room temperature. The following day 200 ul of BSA (2%) was added to the wells containing the digest. After 2hrs. the wells were washed 15 times with PBS and stored in the freezer until use.

ELISA Screening Protocol:

To detect antibody production an ELISA protocol was developed. A primary reactant (antibody or antigen) was coated to the wells of a 96 well microtiter plate and allowed to incubate for 1 hr. The plates were counter sensitized with human serum albumin (HSA) (20mg/ml in PBS) overnight and washed with PBS (0.05% Tween 20). The secondary reactant (antibody or antigen) was added to the wells and incubated for 1 hr. After washing with PBS (0.05% Tween 20), 100ul of peroxidase labeled goat anti-mouse immunoglobulin (Hyclone, Logan, Ut.), diluted 1:6,000 in PBS (0.5% HSA) was added to each well and incubated for 1hr. A second PBS (0.05% tween 20) wash was followed by the addition of 150ul of an equal volume mix of 2,2'-azinodi(3-ethylbenzthiazoline sulfonic acid) (Sigma, St. Louis, Mo) (0.36mM in 0.1M Citrate buffer pH 4.1) and 0.003% H_2O_2 (in PBS, pH 7.4). The indicator developed a blue green color which was read after 20 min. at 405nm in a microELISA scanner.

Sensitization of Mouse Splenocytes:

On day one, Balb/c mice (Cox Laboratory) were immunized intra-peri-toneally with 0.1 ml of whole SCM in saline (1mg SCM/5ml) and intradermally with 0.1 ml of SCM in complete Freunds adjuvant (1mg

SCM/5ml adjuvant). After two weeks the mice received an IP injection of the SCM/saline solution, and a 0.1 ml. intramuscular injection of the SCM/ incomplete Freunds adjuvant solution. The animals were sacrificed three days following the second injection, and the blood was immediately collected. Formed elements were removed from the blood and the remaining serum was tested by ELISA for levels of anti-SCM and anti-GBM antibodies. Serum from clone baring mice was added (100ul/well) to SCM and GBM coated microtiter plates and incubated for 1hr. The remaining procedure for detection of anti-SCM and anti-GBM followed the standard ELISA protocol. Every two weeks for 28 weeks the spleens of 2 animals (with appropriate serum antibody titers) were used in the fusion experiments. The spleen was aseptically recovered for fusion. Heart, kidneys and lungs were also removed for histological evaluation. Control animals were immunized with either complete Freunds adjuvant or incomplete Freunds adjuvant without antigen for the production of control hybrids.

Cell Fusion:

The process of cell fusion was completed under sterile conditions following the protocol of Goding (1980). Spleens aseptically removed from the treated mice, were pushed through a 100 mesh sterile screen which was placed in a petri plate containing 10 ml of Hank's balanced Salt Solution (HBSS)(GIBCO, Chagrin Falls, Oh). The HBSS containing the single cell suspension of splenocytes was placed on top of 5 ml of Ficoll to separate the lymphocytes. The cell suspension was centrifuged for 20 min. at 1,500 rpm. Lymphocytes were removed, washed twice with HBSS, then centrifuged for 5 min. at 1,000 rpm. The lym-

phocytes and P3-X63-Ag8 plasmacytoma cells (X63) were counted and mixed together in a ratio of 1:5 respectively. This cell suspension was centrifuged for 5 min. at 1,000 rpm. The resulting pellet was mixed gently with 0.5 ml of Polyethyleneglycol (PEG) (Sigma, St. Louis, Mo) (40 % mw 6000) for 30 sec., followed by a 6-7 min. centrifugation at 600 rpm. Pelleted cells were then carefully over layered with 5 ml. of HBSS, and 5 ml. of M alpha+ H media (GIBCO, Chagrin Falls, Oh.; (Hybri-Sure, Dutchland Laboratories, inc. Denver.Pa.)) and incubated for 1 min. at room temperature. The cells were then gently swirled, followed by a 5 min. centrifugation at 1,000 rpm. After removal of the supernatant the cells were over layered with 5 ml of M alpha+ media and incubated for 8-10 min. at room temperature. The cells were gently re-suspended and the volume of the culture was increased to 20 ml using M alpha+ H media. The cell suspension was then distributed into a 96 well plate (100ul/well) containing a macrophage feeder layer. Two to four plates were prepared for each fusion. Cells were distributed at concentrations of approximately 10^5 cells/well. Macrophages were obtained by flushing the peritoneal cavity of DBA mice with 10 ml of alpha+ media. Recovered macrophages were counted and resuspended at a concentration of 2 to 3 x 10^5/ ml; 24 hrs. prior to use 50 ul were transferred to each well of the 96 well plate. Each plate was checked for sterility prior to filling with fusion cells. On each of the next three days following the fusion, 100 ul of supernatant was removed from each well and replaced with an equal volume of HAT (Hypoxanthine, Aminopterin, Thymidine) media(GIBCO, Chagrin Falls, Oh). After the third day the

plates were allowed to stand for one week and again fresh HAT media was added. Supernatant from those wells that showed growth of the hybridomas (after 3 weeks) was screened by ELISA for the presence of immunoglobulins. Wells with high immunoglobulin levels were then tested for antibody to either SCM antigens or GBM antigens using the ELISA method.

ELISA Screening of Hybridomas for Ig production:

The screening of hybrids followed the method of Voller (1980) where microtiter plates (96 well) were sensitized at 1:1,300 in PBS with goat anti-mouse immunoglobulin (TAGO Code 4143 u+gamma+light chain specific-ity). Plates were counter sensitized with human serum albumin (HSA) (20mg/ml in PBS). Immunoglobulin production was detected via affinity purified peroxidase labeled goat anti-mouse Ig (gamma, mu+light chain) (HyClone, Logan, Ut). This was prepared at 1:3000 in PBS containing HSA (2g/L) and Tween 20 (0.05%) (J.T. Baker Co., Phillipsburg, NJ), 150ul being added to each well for 1 hr. The remaining procedure for detecting Ig followed the ELISA protocol. Those hybrids which produced a strong positive reaction (positive for Ig production) were tested for the presence of antibodies to both SCM and GBM.

Detection of SCM and GBM Reactive Hybridomas:

Supernatant from Ig positive hybridomas was added (100ul/well) to SCM and GBM coated microtiter plates and incubated for 1hr. The remaining procedure for detecting SCM and GBM reactive hybridomas followed the standard ELISA protocol.

Ascites Preparation:

Balb/c mice were injected IP with 1 ml. of pristane (2,6,10,14 Tetra-methylpentadecane, Aldrich Chemical Company,Milwaukee Wis). On the morning of the seventh day of treatment the mice were injected with a second dose (1 ml) of pristane. In the afternoon the mice received 5 x 10^6 SCM-positive, GBM-positive hybridoma cells IP. On day 10-12 the animals were sacrificed,at which time the ascites fluid and cells were drawn up into a syringe, and the heart, lungs and kidneys were removed for histology. A running total of ascites producers was kept in a log. Information included: the number of uninjected mice; the number of mice injected with X-63 (control); and the number of mice injected with cells (hybridomas). Besides the total number of mice, a score was kept of the number of mice that were: maintained until the time of sacrifice (live); and died before the time of sacrifice (dead).

Limiting Dilution of Hybridomas:

Hybridoma cells (IIIC3) made from SCM induced lymphocytes were injected into a mouse. The mouse was sacrificed 10 days later, and the ascites fluid was checked for antibodies to SCM and GBM using the ELISA assay. In an attempt to isolate those hybridomas responsible for anti-GBM antibody production, the ascites producing cells were collected and diluted to a concentration of 1 cell/well. The cells were allowed to propagate in the wells, and the supernatant from these wells were tested for GBM and SCM antibody production. Cells that produced a positive supernatant for GBM and SCM antibody were dispensed in a 96 well plate at a concentration of 10 cells/well and allowed to propagate. Again the supernatant from these wells were

tested for antibody production against GBM and SCM. The cells producing the positive supernatant were then diluted to a concentration of 1 cell/well and allowed to propagate. This last step was carried out an additional two times. Visual scoring of each well confirmed actual cell content. Only wells which started with a single cell were labeled as cloned.

Isotyping Monoclonal Antibodies:

Isotyping of the cross-reactive monoclonal antibody was accomplished using a kit supplied by Hyclone laboratories. Essentially the manufacturers directions were followed. A stock of PLATE COATING SOLUTION was diluted 1 to 10 using distilled water. For each 10 ml of diluted PLATE COATING SOLUTION, 100 ul of Goat Anti-Mouse Immunoglobulin was added. The contents were mixed thoroughly and 100ul was added to each well of a 96 well microtiter plate. The plates were wrapped in plastic and incubated for 24hrs. at 4^{o}C. Plates which were not used immediately had their contents drained, were wrapped in plastic and stored in the freezer.

Microtiter plates were washed three times with PBS containing 0.5ml SURFACTANT per liter of buffer. Each well then received 50ul of PBS-SURFACTANT, followed by the addition of 50ul of the monoclonal antibody of interest at a dilution of 1:500. The antibody was added to one column of 8 wells and incubated at room temperature for one hour. One column of wells was incubated with normal mouse serum in place of our monoclonal antibody providing us with a positive Ig control.

After incubation the plates were washed twice with PBS and patted

dry with a paper towel. Each well of the 8 well column received two drops of one of the TYPING ANTISERA (Rabbit Anti-Mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA). Negative controls were provided by adding 100ul of PBS-SURFACTANT to any well that did not receive antiserum. The plates were incubated for one hour at room temperature. Upon completion of incubation, the plates were washed three times with PBS and patted dry with a paper towel. The wells were then incubated with 100ul of diluted conjugate (peroxidase labeled Goat Anti-Rabbit antibody diluted 1:4000 with PBS-SURFACTANT) for one hour at room temperature.

The plates were washed three times with PBS and dried. The SUBSTRATE REAGENT (citrate buffer containing 1% urea peroxidase) was diluted 1 to 10 using 9.0ml of distilled water. Next, two CHROMAPHORE tab-lets were added to each 10ml of diluted substrate, dissolved and mixed thoroughly. Each well received 100ul of SUBSTRATE-chromaphore, and the plates were incubated at room temperature for 20 min. Isotype was determined by the development of a yellow, amber color in the well identified by the specificity of the isotype anti-serum.

Hematoxylin and Eosin Staining of Mouse Tissue:

Blocks of tissue were cut from the lung and kidney of normal mice as well as from mice baring the specific clones (X-63, IIF4, IIH1, ID8, IB5, IG10, IF3, and IIC3) and stored in Sorensen's phosphate buffered glutaraldehyde. The tissues were processed by placing them in the following solutions for 1hr each: 80% ethanol, 80% ethanol, 95% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, toluene, toluene, paraffin, paraffin. Tissues were then placed in pans of melted

paraffin and allowed to set. Sections were cut at a thickness of 3um, fixed to glass slides and stained using the following procedure. First, the sections were washed for 5 min. in each of the following solutions: xylene, xylene, 100% ethanol, 100% ethanol, 95% ethanol, 95% ethanol, distilled water. Next, the sections were stained for 7-10 min. in hematoxylin and rinsed in tap water for 2 min. After the rinse the sections were dipped 10-15 times in 1% acid alcohol and rinsed again in tap water for 3min. Sections were counter stained with 20 dips in eosin then dipped 12 times in the following solutions: 95% ethanol, 95% ethanol, 100% ethanol, 100% ethanol. Finally the sections were passed through three washes of xylene for 3 min. each and coverslipped. Lung sections were submitted for light microscope evaluation. These evaluations were made in a single blind study employing numbers as code identification for each animal. Histologic evaluations were then compared with gross appearance of tissue.

mAb Recovery from Reactive Basement Membrane:

GBM and LBM were isolated from all animals by the method of Carlson et al., (1978). Recovered, purified GBM and LBM were extracted three times with 0.5ml glycine hydrochloride (0.02M, pH 2.8). The extracts (containing mAb) were immediately neutralized with 0.2 M buffered borate, pH 8.6. Eluted antibody was assayed by ELISA for reactivity to trypsin digested SCM and GBM.

Morphologic Response to Monoclonal Antibody (mAb):

The gross effects of antibody production on the lung of clone bearing mice was investigated. Lung pathology was evaluated using a qualitative grading system (negative, +1, +2, +3, +4), based on the

amount of observable hemorrhage. Next, the effect of a specific clone (IIF4) on gross lung pathology was investigated. A comparison of pathology was based on the number of cells injected and the sex of the animal. Animals scheduled for injection of clones were pretreated 7 days earlier with 1ml of pristane IP. Clone IIF4 or control X-63 hybrids were injected IP at cell densities of 10^5, $2X10^5$ or 10^6. Male and female litter mates were employed for each grouping. All animals were sacrificed on day 10 or 11. All experiments were done in duplicate.

In a separate experiment, non-pristaned animals were injected IP with X-63 control, or mAb IIF4 ascitic fluid. Animals received the ascitic fluid at one of three dilutions (5ul/g, 10ul/g or 20ul/g), were sacrificed at 4 hrs. and examined at the gross level for the extent of lung hemorrhage. Comparisons of lung damage were made with respect to the dose of ascitic fluid and the sex of the animal.

A more quantitative assay for the measure of lung damage was performed using X-63, mAb and normal controls. Parameters examined included; body weight, lung weight, lung/body (L/B) ratios and gross lung pathology. Cells were injected IP and mice were sacrificed on day 10. L/B ratios were then graded based on a table which arbitrarily divided the range of L/B ratios into five groups (<0.008; 0.0081-0.01; 0.011-0.013; 0.0131-0.016;>0.016).

Quantitation of Ascites IgM levels:

Each 96 well microtiter plate was coated with goat anti-mouse IgM (0.1ug/ml) and blocked with 1% HSA in PBS. The TEPC 183 IgM_K ascitic fluid (Sigma, St. Louis, Mo) was serially diluted (10ug/ml-

10,000ug/ml) in PBS and incubated in the microtiter plate for 1 hr. Separate wells received serial dilutions of one of the following ascitic fluids (100ul/well): X63 (133-5) (1:10-1:10,000); IIH1 (147-12) (1:5,000-1:64,000); IIF4 (115-3) (1:5,000-1:64,000); IIC4 (147-10) (1:5,000-1:64,000). The remaining procedure for quantitation of IgM levels followed the ELISA protocol. A standard curve was generated from the TEPC 183 dilutions. Based on the standard curve protein quantitation of all ascitic fluids were determined.

Cross-Reactivity of mAb:

Ascites fluid from mice bearing one of several clones was tested by ELISA for reactivity to trypsin soluble murine lung basement membrane (LBM). Ascitic fluid from 5 B cell Hybrid clones (IE4, IIC4, IIF4, IC4 and IIH1) was diluted 1:2,000 with PBS (0.05% tween 20) and incubated (100ul/well) in LBM coated microtiter plates for 1hr. X-63 ascitic fluid provided a negative control. The remaining procedure for demonstration of the relative reactivity of several clones with LBM followed the standard ELISA protocol. Clone IIC4 was then simultaneously tested for reactivity to SCM, GBM, LBM and myelin. Ascitic fluid from a positive immunoglobulin secreting CFA hybridoma was tested against these antigens as a second control for antibody binding. Ascitic fluid was diluted (1:2,000, 1:4,000, 1:8,000, 1:16,000 and 1:32,000) in PBS (0.05% tween 20), and 100ul of each dilution was added to each of the 4 antigen coated microtiter plates. The plates were incubated for 1hr, then washed with PBS (0.05% tween 20). The remaining procedure showing the crossreactivity of IIC4 followed the standard ELISA protocol.

SCM Inhibition of Antibody Binding:

Ascitic fluid containing monoclonal antibody IIC4 was diluted 1:5,000 in PBS (1% HSA) and incubated for 2hrs. at 37°C with varying amounts of intact SCM (0, 2.7, 8.1, 16.2 mg/ml). The mix was then incubated overnight at 4°C. Intact SCM was removed by centrifuging the mix for 10 min. at 4,000 rpm. Absorption of the monoclonal antibody was indicated by ELISA which was run on microtiter plates coated with SCM, GBM or LBM (4ug/ml). Absorption of antibody with heat killed whole Streptococcus mutans was used as a negative control.

Fluorescent Demonstration of Cross Reactive Ab:

Small pieces of kidney and lung from normal mice as well as clone bearing mice were cut and mounted on dry ice in buttons of O.T.C. embedding media (Miles Scientific, Il). Tissue sections, 2 um thick, were cut, placed on glass slides and fixed with cold acetone for 5 min. After three 5 min. washes with PBS, a drop of Fluorescein Isothiocyanate (FITC) labeled goat anti-mouse antibody (HyClone, Ut) was applied to each section at the appropriate concentration and left to incubate for 1 hr. FITC labeled goat anti-mouse antibody was diluted (1:10 for kidney sections; 1:40 for lung sections) with PBS (pH 7.4) before being added to the tissue sections. Sections were washed three more times for 5 min. in PBS, and covered with p-phenylenediamine in glycerol buffer mounting media (Johnson and Araujo, 1981) and a cover slip.

Electron Microscopic Evaluation of Kidney and Lung:

Blocks of tissue were cut from the lung and kidney of normal mice as well as from clone bearing mice and stored in Sorensen's phosphate

buffered glutaraldehyde. Before embedding, tissue blocks were washed over night in Millonig's buffer to remove the glutaraldehyde. Tissue was then fixed with a 1% solution of osmium tetroxide in distilled water for 2 hrs. After a brief wash with Millonig's buffer to remove the osmium, the tissues were run through a graded series (25%-100%) of acetone. Epon was gradually infiltrated into the tissue by placing the tissues into epon/acetone mixes of increasing epon concentration (1:3; 1:1; undiluted epon). Tissue was left in each concentration for 24hrs. The tissues were then put into capsules containing fresh undiluted epon and placed in a 65^{o}C oven for 24 hrs. Sections, approximately 0.06 um in thickness, were cut by glass knife, floated on to copper grids, and stained with a saturated solution of uranyl acetate in distilled water for 3 min. The grids were counterstained for 5 min. with Reynold's lead citrate then viewed in a Hitachi H-600 transmission electron microscope. Electron micrographs were taken at 17,000X and enlarged 5 times at printing.

Ammonium Sulfate Fractionation of Ascites:

Monoclonal antibody was isolated from ascitic fluid by two consecutive precipitations in a 33% solution of ammonium sulfate. The entire precipitation procedures was carried out at 4^{o}C. Monoclonal antibodies were precipitated by slowly adding one part saturated ammonium sulfate to two parts ascitic fluid with constant mixing. The mixing was allowed to continue over night. The next day the mix was centrifuged, the pre-cipitated antibodies collected and redissolved in a volume of 0.9% NaCl equal to the original volume of ascitic fluid. Saturated ammonium sulfate was added in a ratio of two parts

redissolved antibody to one part saturated ammonium sulfate to again produce a 33% ammonium sulfate solution. The precipitated antibody was isolated by centrifugation and redissolved in 0.1 M phosphate buffer (pH 7.0), in a volume equal to the volume of the original ascitic fluid. Ammonium sulfate was removed by passing the solution over Sephadex G25 (Pharmacia,NJ) at a flow rate of one drop every 15 sec. The antibody was eluted with 0.1M phosphate buffer pH 7.0 and collected as it came off the column. Separation of the immunoglobulin from the ammonium sulfate was detected spectrophotometrically by changes in light absorption at 278nm. Purity of the antibody in the double precipitation procedure was verified by electrophoresis on cellulose acetate strips. The purified antibody was frozen until quantities sufficient for the production of an affinity column was available. Subsequent IgM purifications were performed by euglobulin precipitation (Garcia-Gonzalez et al., 1988) from ascites fluid and pooled serum of mice carrying one particular clone. Following dialysis against two changes distilled water for 20 hours at 4^{o}, the recovered IgM-rich precipitate was solubilized in borate buffered saline, pH 8.4.

Electrophoresis of Monoclonal Antibodies:

A Sephraphore III cellulose polyacetate electrophoresis strip (Gelman, Pa) was saturated with Barbital buffer (0.075M) pH 8.6. The strip was then blotted to remove excess buffer then inserted into a Beckman Micro-zone electrophoresis chamber containing Barbital buffer (0.075M) pH 8.6. Whole ascitic fluid as well as its 33% ammonium sulfate precipitable fraction were applied to separate sites at the

center of the strip (0.6ul) with a sample applicator (Beckman, Ca). The Micro-zone was connected to a power source (Shandon, type 2541, Colab Products, Chicago Heights, Il) and run at a constant voltage of 250v for 20 min. The cellulose acetate was placed in Ponceau S dye for 10 min, and rinsed in two washes of 5% acetic acid. When the dye stopped dripping, the strip was allowed to dry.

Activation and Coupling of Sepharose 4B

Activation of the Sepharose gel and ligand coupling followed the procedure of March et al.(1974). Briefly, Sepharose is washed three times with three volumes of distilled water, then centrifuged at 200g for 1 min. After the final wash an equal volume of distilled water is added to make the working slurry. The Sepharose slurry is added to an equal volume of 2M sodium carbonate and mixed thoroughly. Cyanogen bromide(CNBr) in acetonitrile(CH_3CN) (2g CNBr per ml. of acetonitrile) is added to the slurry mixture with continuous vigorous mixing for 2 min. The resulting mixture is vacuum filtered employing a coarse sintered-glass funnel and washed in succession with 0.1M $NaHCO_3$ (pH 9.5), deionized water and 0.2M $NaHCO_3$ (pH 9.5).

The activated Sepharose is placed in 0.2M $NaHCO_3$ (pH 9.5) to which is added monoclonal antibody (1mg/ml of 0.1M PBS) in a ratio of 1ml. antibody to 1ml packed gel. The mixture is refrigerated at 4^oC for 20 hrs. with constant stirring. After 20 hrs. 1M glycine is added and mixing is continued for 4 more hrs. at 4^oC to neutralize unreacted cyanogen sites. The coupled slurry is poured into a sintered-glass funnel vacuum filtered and washed successively with 20 volumes of 0.1M sodium acetate (pH 4.0), 1 liter of 2M urea and 1 liter of 0.1M sodium

bicarbonate (pH 10). The concentration of Sepharose bound antibody was estimated by comparing absorbance of light (278nm) by the monoclonal antibody with absorbance of a known amount of normal mouse serum. The washed coupled gel was then poured into a glass chromatography column (Pharmacia, NJ).

Affinity Purification of Membrane Antigen:

Samples of the trypsin digested SCM, or lung were passed over the antibody coupled column, followed by a wash with 0.05M PBS (pH 7.4) (6 drops/min.). Column was washed to insure no further protein eluted. Antibody reactive constituents of the solubilized membrane were eluted from the column with 0.2M glycine hydrochloride (pH 2.7) and detected by UV spectrophotometry at 280nm. The eluted fractions were immediately neutralized with 6N NaOH. Collected fractions were placed in dialysis tubing (>3,500 cut off) and dialyzed against distilled water for 72hrs. After dialysis the eluted fractions were lyophilized then redissolved in distilled water to a volume equal to that which was originally loaded on to the affinity column. Samples were frozen until needed.

High Pressure Liquid Chromatography:

A Waters (Millipore, Bedford Ma) high pressure liquid chromatographic (HPLC) system fitted with a Waters I-125 column, a bonded silica column (7.8mmX30cm) with a protein separation range of 2,000-80,000d,was employed for antigen fractionation. Aqueous solubilized proteins with acidic, neutral or mildly basic pI's were adequately separated on this column. Just prior to use the column is flushed with HPLC methanol, followed by water and equilibrated with running

buffer (0.05M NaH_2PO_4). All samples were centrifuged to remove particulate matter. A standard curve was prepared using the HPLC Molecular Weight Marker Kit (United States Biochemical Corp., Cleveland, OH), which contained lactate de-hydrogenase MW 140,000; enolase MW 67,000; adenylate kinase MW 32,000 and cytochrome C MW 12,000. The samples were prepared in 0.05M Na_2HPO_4 (pH 7.2-7.4) at a concentration of 40ug/ml. The injected volume was varied from 25ul to 100ul and the absorption sensitivities were either 0.005, 0.01 or 0.02. The flow rate was 2ml/min and the chart speed was 2cm/min. Values were plotted at log MW vs the retention time of the protein (in minutes).

Glomerular Response to mAb Exposure:

Cellular proliferation was investigated as an indicator of the response of glomeruli to the presence of mAb (Jennings and Earle, 1961). The cell number per glomerulus in H and E sections of kidney was determined by counting the total number of nuclei in 20 glomeruli. An average number of cells/glomerulus was then determined. Kidneys were chosen from clone bearing mice as well as kidneys from control and X63 bearing mice.

CHAPTER III

RESULTS

Immunization and Spleen Fusion

Five non-immunized mice were selected at random and evaluated for serum antibodies. The maximum serum titer (OD at 405 nm in a range of 0.020 to 0.030 OD units) was 1:5 to 1:10 for anti-SCM and 1:2 to 1:4 for anti-GBM. This is in contrast to the 30 mice immunized with SCM, 28 of which on their day of sacrifice showed anti-SCM titers ranging from 1:320 to 1:640 and anti-GBM ranging from 1:16 to 1:64 (OD at 405nm were >0.100 OD units). The two negative mice were essentially as the normal controls. Spleens from each of the 28 positive animals were used in the fusion procedure to develope the desired cross-reactive hybridomas; however 27 were lost due to contamination and mechanical failures. Control animals immunized with complete Freunds adjuvant (CFA) alone showed serum responses comparable to the non-immunized animals and provided spleens for immunoglobulin secreting control hybridomas. All experiments resulted from the one successful fusion of the splenocytes from the 28th animal.

ELISA Screening of Hybridomas For Ig Production

Hybridomas, resulting from the 28th fusion of anti-SCM primed B cells and X 63 myelomas, grew to confluence in 4 separate 96 well microtiter plates. All supernatants generated by these polyclonal hybridomas were tested for Ig production. Of the supernatant tested

from the fusion experiments, approximately 80% showed the presence of immunoglobulin secreting cells. About 20% of these cultures had cells which were described as high immunoglobulin secretors (O.D.>0.200). The wells that had the highest levels of immunoglobulin were, plate III, column 3, row C (III3C), III4B, IV2F and IV7A as judged by ELISA.

Detection of SCM and GBM Reactive Hybridomas

All high immunoglobulin secreting hybridomas (O.D.>0.200) were propagated in 1.0ml cultures and retested for the presence of immunoglobulins, as well as anti-SCM and anti-GBM reactivity. Approximately 55% were positive (O.D.>0.100) for anti-SCM and about half of these or 25% were positive for anti-GBM reactivity. It must be stressed at this point that while some of the SCM positive (anti-SCM) hybrids were also reactive with GBM (anti-GBM), no hybrids were found which were positive for anti-GBM and negative for anti-SCM activity. None of the fusion products derived from control immunized animals showed elevated anti-SCM or anti-GBM reactivity, although high immunoglobulin secretor populations of cells were found. These comprised about 20% of the fusion wells.

Table 1 summarizes the anti-SCM and anti-GBM titers of supernatant, from wells which grew the four highest immunoglobulin secreting hybrids. Ascitic fluid from X-63 cells did not contain elevated titers of anti-SCM or anti-GBM. Ascitic fluid from control animals, immunized with complete Freunds Adjuvant (CFA) alone (I3C), though containing 3-5 fold more immunoglobulins than X-63 ascitic fluid, was also negative for anti-SCM and anti-GBM. This result is in contrast with SCM immunized uncloned cells; III3C, II4B, IV2F and

TABLE 1

TITRATION FOR ASSESSMENT OF Ab REACTIVITY[a]

FUSION PRODUCT	ANTIGEN	TITER
X-63	SCM	<20
	GBM	<16
IC3[b]	SCM	<160
	GBM	<160
III3C	SCM	>20,480
	GBM	>20,480
III4B	SCM	>20,480
	GBM	>20,480
IV2F	SCM	2,560
	GBM	1,280
IV7A	SCM	2,560
	GBM	1,280

a Measured by ELISA

[b] Control fusion spleen. Mouse was challenged with CFA only, no antigen. The highest immunoglobulin secretor well was harvested for injection into a mouse as a second negative control ascites producer.

IV7A. Polyclones II4B, IV2F and IV7 were frozen away for future investigation.

Limiting Dilution Analysis and Isotyping of Clones

Polyclone II3C which met the criteria of reactivity with SCM and GBM underwent three limiting dilutions, resulting in the isolation of 20 cloned cell lines. The cloned cells were labeled somewhat contrary to the polyclone hybrids. The monoclonal antibody producing cells were labeled by plate, row then column number (eg. IIH1). These clones were used to generate ascitic fluid. Over 300 animals including controls (Table 2) were employed during a one year period to generate the required ascitic fluid and mAb. All subsequent studies were performed using mice from this large population.

Isotyping using the Hyclone laboratories kit was carried out on most of the antibodies produced by the clones of interest. The procedure was run in duplicate and results indicate that the predominate isotype of all generated clones was IgM (Table 3).

Histologic Evaluation of Specific mAb

The X-63 controls provide for an accumulation of ascitic fluid which contained essentially normal levels of mouse serum proteins and provided the negative control for ascitic fluid antibody testing (ELISA) and tissue pathology. Placement of hybridoma cells in some animals (6 in all) caused death due to what appeared to be and associated autoimmune tissue pathology (Table 2). The premature deaths in Experiment Group II, were animals which carried clone IIF4 and exhibited severe lung hemorrhage. The finding that these were associated with one clone prompted us to review all subsequent animals

TABLE 2
SUMMARY OF THE TOTAL NUMBER OF EXPERIMENTAL ANIMALS

Experimental Group	Normal Controls	X-63 Controls	Hybridoma Injected	Dead*	total
I	4	3	36	0	43
II	0	11	67	2	78
III	3	4	68	0	75
IV	6	2	92	4	100
Total	13	20	263	6	296

*Animals which died between days 10 to 12 during ascites production.
These animals showed sever autoimmune reactions.

TABLE 3

ISOTYPE AND ELISA TITERS OF GENERATED ASCITES EMPLOYED IN STUDY.

Ascites Identify	Isotype*	Antigen Plates Pool SCM	Pool GBM	Pool LBM
X-63	Mixed	≤100	≤100	≤100
IIF4	IgM	64000	64000	64000
IIH1	IgM	32000	64000	6000
IIF5	IgM	64000	8000	8000
IB5	IgM	64000	128000	64000
IF3	IgM	32000	64000	128000
IG8	IgM	64000	128000	32000
IF7	IgM	32000	128000	32000
IB5	IgM	64000	128000	16000
IIC4	IgM	64000	64000	64000
ID1	IgM	64000	64000	32000
IIF5	IgM	64000	8000	8000
IG10	IgM	64000	64000	32000
ID8	IgM	32000	32000	16000
IIIC3	IgM	128000	128000	128000

*Isotyping was determined on serially diluted ascites. For X-63 no single isotype was seen to have predominate concentration; findings were as for a normal serum. For hybridoma ascites IgM was only detectable isotype at 1:8000 and higher.

for lung hemorrhages. In vitro assays were employed in order to relate gross lung hemorrhage to tissue binding of cross-reactive monoclonal anti-SCM antibodies. LBM was considered the most likely site for an antigen shared by SCM, GBM and lung.

Table 3 presents data on select mAbs for their reactivity to SCM, GBM and LBM solubilized antigens. It should be noted that the control ascites generated by X-63 plasmacytoma cells did not show appreciable reactivity to these test antigens. In contrast, mAb generated by the hybridomas produced strong anti-SCM binding and variable cross-reactivity to the solubilized pooled GBM or LBM antigen. While the selection of cross-reactive clones was originally performed on GBM the subsequent testing on LBM produced these surprisingly variable results, that is, some mAb displayed greater or lesser reactivity to LBM at the time the assessment was made. While IIF4, IB5 and IF3 were strongly reactive with trypsin digested lung, IIH1 bound the antigen only weakly. Table 4 presents selected experiments of the 297 animals to indicate the range in pathology induced by the different clones. Evaluation of gross lung pathology indicated that clones IIF4, IG10, and IF3 were able to produce dramatic lung hemorrhages. Typical gross pathology is seen in Fig. 1. The amount of lung pathology reflected in the degree of edema and hemorrhage appeared to be dependent on both the number of cells the animal received and the sex of the animal. A comparison of the lungs of the mice who received $5X10^5$ cells (two top lungs) with the lungs of the mice who received $1X10^5$ cells (the bottom lungs) shows that while the lungs of both sets mice were edematous and hemorrhagic, the mice who were dosed with the larger cell number

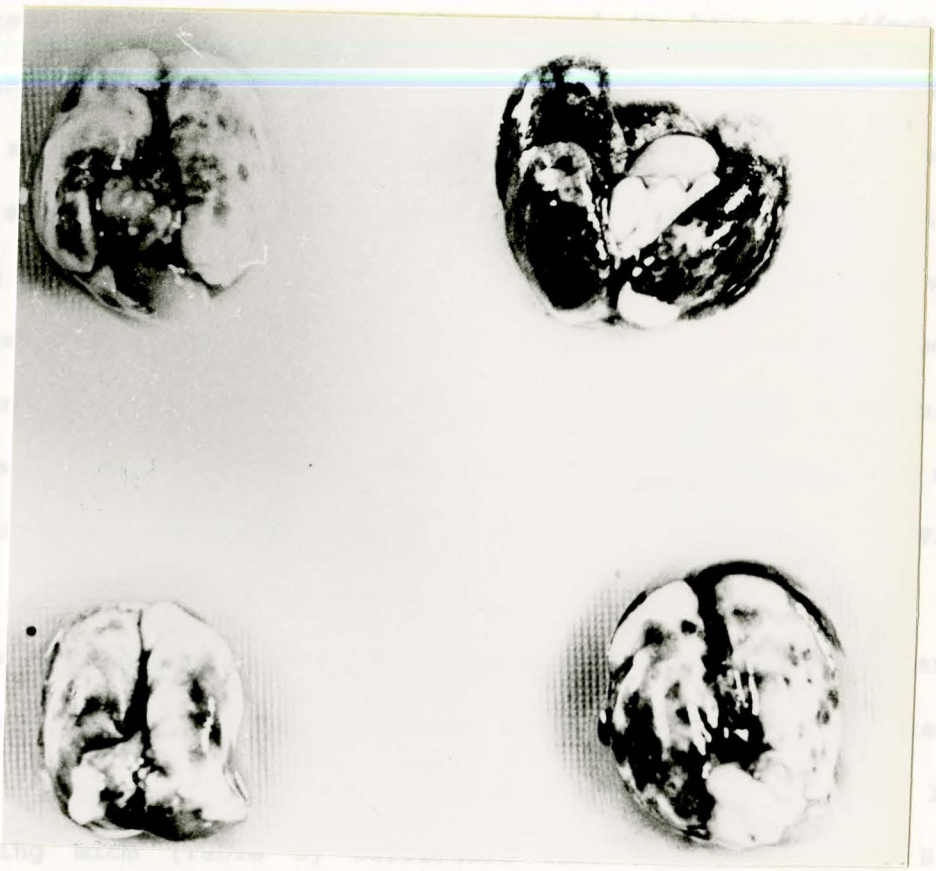
TABLE 4

BLIND STUDY OF LUNG PATHOLOGY*

Animal No.	Clone	Gross Evaluation	Histologic* Evaluation
145-15	-	normal	normal
147-14	-	normal	normal
133-5	X-63	normal	focal/normal
115-3	IIF4	+4 hemorrhagic lesion	massive hemorrhage
139-6	IIF4	+3 hemorrhagic lesion	massive hemorrhage
147-12	IIH1	normal	normal
147-13	IIH1	normal	normal
105-18	ID8	+1 hemorrhagic lesion	focal
115-2	ID8	+1 hemorrhagic lesion	focal
105-17	IB5	+1 hemorrhagic lesion	focal
147-12	IB5	normal	normal
105-19	IG10	+1 hemorrhagic lesion	focal
145-14	IG10	+4 hemorrhagic lesion	massive hemorrhage
133-8	IIIC3	+4 hemorrhagic lesion	massive hemorrhage
133-12	IF3	+3 hemorrhagic lesion	massive hemorrhage

* Selected tissues were submitted for light microscopic evaluation. These evaluations were made on a blind basis employing animal numbers as code identification

Figure 1. IIF4 clones were injected into the peritoneal cavity of pristane primed mice. These were lungs from litter mates, 2 males (left) and 2 females (right). The lungs on top were from mice receiving $5X10^5$ cells, and those on the bottom were from mice injected with $1X10^5$ cells. The Ten days later the mice were sacrificed, the ascitic fluid removed and the cloned cells retrieved. Gross evaluation of the lungs showed overwhelming hemorrhage. Note that the lungs from the females appear larger and more hemorrhagic.



X1

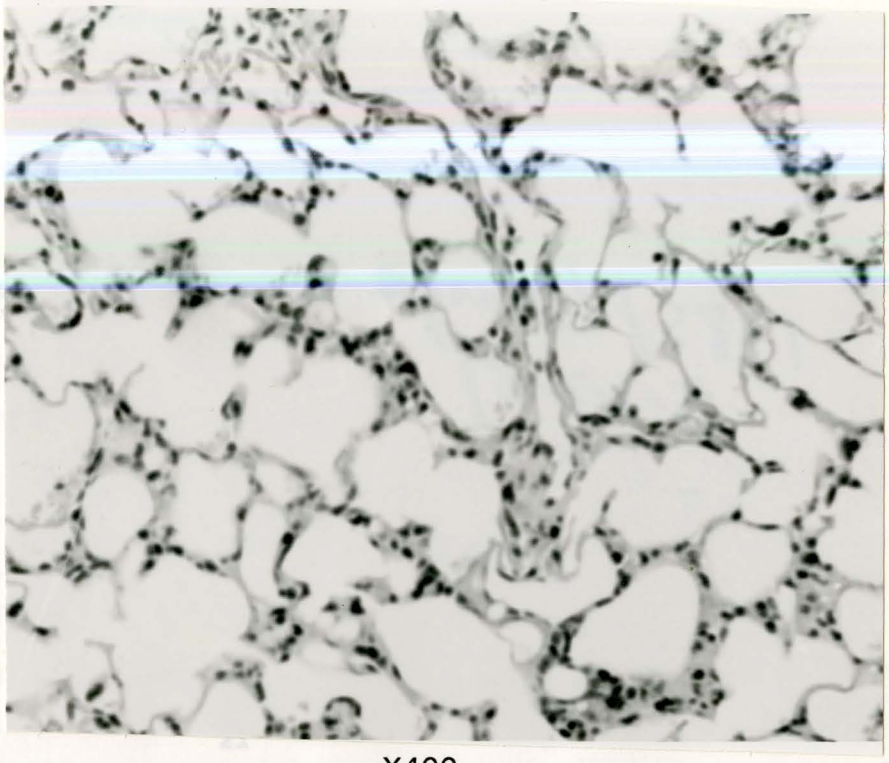
presented with lung which showed a greater degree of damage. A similar comparison was drawn by comparing lungs from male animals (left) with lungs from females (right). Lungs from the female mice seem to be traumatized by the clone generated antibodies to a greater extent. Both IIH1 and X-63 appeared to have no effect on lung pathology, at least at the gross level. Hematoxylin and Eosin (H and E) stained lung sections were then examined for histologic evaluation in a single blind study employing a number code for identification of the animal. Histologic and gross evaluation correlated well with regards to antibody induced lung damage. H and E lung sections of clone baring mice showed a classic inflammatory response. Lungs showed congestion with infiltrates of neutrophils and some monocytes indicating a progression from an acute to a chronic state (Fig. 2).

An evaluation of gross lung pathology was made on all mice receiving generated clones (Table 5). The grading was quantitative (negative to +4), based on the extent of hemorrhaging noted in the lung tissue. An overview of lung pathology of the final 140 clone baring mice (Table 5) correlated well with the H and E stained sections which indicate typical antibody induced lung damage. H and E stained sections of corresponding kidney tissue again showed a nephritic state presumably induced by the presence of clonally derived antibodies.

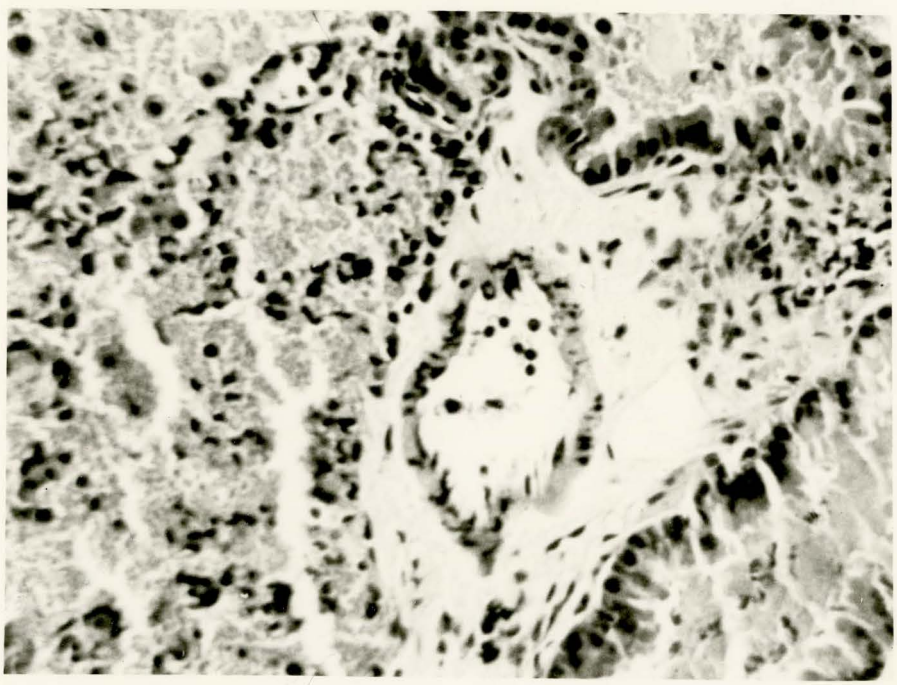
In Vivo Results of mAb or Hybridoma Challenge

Table 6 presents the selected results of an experiment designed to assess the effect of cell dose and animal sex on lung pathology. Again a qualitative assessment of lung pathology was made. In all,

Figure 2. H and E staining of a frozen lung section from a mouse bearing clone IIH1 (top) was histologically the same as that from a control mouse. Lung from a mouse baring the IIF4 clone (bottom) showed massive hemorrhage and infiltrate into the alveolar spaces. The lung from a mouse bearing the IIH1 clone was essentially normal.



X400



X400

TABLE 5

EVALUATION OF GROSS LUNG PATHOLOGY

		GRADE				
ID No.	No. Mice	Neg.	+1	+2	+3	+4
13	19	4	3	0	9	3
19	13	4	6	0	3	0
105	10	0	6	0	1	3
111	8	7	0	0	1	0
139	9	1	2	2	2	2
145	21	3	11	0	5	2
147	21	6	2	3	7	3
149	20	3	8	2	7	0
151	19	6	10	0	3	0
Totals	140	34	48	7	38	13

Murine lungs were graded qualitatively based on observable tissue damage.

TABLE 6

PROPAGATION OF HYBRIDOMA CELL IN ANIMALS AND RESULTING PATHOLOGY*

EVALUATION TREATMENT	SEX & AGE	SACRIFICE TIME**	GROSS LUNG PATHOLOGY^
X-63 PLASMACYTOMA			
10^6 cells	M-6 wks	11D	None/None
	F-6 wks	11D	None/None
IIF4 HYBRIDOMA			
10^5 cells	M-6 wks	10D	±/±
	F-6 wks	10D	+2/+2
2 x 10^5 cells	M-6 wks	10D	±/±
	F-6 wks	10D	+4/+4
ID8 HYBRIDOMA			
10^5 cells	M-6 wks	10D/11D	+1/+1
	F-6 wks	10D/11D	+2/+2
5X10^5 cells	M-6 wks	10D/11D	+2/+3
	F-6 wks	10D/11D	+3/+3
10^6 cells	M-6 wks	10D/11D	+2/+2
	F-6 wks	10D/11D	+4/+4
IB5 HYBRIDOMA			
5X10^5 cells	M-8 wks	10D/11D	+/+
	F-8 wks	10D/11D	+4/+4
10^6 cells	M-8 wks	10D	+/+
	F-8 wks	10D	+2/+2
10^7 cells	M-8 wks	10D	+4/+4

* Summary of two trials

** In cases indicated second set of animals were sacrificed on Day 11, Gross observations were not necessarily at variance to Day 10 group

^ ± observed hemorrhage may be real or due to aspirated blood
1+ small isolated hemorrhages
2+ many hemorrhages
3+ all hemorrhages greater than 3 mm in diameter few clear areas
4+ lungs totally hemorrhagic

forty animals were evaluated and in all cases the gross pathology seen in these animals was greater in the female than in the male litter mate. Data from selected clones was shown. Higher doses of injected cells induced a greater amount of lung hemorrhage than did the lower doses. Since ascites generated by X-63 cells or IC3 (CFA clones) did not produce any gross changes it is assumed that ascites per-se was not a factor. In animals with overt pathology the high level of mAb generated by the hybridoma cells made interpretation of dose dependent correlations somewhat equivocal. To obtain such correlations a direct injection of measured amounts of mAb was employed. IIF4 was selected and injected on a volume per body weight basis. The antibody concentration was determined by comparing dilutions of ascitic fluid to a TEPC standard curve (Fig. 3). For these experiments all animals (controls and experimentals) were the same age and were sacrificed 4 hrs after an intraperitoneal injection of the mAb containing ascitic fluid (Table 7). These short term experiments demonstrate the rapidity of the uptake of antibody from the peritoneal cavity and its binding at the tissue site. Here too, as in Table 6, the concentration of mAb in the host is directly proportional to the observed severity of the gross pathology as illustrated in Fig. 1. Again we found the severity of the hemorrhages was greater in the female than the male. Mice injected with X-63 ascitic fluid showed no gross pathology.

At the time of sacrifice the animals were examined for gross lung pathology. Body weight, lung weight and lung to body weight (L/B) ratios were recorded for a more quantitative estimate of lung

Figure 3. Optical densities were determined for a known concentration of IgM produced by TEPC mouse myeloma. IgM concentrations of ascitic fluid generated by mice baring appropriate clones was determined from this standard curve.

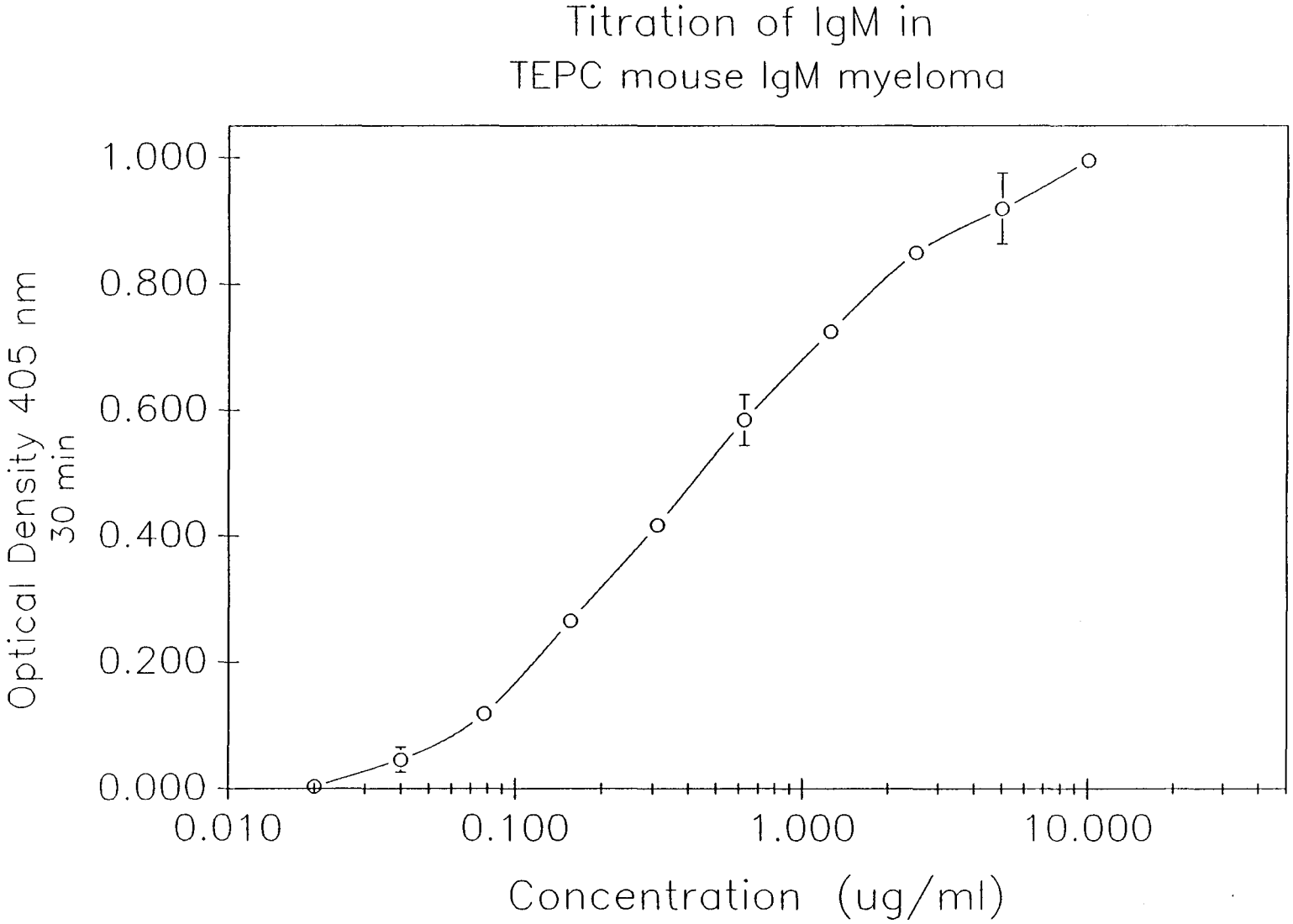

Titration of IgM in
TEPC mouse IgM myeloma
Optical Density 405 nm
30 min
1.000
0.800
0.600
0.400
0.200
0.000
0.010
0.100
1.000
10.000
Concentration (ug/ml)

TABLE 7

PATHOLOGY GENERATED BY THE INJECTION OF mAb INTO ANIMALS

TREATMENT	SEX & AGE	SACRIFICE TIME	GROSS EVALUATION LUNG PATHOLOGY*
IIF4 mAb			
5 μl/g (24ug/g)	M-10 wks	4 hrs.	-
10 μl/g (48ug/g)	M-10 wks	4 hrs.	-
20 μl/g (96ug/g)	M-10 wks	4 hrs.	±
IIF4 mAb			
5 μl/g (24ug/g)	F-10 wks	4 hrs.	±
10 μl/g (48ug/g)	F-10 wks	4 hrs.	2+
20 μl/g (96ug/g)	F-10 wks	4 hrs.	3+

() - micrograms of immunoglobulin per gram body weight
* mice injected with X-63 ascitic fluid showed no gross pathology
1+ small isolated hemorrhages
2+ many hemorrhages
3+ all hemorrhages greater than 3 mm in diameter few clear areas
(2 mice/group)

pathology. Again, out of forty animals, selected data is presented to contrast results (Table 8). Cell line IIF4 induced lung hemorrhages. Lung damage was also indicated by a large absolute lung weight (0.274g) as well as a large lung weight to body weight ratio (0.01-19g). Note that ascites production alone does not produce any lung changes as shown for the X-63 cells. Correlation to ELISA data as well as that presented in previous Tables is maintained. The grade +2 which indicates the amount of lung damage in the IIF4 clone bearing mouse, was derived from a table in which L/B ratios were divided into 5 groups. A total of 36 mice was reviewed (Table 9). The lung/body weight ratios seemed to cluster around certain values. The cut off value for each group was arbitrarily assigned based on the clustering of these values. In this way a range of values was generated and a numerical value indicating the amount of tissue damage was assigned. The values listed under "Lung Pathology" and "Average Grade" in tables 7 and 8 respectively were thus generated.

Witebsky's criteria provides guidelines for the investigation of autoimmune phenomena. Following these criteria, an attempt was made to recover mAb from all the target organs. Purified BM was isolated from lungs and kidneys and acid extracted. Extracted mAb was tested by ELISA for reactivity to SCM and GBM. Any dilution of antibody giving a reading above the standard deviation of the control wells was considered to have positive reactivity. IIF4 and IF3 produced titers in the range of 1:4 to 1:8 to SCM and GBM (Table 10). Though the titers are low, the cross reactivity of the positive reactors is evident. All control extracts from X-63 or normal animals were

TABLE 8

LUNG PATHOLOGY RESULTING FROM INJECTION OF HYBRIDOMA CELLS INTO MICE

Cell line	Body weight	Lung Gross pathology *	Lung weight	L/B**	Average	Grade~
Normal	32	pink	0.1668	0.0052	0.0066^	N
	30	pink	0.2403	0.0079		
X-63	21.5	clear	0.158	0.0074	0.0072	N
X-63	21.5	clear	0.149	0.0069		
IIF4	23.0	hemorrhaged	0.2740	0.0119	0.0112	+2
IIF4	23.0	hemorrhaged	0.2411	0.0105		

* Confirmed by EM and/or fluorescent microscopy.

** Lung wt/body wt ratio

~ Grades N; Normal, +1 approx 50% wt gain, + 2 100% gain or greater over normal ratio.

^ Published value for normal L/B ratio is 0.007 ([illegible], [illegible])

TABLE 9

LUNG/BODY WT. RATIO

RANGE	GROSS PATH.	No. MICE
< 0.008	-	10
0.0081-0.01	+1	10
0.011-0.013	+2	8
0.014-0.016	+3	5
> 0.016	+4	3

TABLE 10

ELISA OF RECOVERED mAb IN TISSUE ELUATES

CLONE	ORGAN	SEX	VOL. ELUATE	TITER SCM	TITER GBM
IIF4	Kidney	M	3.0	1:4	1:4
	Lung	M	3.0	-	-
IIF4	Kidney	F	3.0	1:4	1:8
	Lung	F	3.0	-	-
IIF4	Kidney	F	2.0	1:4	1:4
	Lung	F	2.0	-	-
IF3	Kidney	F	3.0	1:4	1:4
	Lung	F	3.0	-	1:4
IF3	Kidney	F	2.0	1:8	1:8
	Lung	F	2.0	1.4	-

negative under the same testing conditions.

Cross reactivity and Specificity of mAb

Several clones (IE4, IIC4, IIF4, IC4, and IIH1) were screened by ELISA and shown to have varying reactivity to trypsin digested murine lung at a dilution of 1:2000. Ascitic fluids IIF4 and IIC4 had the strongest reactivities (Fig. 4). To support these observations ascitic fluids were run in an ELISA simultaneously testing for reactivity to SCM, GBM, and LBM. The monoclonal antibody in the ascitic fluid showed strong reactivity with the all three antigens (GBM, SCM and LBM) (Figs. 5a,b,c). Monoclonal antibody was run on ELISA plates at several dilutions (1:200 to 1: 32,000). Antibody binding is shown to decrease in a concentration dependent manner. The solubilized membranes each reacted with monoclonal antibody IIC4 at titers as high as 1:25,600. The monoclonal antibody reacted weakly with myosin (an antigen negative control) at the lowest dilutions (1:1,600). Ascitic fluid from a CFA hybridoma bound minimally to the antigens, coated on microtiter plates, thus providing an antibody negative control (Fig. 6)

In an ELISA study using the same three antigens a comparison was made of the reactivity of clones IIF4, IIC4, and IIH1. Since levels of antibody can vary from clone to clone, IgM levels were determined for the aliquots of ascitic fluids used in these ELISAs. The following levels of IgM were recorded: X-63 (58.6ug/ml); IIF4 (4,800ug/ml); IIC4 (5,000ug/ml); IIH1 (10,500ug/ml). Titers of equivalent concentrations were compared and IIF4 and IIC4 showed similar binding to the antigens (Fig.7). While IIH1 reacted strongly

Figure 4. An ELISA was carried out to determine the reactivity of mAbs IE4, IIC4, IIH1, IIF4 and IC4 to trypsin digested lung. Ascitic fluid was run at a single dilution (1:2000). Clones IIC4 and IIF4 were determined to be two of the strongest lung reactive clones.

ELISA OF mAB ASCITIC FLUID

DILUTED 1:2000

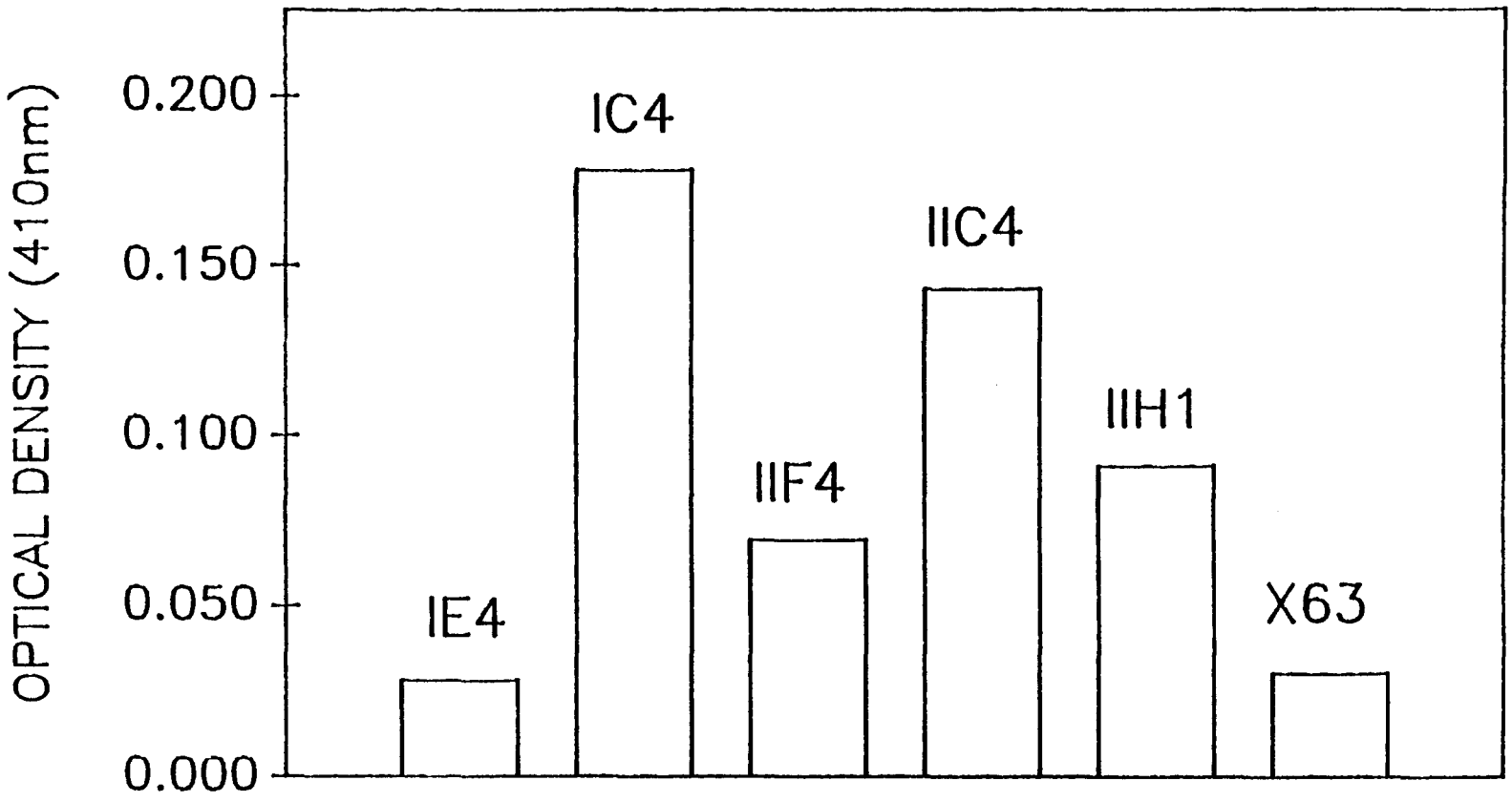
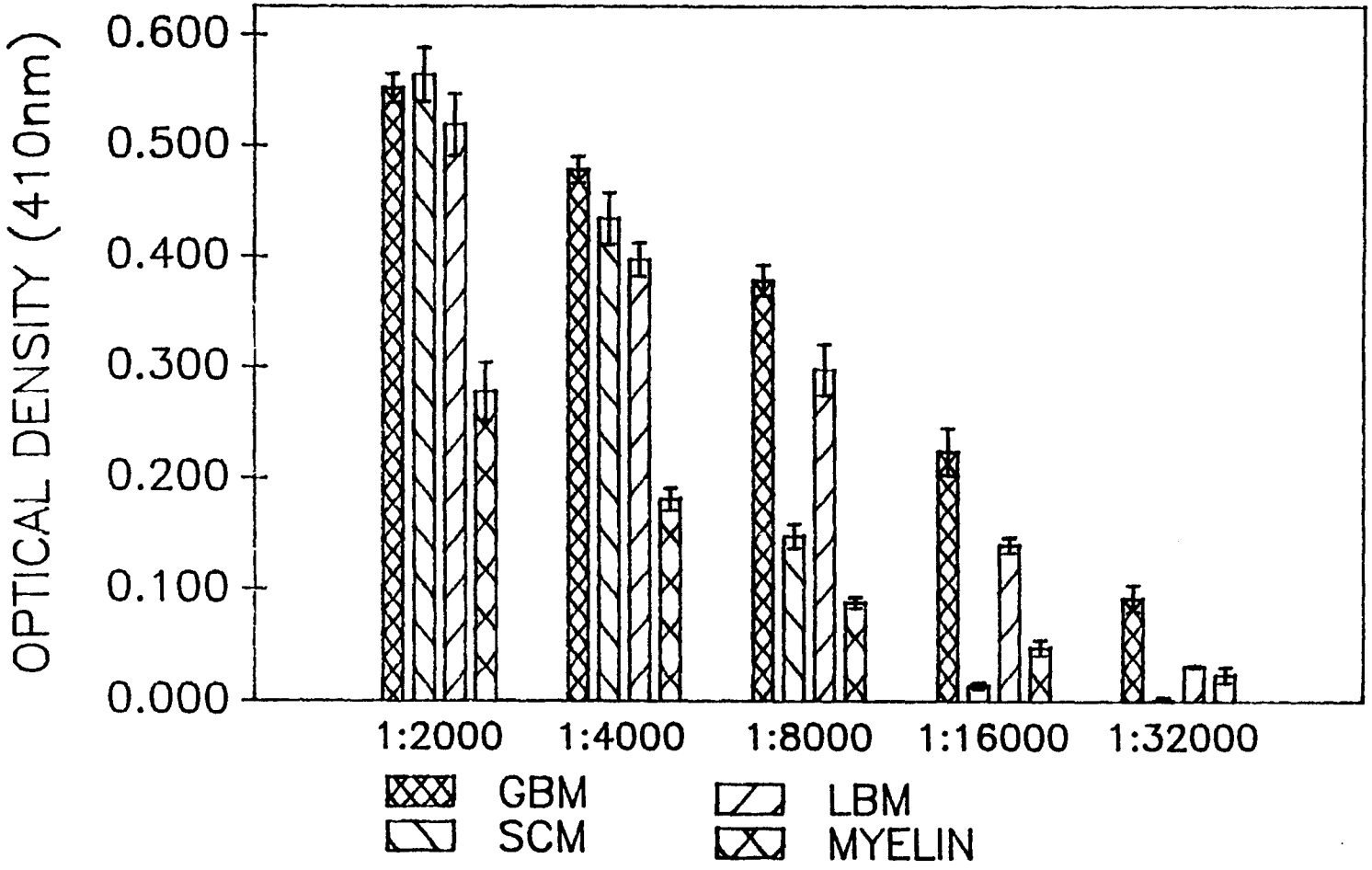


Figure 5a,b,c. Monoclonal antibody IIC4 was tested by ELISA for simultaneous reactivity with SCM [\\\], GBM [xxx] and LBM [///]. IIC4 reacted strongly with the three membrane antigens but weekly with myelin [x x] the control antigen. Variance in the activity of unstable substrates was negated by showing the results as separate experiments. The antibody was run in quadruplicate for each dilution.

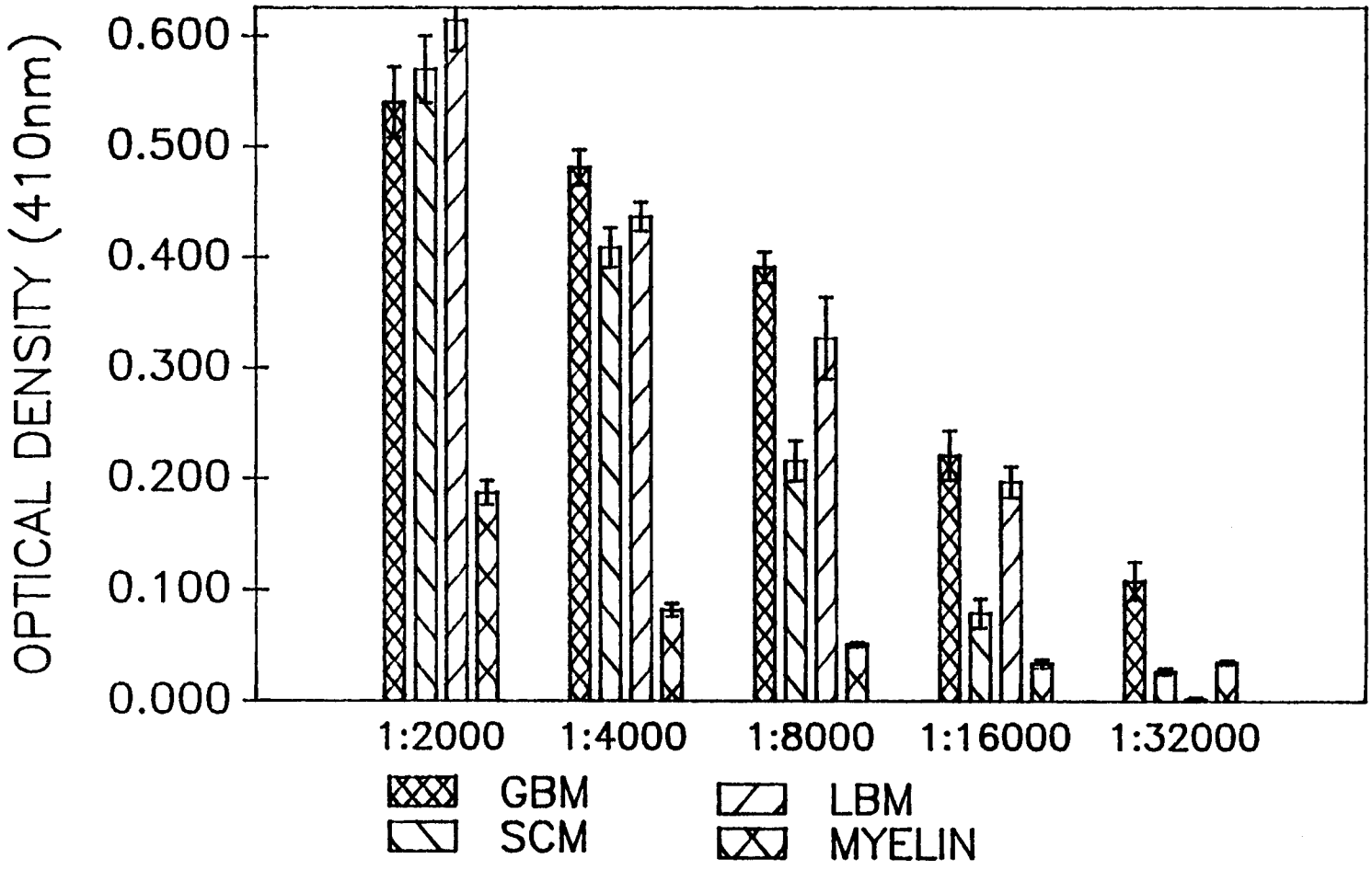
ELISA OF mAb IIC4

EXPERIMENT I



ELISA OF mAb IIC4

EXPERIMENT II



ELISA OF mAb IIC4

EXPERIMENT III

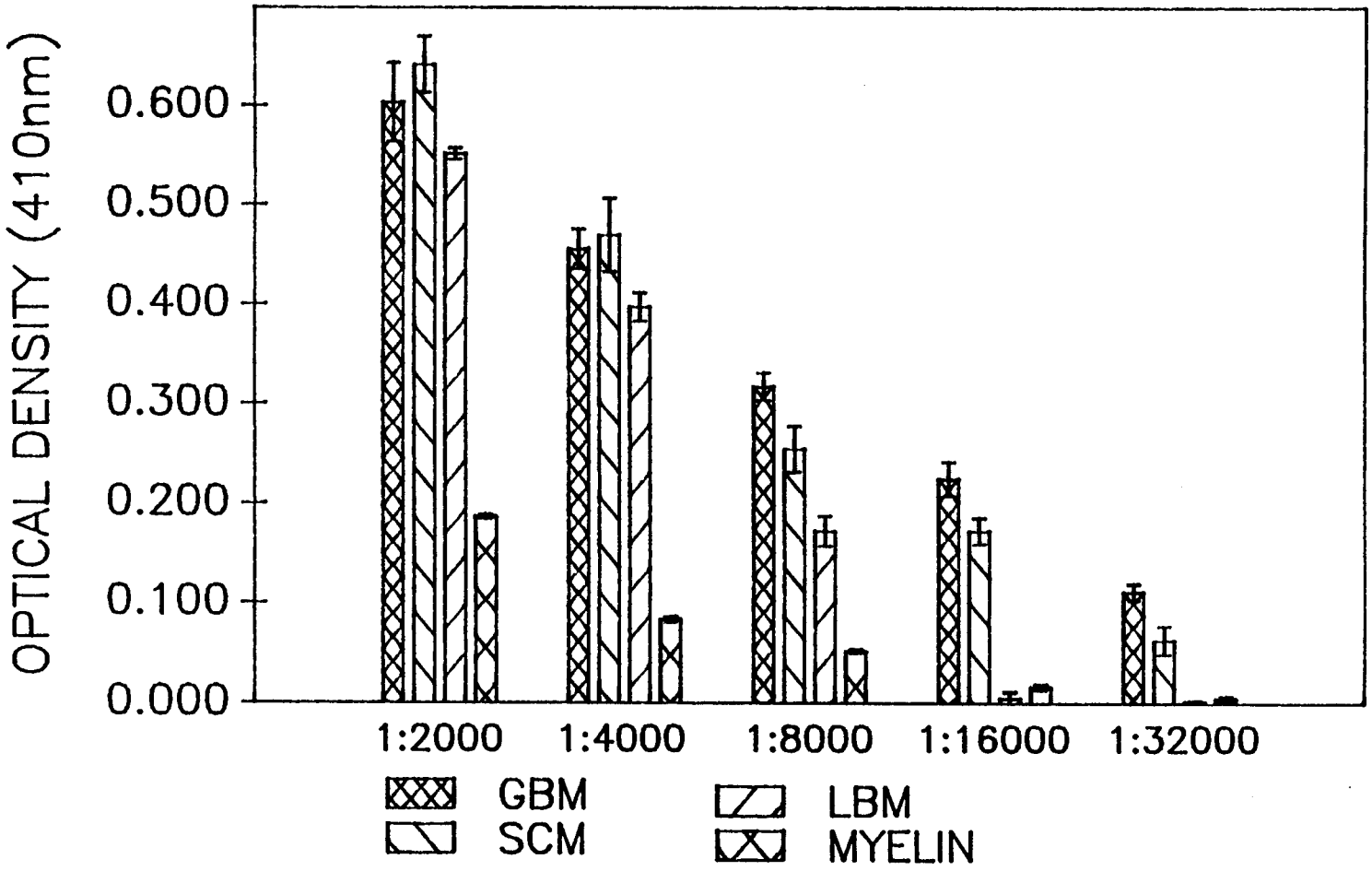


Figure 6. Ascitic fluid from hybridoma IC3 (a CFA hybridoma) was used as a negative control for non-specific reactivity of fluid constituents. IC3 ascitic fluid showed no substantial binding at any dilution to SCM [\\\\], GBM [xxx], LBM [///] or myelin [x x].

ELISA OF TISSUE ANTIGENS

ASCITIC FLUID FROM HYBRIDOMA CFA

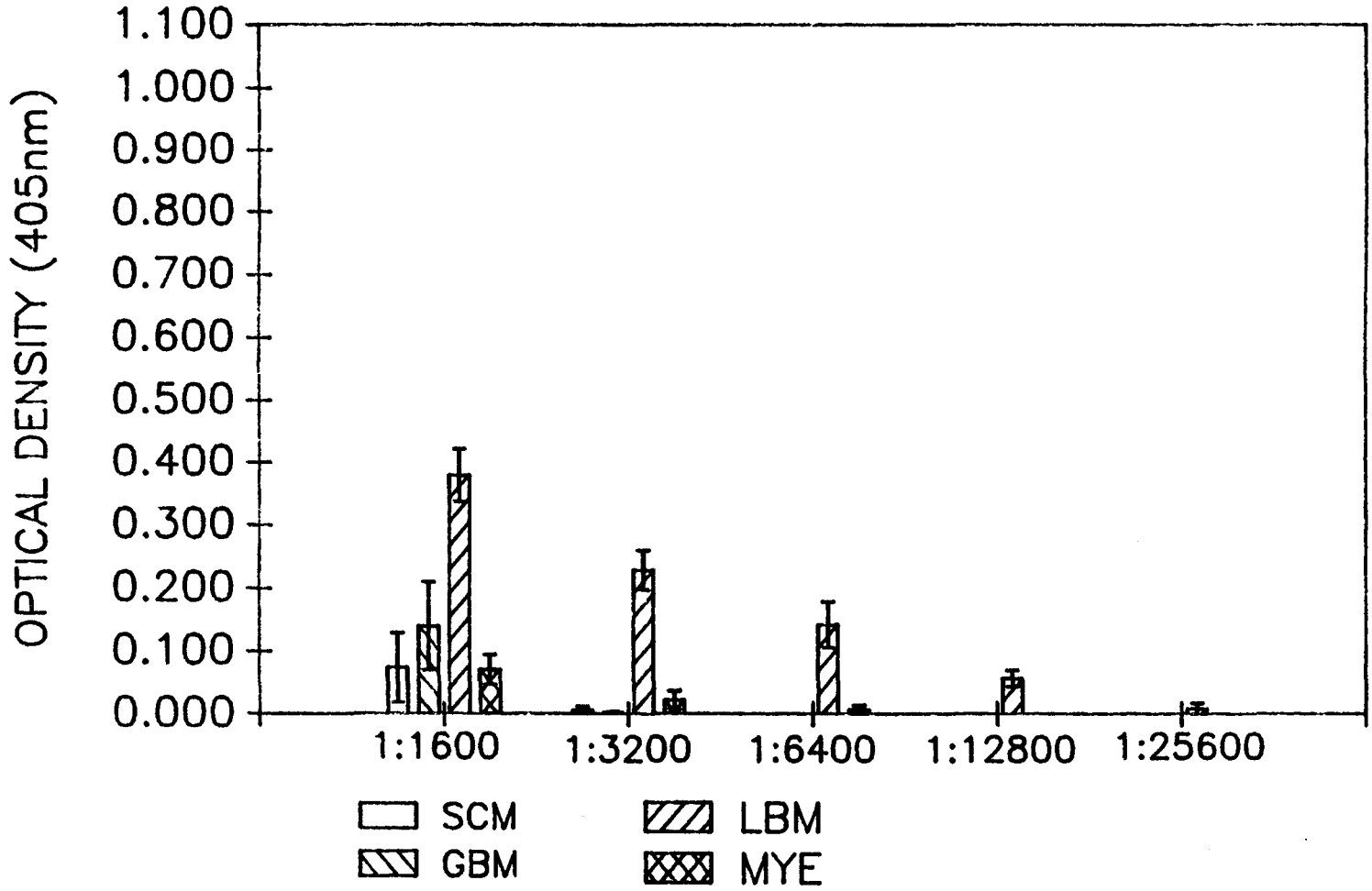
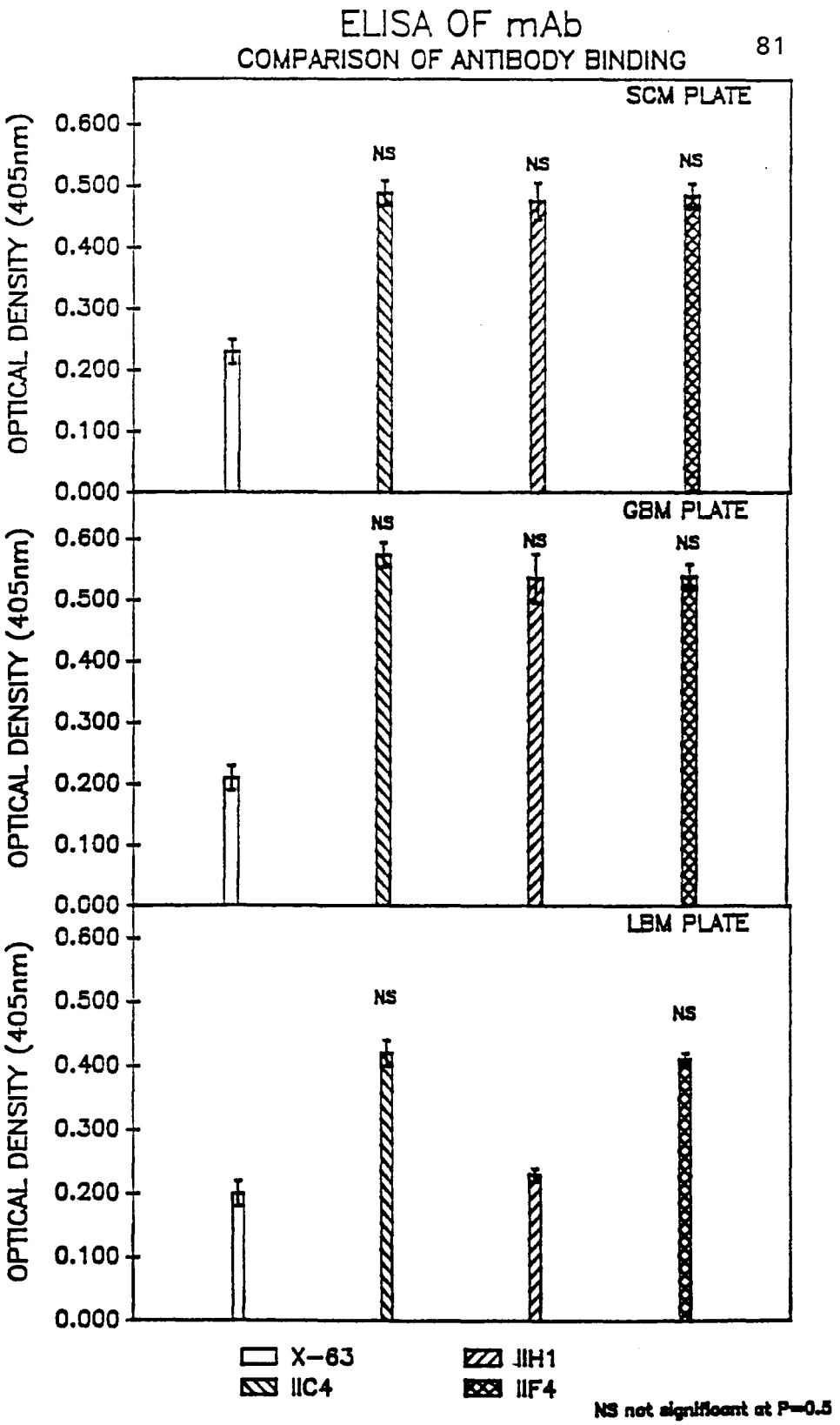


Figure 7. Clones IIF4 [///], IIC4 [xxx] and IIH1 [\\\] were corrected for antibody concentration and simultaneously tested by ELISA for reactivity to SCM, GBM and LBM. While ascites from all three clone reacted with SCM and GBM with equal affinity, only IIF4 and IIC4 reacted strongly with LBM. IIH1 showed only minimal binding to LBM.


ELISA OF mAb
COMPARISON OF ANTIBODY BINDING
SCM PLATE
GBM PLATE
LBM PLATE
OPTICAL DENSITY (405nm)
0.600
0.500
0.400
0.300
0.200
0.100
0.000
NS
X-63
IIC4
IIH1
IIF4
NS not significant at P=0.5

with SCM and GBM, it was only slightly reactive with LBM. This data support the effects of these clones observed at the gross level (Table 4).

SCM Inhibition of Antibody Binding:

Binding of IIC4 mAb to trypsin solubilized SCM, GBM and LBM coated microtiter plates was inhibited by premixing the IIC4 ascitic fluid with intact SCM (Fig. 8). A dose dependent response was generated by increasing the concentration of intact SCM. Whole S. mutans did not inhibit binding of the mAb to SCM.

Fluorescent Demonstration of Cross Reactive Antibodies:

Fluorescent antibody tests of the kidneys of all clone baring animals indicated that those animals receiving clones IIF4, IIC4,and IIH1 showed positive findings (Fig. 9). FITC-labeled goat anti-mouse antibody bound in a granular manner to the GBM of the glomerulus. While lung from IIF4 and IIC4 baring mice showed fluorescence after incubation with FITC-labeled antibody, lung from IIH1 baring mice showed no fluorescence (Fig. 10). All control animals gave negative results under the same conditions of the experimental animals.

Electron Microscopic Evaluation of Kidney and Lung:

Electron micrographs of GBM from X-63 baring mice (Fig. 11) show a basement membrane of uniform thickness and staining intensity. Both IIF4 and IIH1 clone baring mice had basement membranes which contained subepithelial humps. Subepithelial humps are a classic sign of GBM damage appearing as a result of post-streptococcal sequelae. Electron micrographs of lung tissue from IIF4 clone baring mice showed an uneven thickening along the basement membrane. X-63 and IIH1 clones

Figure 8. An inhibition assay using the ELISA protocol was run to assess the specificity of clone IIC4 reactivity with SCM, GBM and LBM. IIC4 was premixed with either intact SCM from S. pyogenes [\\\\] or intact S. mutans [///]. Reactivity of mAb IIC4 with S. pyogenes removed the mAb from the ascitic fluid thus inhibiting antibody binding with membrane antigen. There was no significant difference between the antigen reactivity of IIC4 premixed with S. mutans and antigen reactivity of unmixed IIC4.

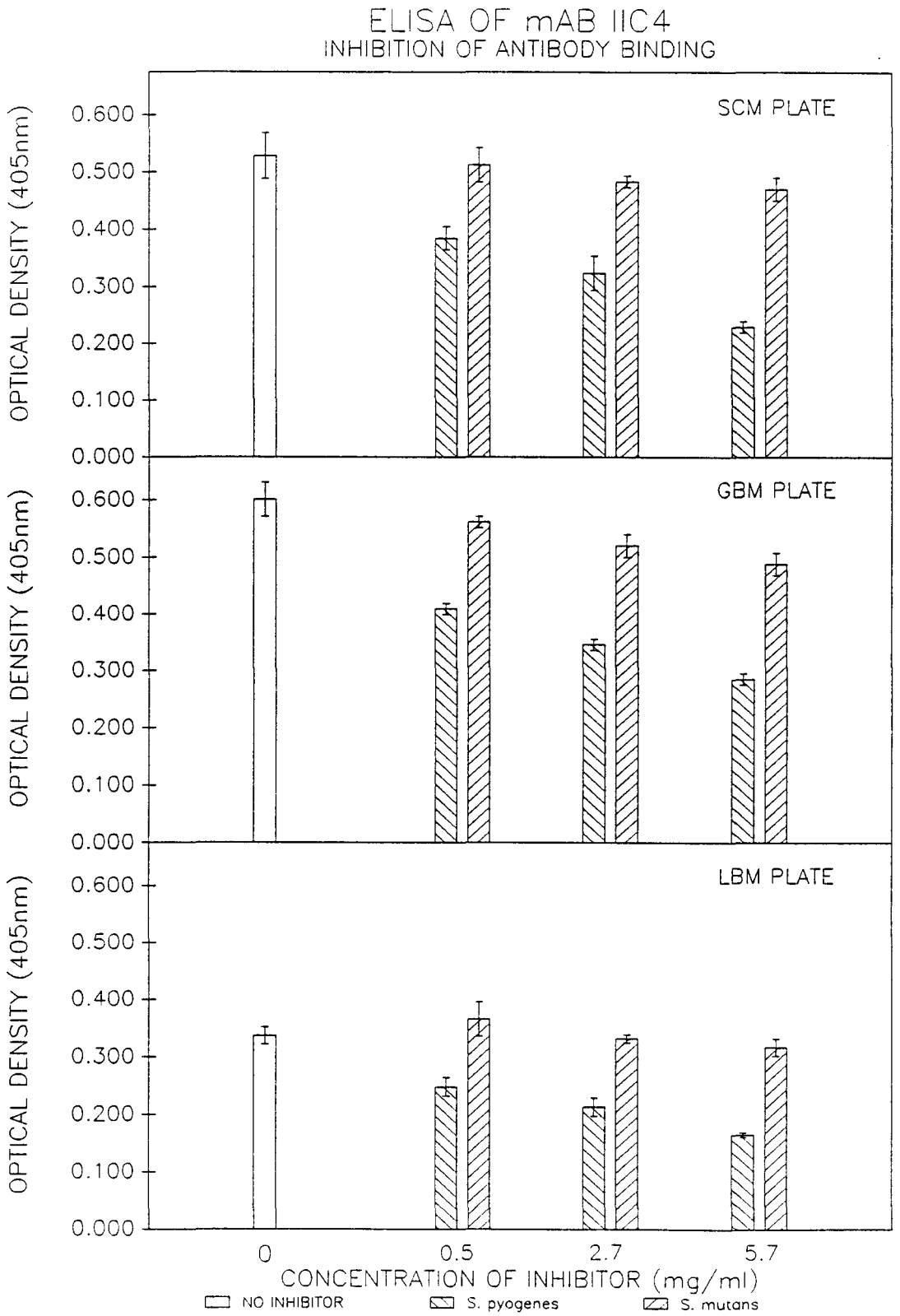
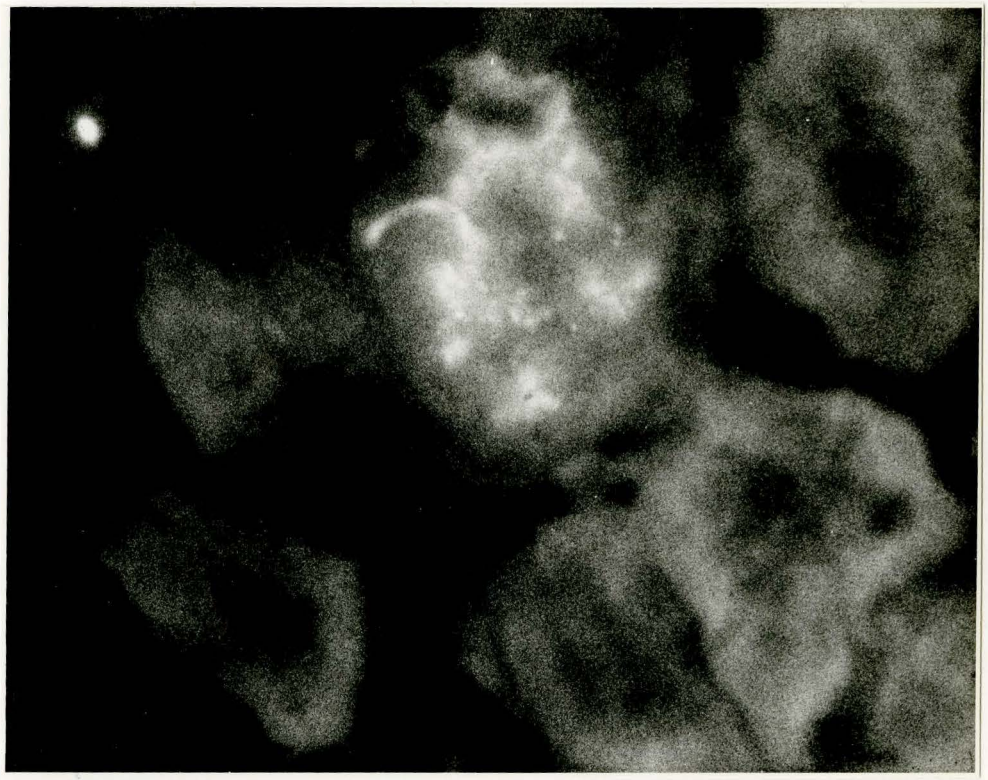
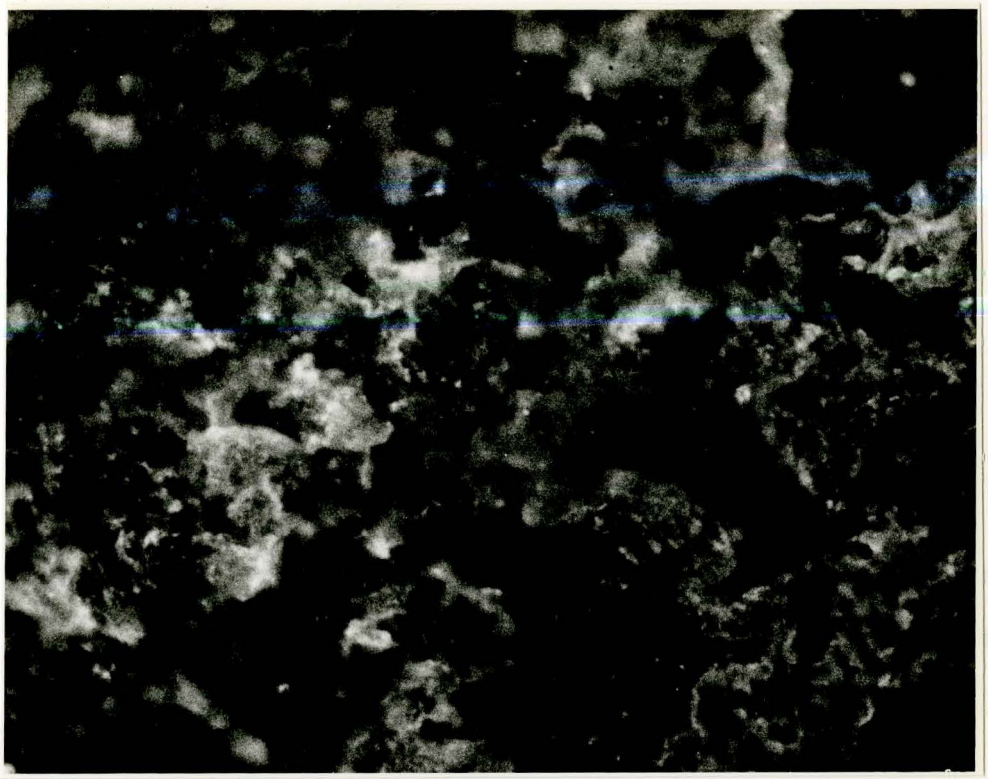

ELISA OF mAB IIC4
INHIBITION OF ANTIBODY BINDING
SCM PLATE
GBM PLATE
LBM PLATE
OPTICAL DENSITY (405nm)
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
0.5
2.7
5.7
CONCENTRATION OF INHIBITOR (mg/ml)
NO INHIBITOR
S. pyogenes
S. mutans

Figure 9. Frozen sections of kidney from mice bearing clone IIC4, IIF4 and IIH1 were tested by direct fluorescence for antibody binding. FITC-labeled antibody show a pattern of granular deposition of immunoglobulin primarily in the capillary tuft of the glomeruli.

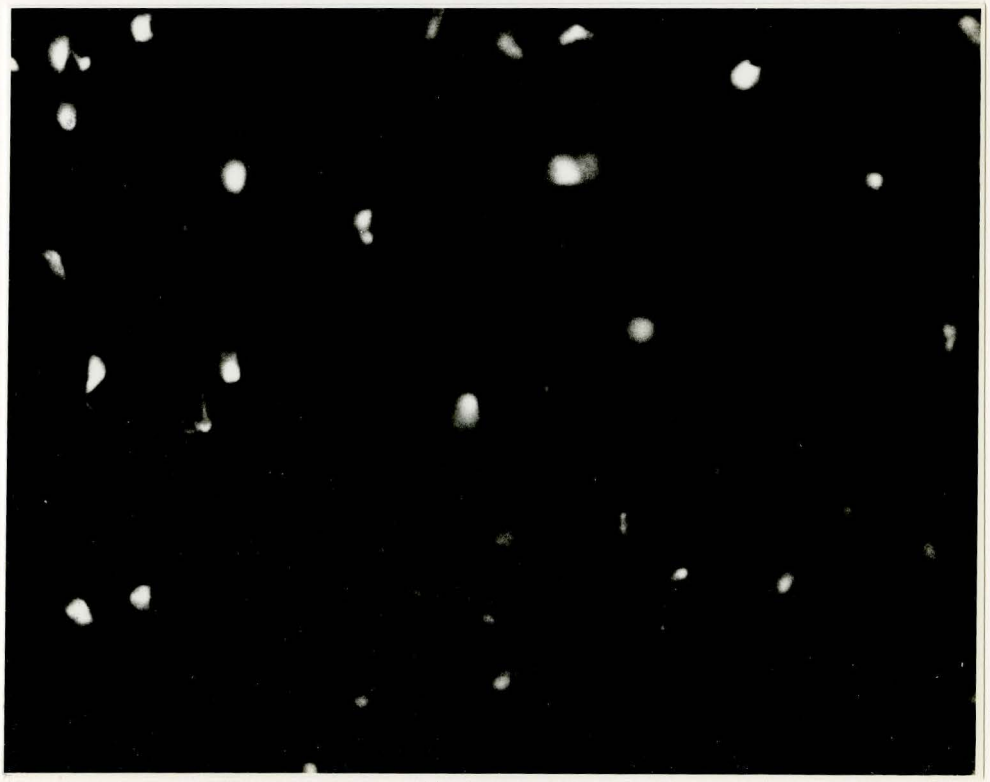


X400

Figure 10. Frozen sections of lung from mice bearing the IIF4, IIC4 and IIH1 clone were tested by direct fluorescence for antibody binding. Clones IIF4 and IIC4 produced antibodies which bound to LBM as indicated by a FITC-labeled secondary antibody. Lung from IIH1 bearing mice showed no indication of mAb binding.

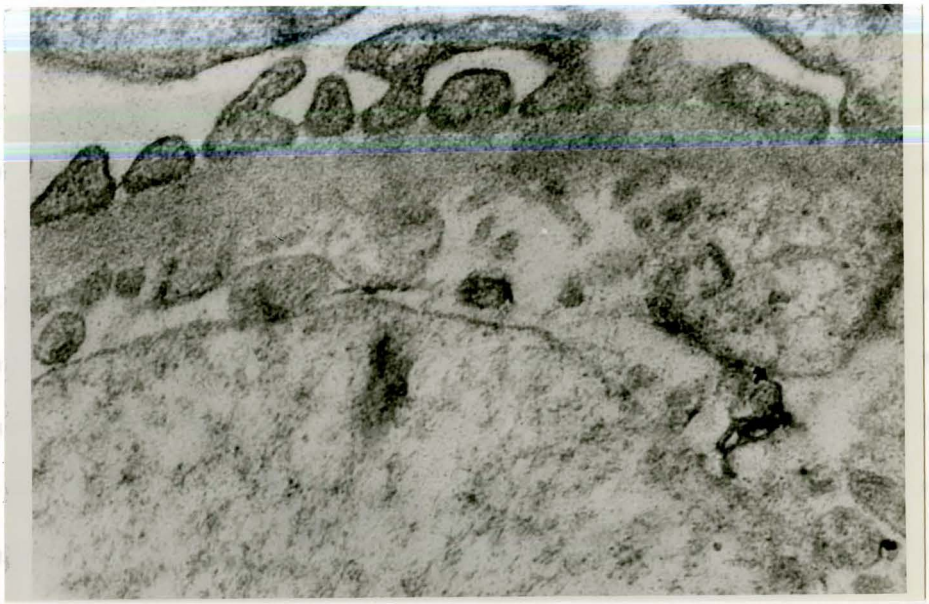


X400

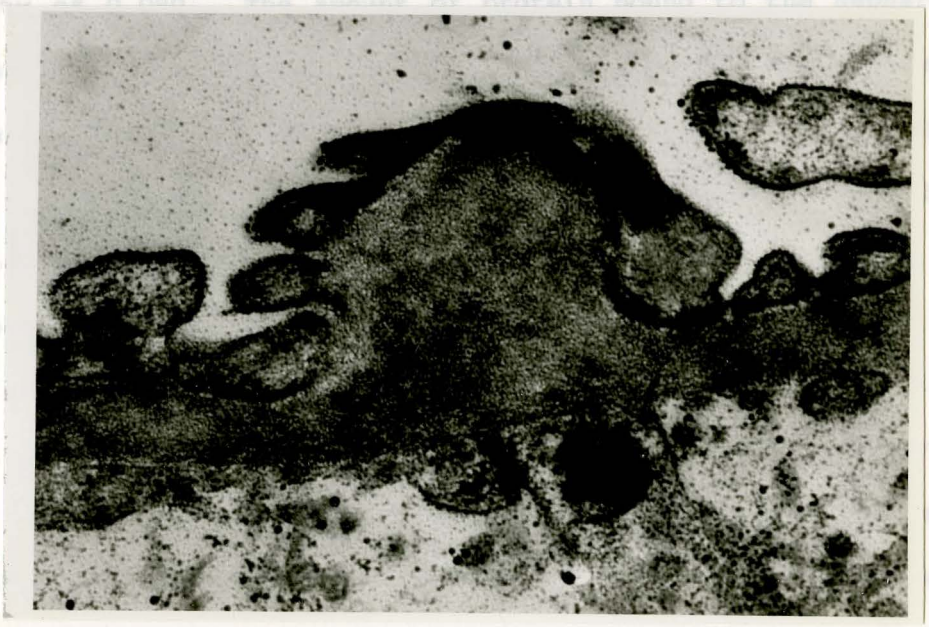


X400

Figure 11. Electron micrographs of GBM from X-63 bearing mice show a normal basement membrane. There is no indication of antibody deposition on the GBM. GBM from mice bearing IIF4 or IIH1 presented with antibody deposition in the form of subepithelial humps.



X51,000



X51,000

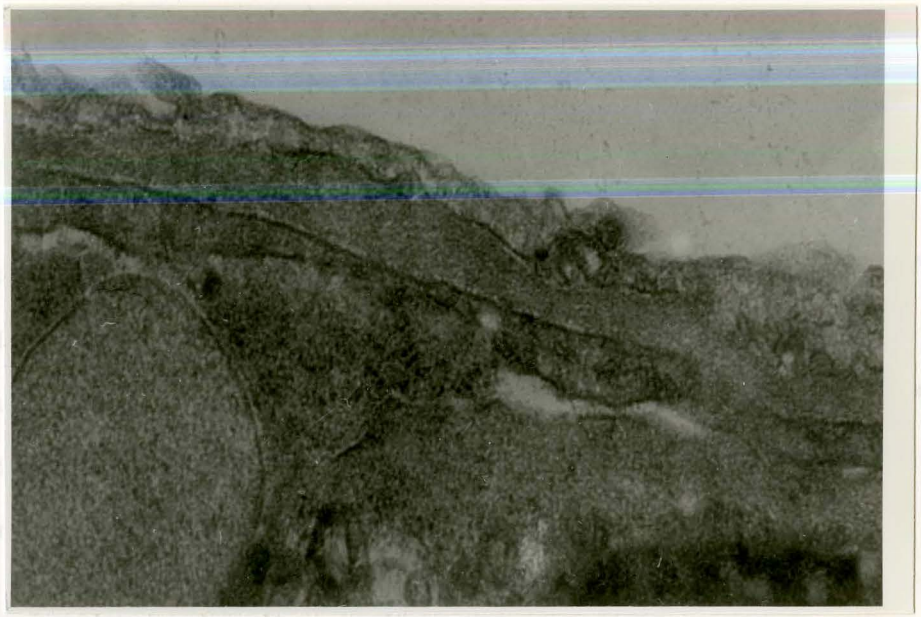
had no effect on the LBM characteristics at this level of detection (Fig. 12).

Affinity Purification of Membrane Antigen:

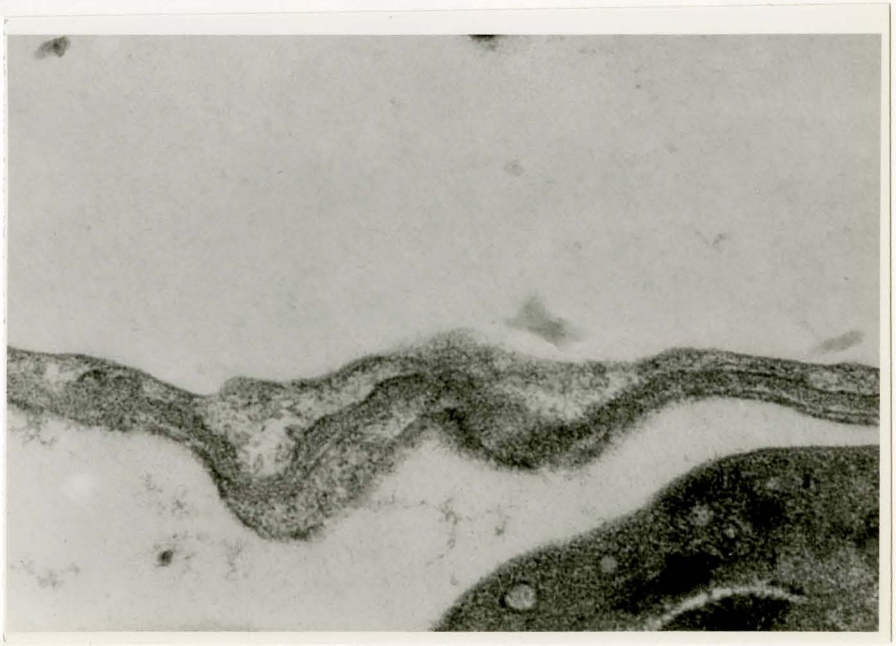
The amount of IIF4 ascitic fluid collected from two mice for affinity chromatography was 7ml. Ascitic fluid contains in addition to the mAb other serum proteins. To isolate a pure sample of antibody from the ascitic fluid, a double precipitation with ammonium sulfate was initiated. The purity of the antibody isolate was necessary for production of the IIF4 affinity column. Purity of the antibody isolate was checked by electrophoresis. After ammonium sulfate fractioning, the elution procedure through G25 sephadex increased the volume to 30ml. The ascitic fluid was concentrated to 20ml. Optical density (278nm) of the ascitic fluid before mixing with activated Sepharose was 0.175; after 20hr. of mixing the ascitic fluid had an optical density of 0.080. The amount of protein bound to the Sepharose was estimated to be 1.4mg protein/ml packed Sepharose. A separate column was make for the fractionation of each of the antigens (SCM, GBM, LBM). The antigen was applied at a concentration of 2mg/ml. The effluent was washed out with 0.05M PBS (pH 7.4), and the bound antigen eluted with glycine HCl.

Each solubilized membrane produced a characteristic plot of its elution profile over the monoclonal antibody (IIF4) affinity column (Fig. 13). When trypsin solubilized lung was eluted two separate peaks were collected from the effluent. The areas under the two peaks appear to be almost equal, though the first peak is consistently higher than the second. It was established that this was a sieving

Figure 12. Electron micrographs of lung tissue from mice bearing clone IIF4 showed abnormally thick and uneven basement membranes. No alteration could be found in the LBM of mice bearing X-63 or clone IIH1.



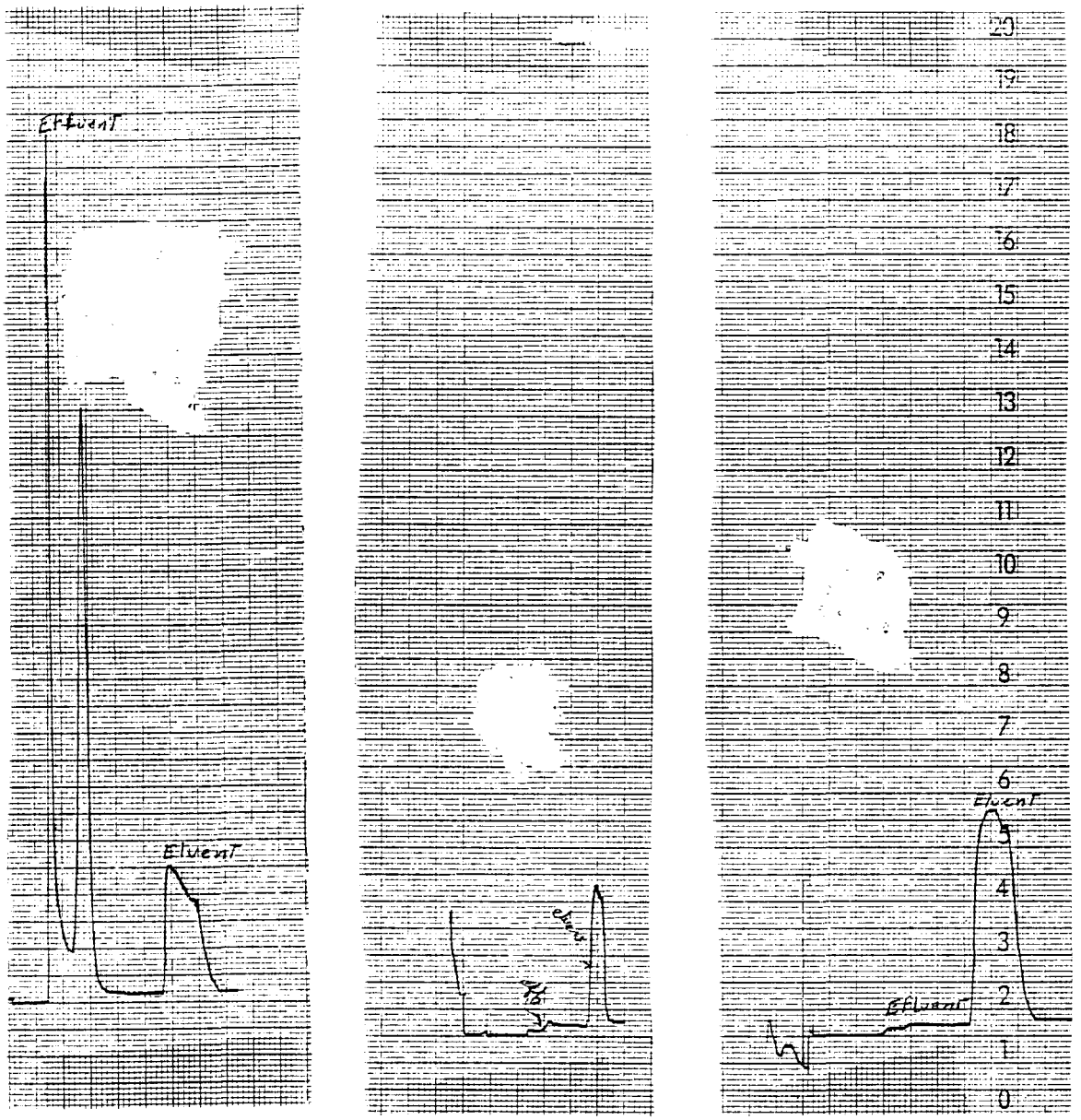
X60,000



X60,000

Figure 13. Trypsin solubilized SCM, GBM or LBM was run over a IIF4 affinity column. The elution profile was unique for each membrane. Column contained 5ml. of Sephadex 4B coupled with IIF4 mAb. The antigen was applied at a concentration of 2mg/ml and elution of column bound antigen was accomplished using glycine HCl pH 2.0.

IIF4 AFFINITY COLUMN



LBM SCM GBM

phenomena of the Sepharose 4B itself because; solubilized LBM which was passed over activated but uncoupled Sepharose also produced the double peaks. The eluent produces a peak that contains an area some what larger than the sum of the two effluent peaks. The solubilized GBM and SCM were similar in that very small effluent peaks were produced as compared to their respective eluent peaks. The GBM eluent formed larger peaks than did the SCM eluent.

High Pressure Liquid Chromatography:

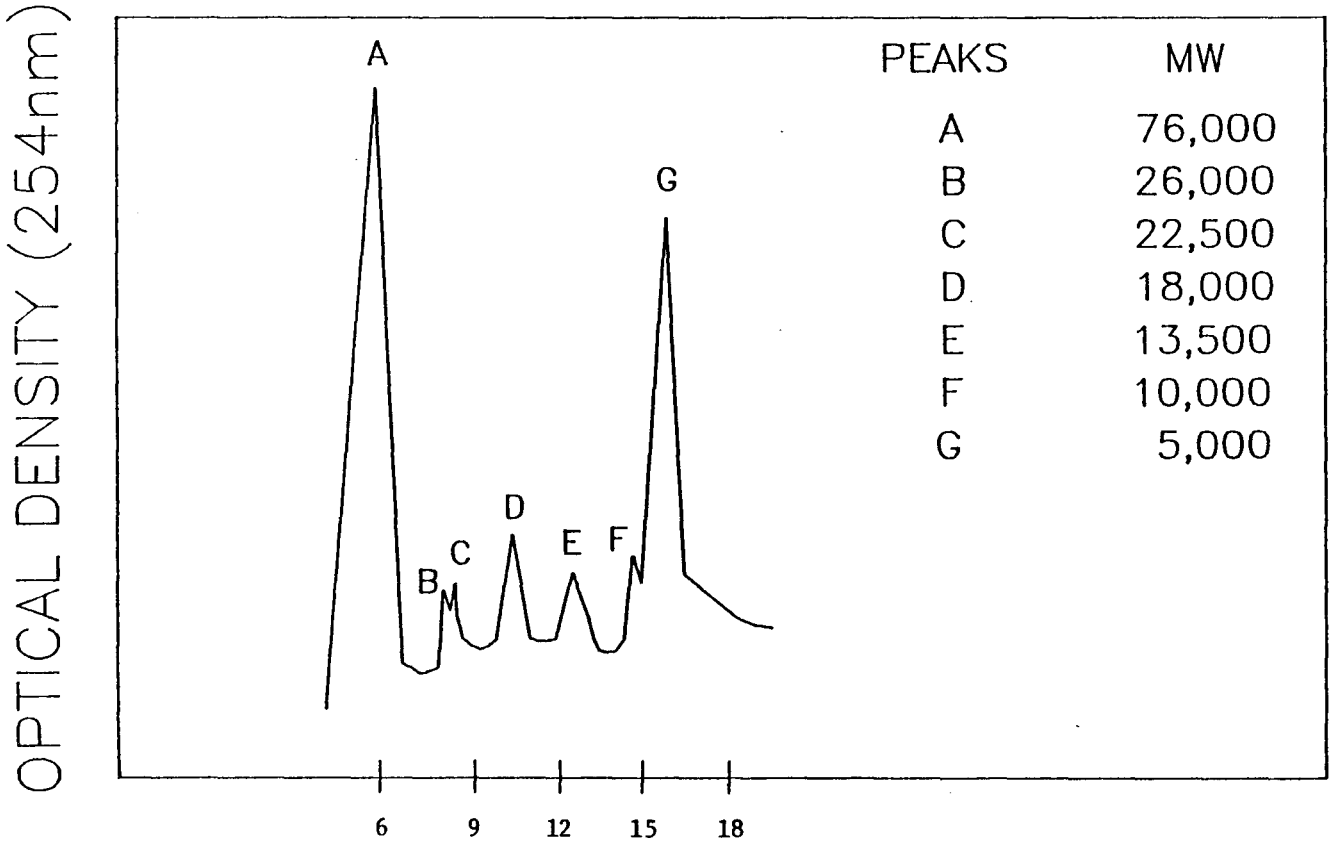
When SCM and LBM were run over the affinity column, the eluent and effluent were collected and passed through the HPLC column. The HPLC tracing of the SCM effluent (Fig. 14) shows six peaks, representing proteins of molecular weights 22.4Kd; 20Kd; 17Kd; 10.5Kd; 5.8Kd and 2.2Kd respectively, as determined from the standard curve (Fig. 15). The IIF4 affinity column eluent appears to be enriched for the 10.5Kd MW fragment (Fig. 16). The presence of the 5.8kd and 2.2Kd peaks is significant in that it indicates that these small components also contain the binding epitopes.

Cellular Proliferation of Glomeruli:

Cellular proliferation, an indicator of glomerular damage, was observed in the H and E stained kidney sections of mice carrying clones IIF4 and IIH1 (Fig. 17). Each clone was injected into two mice. Frozen sections were cut from the kidneys of clone bearing mice, with approximately 5 sections cut per mouse. At least 20 glomeruli per section were counted. Glomeruli from mice carrying clones IIF4 and IIH1 averaged 58 nuclei and 56 nuclei respectively compared to an average of 35 nuclei in the glomeruli of kidneys from non-injected

Figure 14. LBM effluent off a IIF4 affinity column was run through an HPLC column. Molecular weights were determined from a standard curve. A high molecular weight fraction (peak A) seemed to be the major constituent.

AFFINITY COLUMN IIF4
LBM EFFLUENT



PEAKS	MW
A	76,000
B	26,000
C	22,500
D	18,000
E	13,500
F	10,000
G	5,000

Figure 15. A standard curve for molecular weights was generated by running compounds of known molecular weight through the HPLC column and timing their elution speed. Standards include Lactate Dehydrogenase (140,000), Enolase (67,000), Adenylate Kinase (32,000) and Cytochrome C (12,400).

HPLC MOLECULAR WEIGHT MARKERS

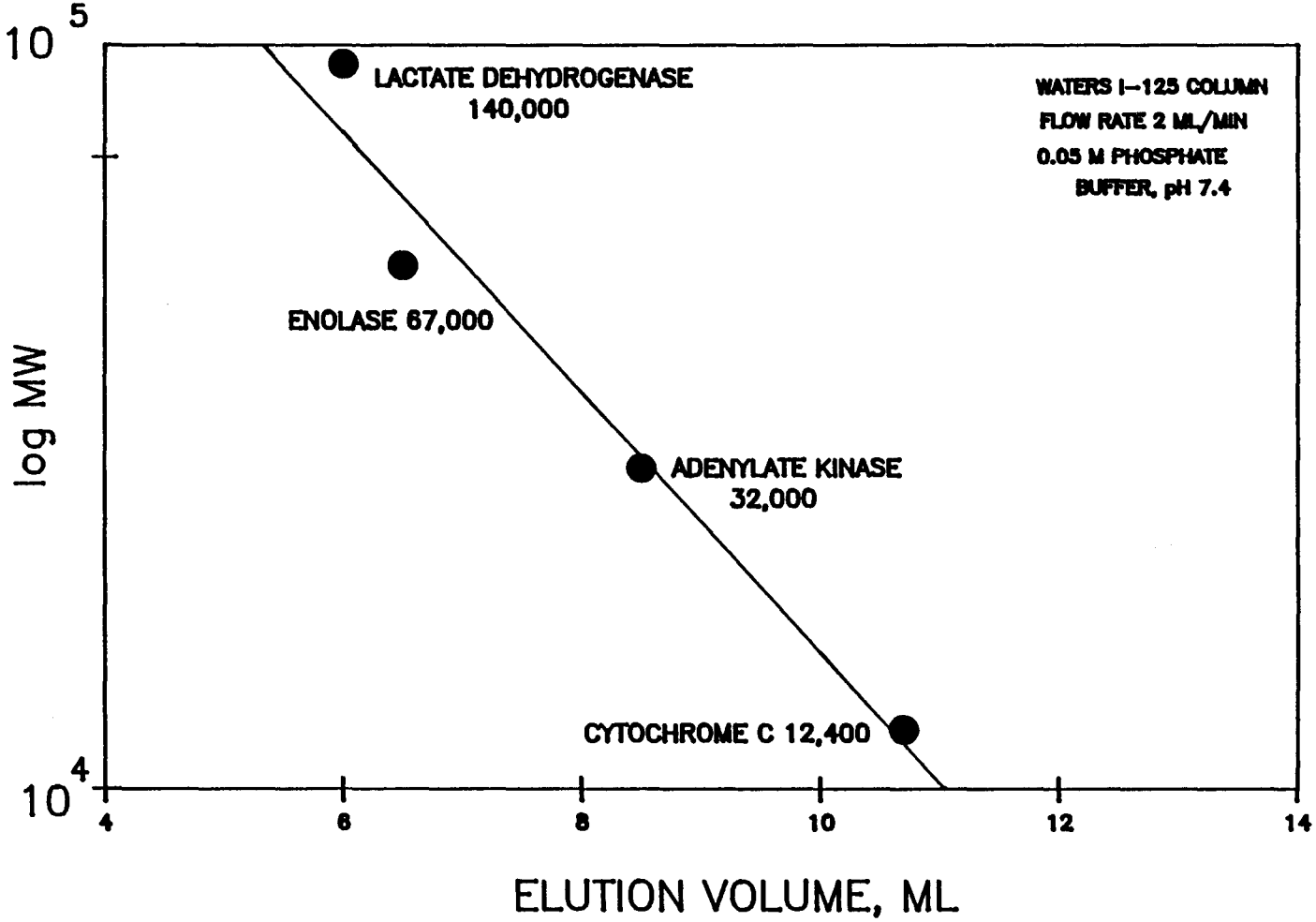
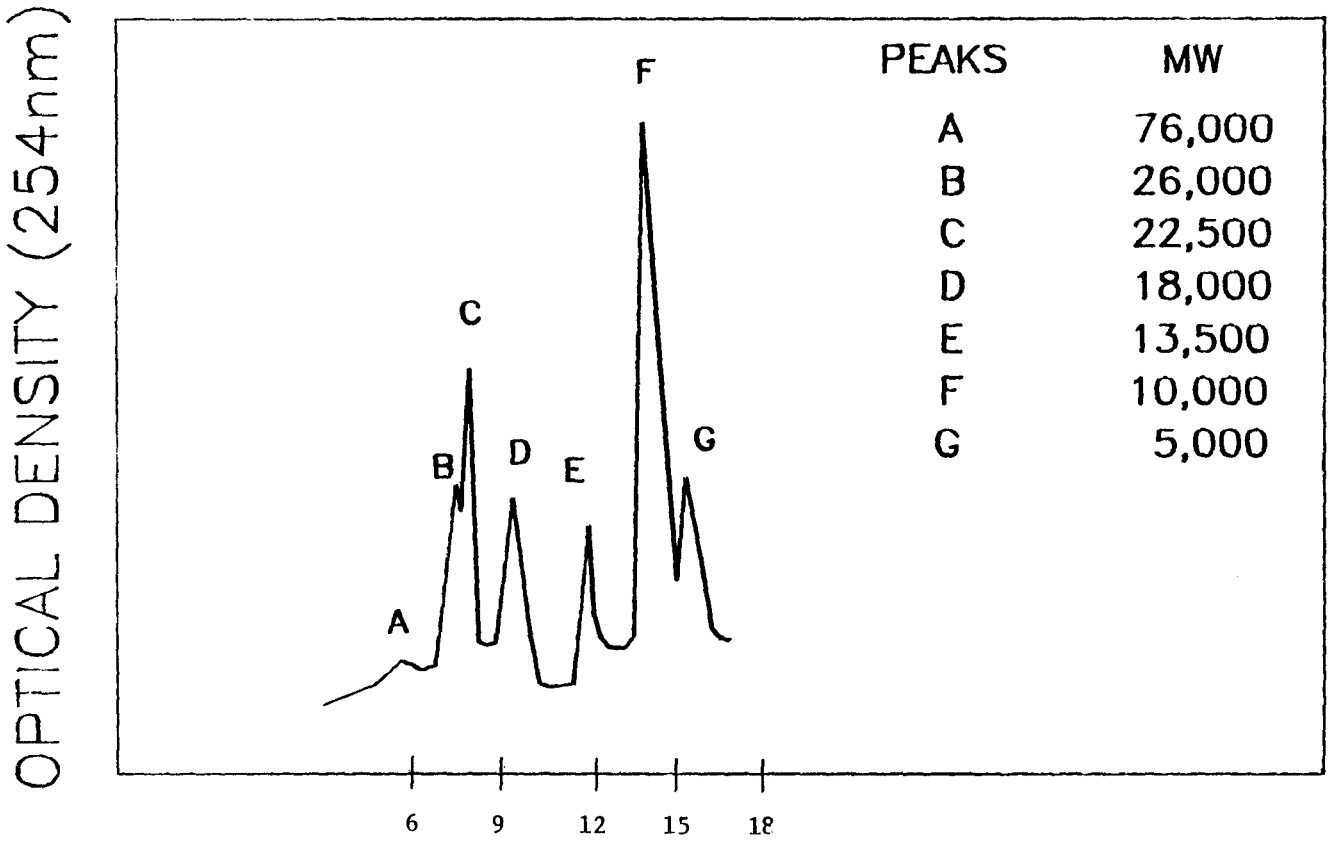


Figure 16. LBM eluent off a IIF4 affinity column was run through an HPLC column. Molecular weights were determined from a standard curve. A low molecular weight fraction (peak F) appears to be the major constituent.

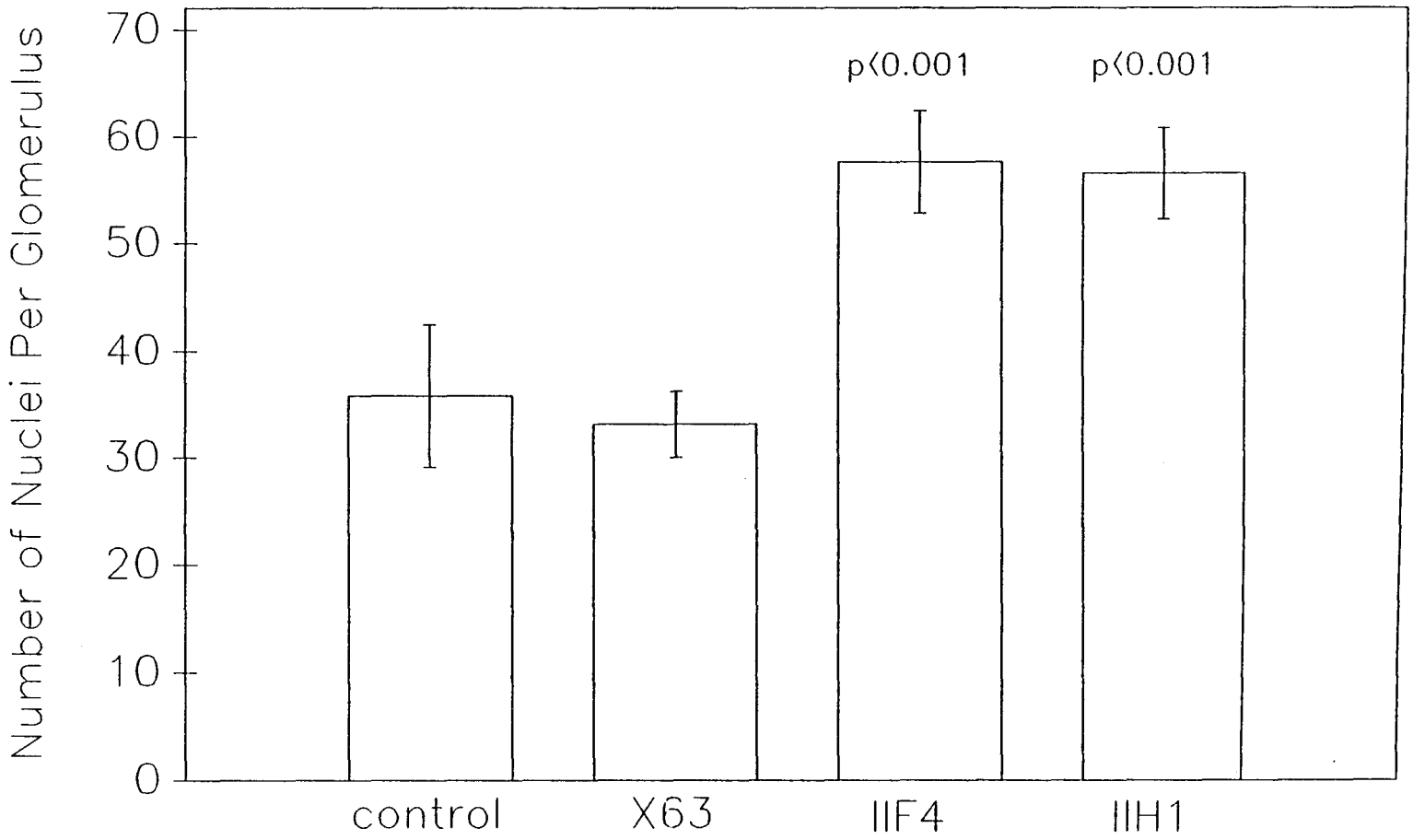
AFFINITY COLUMN IIF4

LBM ELUENT



PEAKS	MW
A	76,000
B	26,000
C	22,500
D	18,000
E	13,500
F	10,000
G	5,000

Figure 17. The number of nuclei per glomerulus was determined. Clones IIH1 and IIF4 induced cellular proliferation in the glomeruli of the kidneys from clone bearing mice. X-63 was unable to induce proliferation. Proliferation is an indication of immunologic damage to basement membrane.


Total Number of Nuclei Per Glomerulus
proliferation in response to mAb
Number of Nuclei Per Glomerulus
70
60
50
40
30
20
10
0
p<0.001
p<0.001
control
X63
IIF4
IIH1

mice. No cellular proliferation was noted in the glomeruli of control or X-63 bearing mice where the average was 35 nuclei/glomerulus.

CHAPTER IV

DISCUSSION

Autoimmune diseases present both problems and opportunities for understanding the immune system and its response to foreign antigens. Normally functioning in a protective capacity, the immune system can also produce antibody which will bind host antigens. Autoimmune diseases are a result of this action. The initial stimulus for the appearance of these auto-antibodies, and their relation to the normal repertoire of antibodies remains unclear. Human anti-DNA antibodies present in systemic lupus erythematosis (SLE) appears to come from germ line genes (Cairns et al., 1989). These antibodies can be found in healthy individuals in low levels (Cairns et al., 1985) demonstrating that the presence of auto-antibodies as determined by in vitro testing is not always indicative of autoimmune disease. Also, the cause of the elevated levels of auto-antibodies is uncertain. Animal models can be of great benefit in the study of cell-cell interaction in autoimmune diseases, their causative factors and pathology. The role of gender, as well as the efficacy of treatment protocols can be investigated using the appropriate animal model.

We originally generated anti-SCM hybridomas in order to investigate mechanisms of post-streptococcal glomerulonephritis associated with Group A beta hemolytic streptococcal infection. Data and observations presented here are consistent with the accepted pattern of post-streptococcal glomerulonephritis. Though initially

not the goal of this dissertation, we have developed a model which reproduces certain aspects of Goodpasture's syndrome using select anti-SCM clones. These clones produce specific mAb which cause pathology reflecting this syndrome. The animals exposed to these antibodies show pulmonary hemorrhages, glomerulonephritis as well as deposition of antibodies into basement membrane. The development of this animal model, therefore, became the focus of this thesis.

The etiology of Goodpastures's (GP) syndrome is still obscure but the presentation of pulmonary hemorrhage associated with a glomerulonephritis and the presence of anti-GBM antibodies in the serum, is well accepted (Benoit et al., 1964). The presumption that the GP antigen is shared by the lung and the kidney is supported by the following discoveries: 1) the occurrence of lung hemorrhaging in patients who produce anti-GBM antibodies; 2) the use of bilateral nephrectomy to halt this hemorrhaging; and 3) the binding of anti-GBM antibodies to lung section. It appears therefore that an antigenic relationship exists between the lungs and kidneys. We have demonstrated that antibodies raised against SCM can produce pathology similar to GP when injected into the peritoneal cavity of Balb/c mice. Of real significance is the fact that the inducing antibody is an anti-streptococcal antibody rather than a heterologous antigen. Much has been written about the heterogeneity of Goodpastures's antigen but little is known about the inducing immunogen responsible for the anti-GP antibody (Price and Wang 1988). A rat model for GP has been described wherein the challenging immunogen is bovine GBM (Sado et al., 1984). This study employed only female animals and was able to

mimic a GP, after 10 weeks, in animals receiving a high (1 mg) challenge of antigen. Our study has a significant difference in that the inducing immunogen is a streptococcal antigen not a heterologous but related tissue antigen. Further, since the host animals only see or are exposed to homologous cells or antibody, we believe this represents an autoimmune induced GP with a post-streptococcal sequelae implication.

At this time there is no good evidence for a post-streptococcal etiology for GP however the results obtained by our work with anti-SCM hybridomas do present an interesting parameter to this human syndrome. Since the causative agent for GP is unknown, any relationship between the eukaryotic and prokaryotic membranes may give insight to factors which initiate autoimmune disease. If the disease state can be mimicked by anti-SCM antibodies perhaps a previous streptococcal infection played some role in the development of this autoimmune disease. The deposition of antibodies has been localized to the basement membrane of the lung and kidney. Immunofluorescence and electron microscopy have shown patterns of immunoglobulin deposition on GBM similar to that seen in PSGN (ie. sub-epithelial granular staining). Linear staining is generally seen in GP. The difference in antibody staining patterns may reflect the difference in the course of the disease induced by the hybridomas as contrasted to the natural development of GP.

Rees et al.(1977) have demonstrated a relationship between intercurrent bacterial infection and relapse of the clinical symptoms of GP. These relapses seem to be independent of a rise in anti-GBM.

Therefore categorizing GP as an anti-GBM disease may be misleading. The extensive involvement of the kidney could merely reflect the greater accessability of the pertinent antigen. The pathology reported in GP may in part be due to "infection stimulated responses occurring in the presence of a pathogenic autoantibody" (Rees et al., 1977). It is hypothesized that local bacterial infections may raise serum levels of tumor necrosis factor (TNF) or IL-1, resulting in a tissue damage far beyond the localized infection. Tomosugi et al. (1989) have shown that small doses of TNF and IL-1β amplifies the severity of injury caused by subsequent injections of heterologous anti-GBM. Tissue damage may thus be the result of autoantibodies in the context of an immune response to foreign antigens.

Swartzwelder et al. (1988) contend that antigens (S. mutans) stimulate the production of naturally occurring non-cross-reactive antibodies (anti-S. mutans and anti-HRA), but not the formation of new cross-reactive antibodies. In our animal model the use of a monoclonal antibody which binds both lung and kidney supports the contention that cross-reactive antibodies do exist. If Swartzwelder et al. (1988) are correct in their assumption, an equally fascinating question is; How can a single antigen (S. mutans) stimulate the expansion of a population of B cells directed at an apparently unrelated antigen (kidney)? Between antigen presentation and effector function some cell must stimulate both populations of B cells.

Although preexisting autoimmune clones, being responsible for the generation of some clones, have not been entirely ruled out in the present study, we believe that these findings represent specific

cross-reactions of anti-SCM to specific tissue antigens and is not simply proliferation of multiple organ-reactive (MOR) clones as described by Prabhaker et al., (1984). It should be noted that these investigators only looked at antibodies employing an indirect fluorescent antibody method. In contrast, we feel that the preparation of carefully selected cross-reactive hybridomas may allow for studies of post-streptococcal sequelae. Further, if pre-existing clones were present it seems likely that the control fusion (IC3) (Table 1) would have shown higher SCM titers. Also, while a certain fraction of SCM hybrids when screened by ELISA were found to be reactive with GBM, there were no GBM hybrids generated which were not reactive with SCM. If we were merely stimulating preexisting autoimmune clones it would seem likely that non-cross reactive anti-GBM hybridomas would have been detected. Selected hybridomas from the fusion of an anti-SCM B cell and an X-63 plasmacytoma were generated in order to draw a relationship between streptococcal infections and PSGN (Table 1). By demonstrating specific reactivity of anti-SCM antibodies with GBM antigen insight was gained as to the bacterial constituent which induced the host reactive antibody and the site of antibody induced tissue damage.

While investigating the relationship of anti-SCM hybridomas and renal pathology, it was noted that on autopsy certain mice were showing profound lung pathology. Because we were using polyclones it was unclear whether separate clones were causing lung and kidney damage or a single anti-SCM clone was reacting with an epitope common to both organs. Only the production of monoclonal antibodies could

resolve this question. Also since pathology was occurring in two different organ systems, and the anti-SCM hybrids were shown to be reactive with GBM, it was felt that a common antigen to both systems would most likely be found in the basement membrane.

The topic of cross-reactive anti-streptococcal monoclonal antibodies has been the subject of other studies (Cunningham, et al., 1983, 1984, 1986 and 1988) who evaluated the reactivity of their mAb by Western analysis without any in vivo workup. The immune response to SCM is polyclonal in nature. The process of limiting dilution results in the production of mAb, allowing comparison of specific epitopes on the SCM and mammalian tissue. The ELISA data supports our original hypothesis that the SCM contains unique epitopes which are responsible for the generation of antibodies that cross react with mammalian antigens. We established by direct ELISA the cross reactivity of several of our SCM mAb to GBM and LBM (Table 3). The ELISA findings from generated ascitic fluid was supported by gross evaluation of the lung and histologic evaluation of alveolar tissue (Table 4). Anti-LBM reactivity translated in vivo into hemorrhagic lesions of the lung. The histologic study revealed a stereotypic inflammatory response. Polymorphic neutrophils were present in response to extensive cellular damage presumably due to the binding of mAb to LBM. The appearance of macrophages is generally considered a sign of the chronic nature of the inflammatory response. As antibodies continue to be produced by the clones and bind to host tissue the macrophage plays a more prominent role in removing damaged tissue.

In addition to noting the lung pathology in some early polyclone bearing mice, the finding of a single clone leading to death of the host animal reinforced the focus of this dissertation on the investigation of Goodpasture's syndrome. Consistently through the course of this investigation both the dose of injected clone (or mAb) and the sex of the animal has played a significant role in the extent of lung damage. A dose dependent response implies a strong correlation between antibody titer and tissue pathology. Table 6 indicates that gross pathology in the lung was not the result of excess peritoneal fluid accumulation but because of the antibody found in the fluid which is equilibrating with blood and finding its way to host tissue. Increasing the loading dose of cells increased the degree of tissue damage. Additionally, equilibration of antibodies between peritoneal fluid and blood was rapid enough to allow injected antibody to reach the lung and cause damage within 4 hrs (Table 7).

Although the role of gender in the prevalence of GP is unclear, it is commonly known that the incidence and severity of certain autoimmune diseases is dependent on the individual's sex. Presumably a change in steroid level is related to this differential response. This may be especially pertinent in the female where hormones fluctuate over a large range and in a cyclic nature. These changes in hormone levels may expose certain relevant epitopes which may directly stimulate the immune system.

The use of monoclonal antibodies has enabled us to investigate similarities between eukaryotic basement membranes (kidney and lung) and the cytoplasmic membrane of Group A, beta hemolytic streptococci.

The fact that anti-SCM antibodies were able to produce GP symptoms (i.e. hemorrhagic lungs and proteinuria) was demonstrated at the gross level by observing lung damage, and histologic staining with H and E. The pathology demonstrated by the H and E sections support an inflammatory response due to the presence of the anti-SCM antibody.

All the experiments performed as part of this thesis involved the placing of either hybrid cells or mAb into normal streptococcal antigen free host; therefore, any binding of the mAb at host tissue sites must be due to an immunologically related tissue antigen. Additionally, since many experiments were performed on litter mates, it is assumed that any infections agent would have equal impact on normal, control (X63), and experimental animals. Furthermore, administration of increasing concentrations of antibody produced increasing levels of mAb binding especially in the female animals which supports the concept of cross-reactivity and rules out antibody binding to prelocalized streptococcal antigen. If a localized streptococcal antigen were to bind the antibody, one would not expect to find the stoichiometry displayed in the results shown in Tables 5 and 6. Likewise, one would expect to see similar results in both sexes, which is contrary to data presented in this thesis. Also, it is likely that a persistent localized antigen would have induced an immune response in the host. No evidence along this line was discovered in any control animal.

The <u>in vivo</u> experiments are a parallel to those of Sado, et al., (1984) employing anti-bovine GBM antibodies. Indeed, most reports of pulmonary hemorrhages seen in experimental models are a result of

heterologous challenge (Steblay and Rudolfsky, 1983 a & b). This we feel is a major shortcoming of many of these studies. Our results support the concept of autoimmune phenomenon arising because of cross-reactivity of host tissue antigens and exogenous but immunologically related microbial antigen, not the result of de novo anti-GBM antibody production.

Deposition of immunoglobulin in the lung results in edema and gross hemorrhagic lesions. Initially, the amount of lung damage was determined by qualitative evaluation. Since this evaluation of gross lung damage due to the presence of mAb tends to be arbitrary in nature, a more quantitative approach was taken. Hemorrhages in the lung resulting from the reactivity of mAb with the basement membrane result in an altered lung weight. By generating an L/B ratio for clone baring mice a more quantitative evaluation of antibody induced damage was developed.

One of the most unique aspects of this research was the finding of two different types of anti-SCM cross-reactive clones. Two clones (IIF4 and IIC4) are able to produce pathology in the lung and kidney; while one clone (IIH1) affected the kidney but left the lung undamaged. Since GP involves glomerulonephritis with intermittent lung pathology, it was thought that appearance of lung hemorrhage depended on the recruitment of the correct antibody. Immune responses against most foreign bodies (including bacterial) is polyclonal in nature. The immune system recognizes a variety of epitopes and produces antibodies against them. Then, those clones which are most effective at binding and eliminating the foreign body are expanded.

While IIF4 and IIC4 recognize an epitope shared by SCM, GBM and LBM, clone IIH1 binds a different epitope seen on the GBM and SCM but not on the LBM. Apparently, the appropriate clone must be generated to cause lung involvement. What conditions precipitate the generation of a lung reactive clone is unclear. Also, the extent to which individuals in a population can generate this specific clone has yet to be determined. The fact that Goodpasture's syndrome irregularly shows lung hemorrhage may be explained by the theory that only a restricted part of a population has the capacity to generate the antibody which cross-reacts with lung. In the rest of the population a hole may exist in their repertoire, thus preventing the generation of this clone.

Witebsky's Criteria regarding autoimmune phenomena parallel Koch's postulate for bacterial etiology. One criteria that must be fulfilled to support the contention of autoimmune disease is that the experimental disease must be transferred to a non-immunized animal by serum or by lymphoid cells. We have used both ascitic fluid and lymphoid cells to reproduce murine lung and kidney pathology. In addition, by eluting antibody off an intact membrane and demonstrating its reactivity to SCM and GBM by ELISA (Table 10), we were able to show that the mAb was able to recognize a specific epitope in the intricate basement membrane. <u>In vivo</u> binding was not simply a case of unusually large quantities of antibody being trapped in the membrane in a non-specific fashion. If it were a non-specific event, ELISA date would not have shown such strong antibody binding. We have shown that the binding to <u>in vivo</u> basement membrane is a specific event.

The ELISA using monoclonal antibody IIF4 showed strong reactivity with SCM, GBM and LBM. The reactivity of the monoclonal antibody with GBM and LBM is interesting since the B cells which were used to form the clone were sensitized only with SCM. The weak binding of monoclonal antibody IIF4 and IC3 to myosin at the low dilution (1:1600) indicates a small non-specific binding phenomena. This data was further supported by reciprocal inhibition of antibody binding. The cross reactive nature of the mAb IIC4 caused its removal from the ascitic fluid when mixed with undigested Streptococcus pyogenes SCM but not with S. mutans. The specificity of the adsorption of antibody by SCM was previously demonstrated by Blue and Lange (1975). Ascitic fluid from clone IIC4 was used for the inhibition assay for two reasons: 1) IIC4 showed a somewhat stronger binding of mAb to trypsin digested murine lung in the ELISA screening of SCM generated clones; and 2) IIC4 seemed to give similar ELISA readings when tested against all three antigens (SCM, GBM, and LBM). Inhibiting the binding of a mAb that shows high reactivity to LBM indicates that the antibody is recognizing a major constituent in the lung. The extent of gross lung damage in clone baring mice supports this contention. Similar ELISA readings gave a clue as to the amount of inhibitor required for adequate demonstration of cross-reactivity.

The concentration of ascitic fluid in the ELISA investigations was stated in terms of dilutions of original aliquots. To account for differences in mAb production between clones IgM levels in the ascitic fluids was determined by generating a standard curve using TEPC ascitic fluid which contained known amounts of IgM. Optical densities

of ascitic fluid from TEPC were compared against the desired clone. Since IIF4 and IIC4 produced comparable amounts of IgM, the amount of antibody used in the ELISA studies was recorded in terms of dilution of original immunoglobulin level.

Direct fluorescence of lung and kidney from mice baring the IIF4 and IIC4 clones showed binding of antibody from the respective clone to the observed tissue. IIH1 while producing a strong fluorescence in the kidney showed no binding to the murine lung. In all cases the fluorescence appeared to be restricted to the basement membrane. This last point however was difficult to assess since the brightness of fluorescence precludes differentiation of basement membrane from cytoplasmic membrane. It was for this reason that electron microscopic techniques were employed. The electron micrographs clearly indicate that pathology observed in gross evaluation, histologic sections, ELISA, and fluorescent studies was located in the basement membrane. It was not surprising that subepithelial humps appeared in the GBM since the clones which produced them were originally developed from anti-SCM B cells. Subepithelial humps are an indication of glomerular damage due to a previous streptococcal infection (Lange and Nayyar, 1987; Nayyar et al., 1985) . The lungs of clone IIF4 baring mice showed uneven thickening of the basement membrane. Whether the altered make up of these basement membranes is due to antigen-antibody complexes or the addition of new collagen matrix is unclear at this time. Regardless, these data indicate that the observed gross symptoms are due to damage of specific organs in the same sites identified in Goodpasture's syndrome.

When Goodpasture's patents show organ pathology in both kidney and lung it is important to determine the nature of the autoimmune response being directed at these elected organs. ELISA data indicates that the immune response is specific and directed at epitopes shared not only between different mammalian organs but also with SCM. We have shown that it is possible to isolate the common antigen of these membranes by binding the cross-reactive mAb to an affinity column and passing the trypsin digests of the membrane over the column. With the common epitopes isolated we may begin to investigate the characteristics and composition of these shared epitopes.

Although both lung and kidney contain type IV collagen the basement membrane's gas exchange in the lungs has necessitated a more diverse arrangement of basement membranes. The wall between two alveoli contains alveolar cells, capillaries and their basement membranes. Each wall has a thin side and a thick side. The thin side is made up of the alveolar epithelium, a basement membrane and the capillary endothelium. The thin side morphology allows gas exchange to occur. The thick side contains both an alveolar basement membrane (ABM) and a capillary basement membrane (CBM) which separates the epithelium from the endothelium. The (ABM) is dense and amorphous, containing 3 to 5 nm long filaments which run perpendicular from the ABM lamina densa to the cell plasma membrane of the epithelial and endothelial cells. The CBM is fibrillar and less compact than the ABM, containing only 1/5 the number of anionic binding sites. The ABM and CBM are in turn separated by interstitial type IV collagen and elastin. It is thought (Huang, 1978) that the thin side is in fact a

unit membrane composed of both ABM and CBM in discreet but tightly packed layers. The position of interstitial collagen with respect to the capillaries in the lung may be significant. The occurrence of pulmonary hemorrhages in Goodpasture's syndrome has been linked to altered permeability of the lung capillaries with respect to immunoglobulins. Donaghy and Rees (1983), attribute this increased permeability to IgG, and the subsequent exposure to normally sequestered antigens to inhalation of cigarette smoke. Altered anti-GBM antibodies, on the other hand, is not a viable explanation for pulmonary hemorrhage in Goodpasture's syndrome (Lockwood et al., 1976; Rees et al., 1977).

During our quest to find cross-reactive monoclonal antibodies we noted some interesting observations. The generation of anti-SCM hybrids which yielded cross-reactive mAb able to react in vitro with GBM antigens was an early accomplishment. When various hybrids were placed in animals to obtain high concentrations of mAb, it was noted that some animals developed severe tissue pathology. In following up that observation we have developed an animal model which mimics Goodpasture's syndrome. Proliferation of anti-SCM antibody producing clones in the peritoneum of mice has resulted in glomerulonephritis and pulmonary hemorrhages. These resultant pathologies would seem to indicate that a previous streptococcal infection could give rise to Goodpasture's events. But in general terms we have a series of mAb able to induce autoimmune mechanisms in the host. Such animal models will be useful in many different studies.

SUMMARY

Initial research was directed toward understanding the autoimmune mechanism of post-streptococcal glomerulonephritis. Hybridoma clones were prepared via the fusion of non-secreting P-X63-Ag8 plasmacytoma cells and splenocytes derived from Balb/c mice which were sensitized with streptococcal cell membrane (SCM). Clones were selected, for simultaneous anti-SCM and anti-glomerular basement membrane (GBM) activity by ELISA using plates sensitized with solubilized membrane of trypsin digested GBM or digested SCM. Pertinent clones were subsequently placed in mice for greater mAb content. At the time of ascites recovery it was noted that some animals with select clones developed severe lung lesions. These animals seemed to have a pathological condition similar to that of Goodpasture's syndrome an autoimmune disorder characterized by anti-GBM antibody induced glomerulonephritis and lung hemorrhage. The cause and predisposing factors of Goodpasture's remain unknown. Group A streptococcal infections precede a number of tissue debilitating immunologic pathologies i.e. rheumatic fever and glomerulonephritis . The primary organs for these sequelae diseases are the kidney and heart and autoantibodies appear to be the major cause of subsequent tissue damage. Goodpasture's syndrome, though never directly linked with streptococcal infections, mediates its pathology through kidney reactive anti-GBM antibodies. Animals exposed to anti-SCM mAb presented with gross pathologies which mimicked Goodpasture's disease. Organ damage included hemorrhagic lungs confirmed by increased lung/body weight ratios and kidneys with observable petechia. Fluorescent and electron

microscopy confirmed localization of mAb at these tissue sites. Severity of damage appears to be related to the specific anti-SCM clone, the dose of antibody administered, and the sex of the animal. Specificity of the anti-SCM clones was confirmed by reciprocal inhibition. The anti-SCM mAb was generated by clones demonstrated by ELISA to have simultaneous reactivity to trypsin digested SCM, GBM, and lung basement membrane (LBM). The induction of a Goodpasture's syndrome by anti-SCM mAb has provided: 1) a possible animal model for this disease; and 2) a novel concept on its induction. This concept being based on cross-reactivity i.e. an anti-SCM antibody reacting with host tissue antigen inducing an autoimmune disease.

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The dissertation is therfore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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