AN INVESTIGATION OF THE ANTIGENIC CHARACTERISTICS OF THE RENAL GLOMERULUS IN THE RAT

by

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To Paddy and Cameron, who bring
so much joy to my life.

Abstract

The finding of a granular deposition of immunoglobulin in the kidney in experimental animal models of glomerulonephritis has been been interpreted as resulting from the random deposition of immune complexes in the glomeruli. Recent data suggests that although immune complex deposition may be an important factor in some forms of glomerulonephritis, the in situ formation of immune complexes between circulating anti-kidney antibodies and fixed glomerular capillary wall antigens may also be a significant factor in the pathogenesis of some animal models of glomerulonephritis.

To examine the characteristics of discontinuously represented glomerular capillary wall antigens in the rat, monoclonal antibodies were generated against a glomerular plasma membrane fraction, depleted of glomerular basement membrane, prepared from isolated Lewis rat glomeruli. A total of 17 hybridomas, generated from the fusion of splenocytes obtained from mice immunised with the glomerular membrane fraction produced monoclonal antibodies which reacted with discontinuously represented antigens in the glomerulus and renal tubules. One further hybridoma secreted a monoclonal antibody which reacted with an antigen present on glomerular and tubular nuclear membranes. No hybridomas were produced which secreted a monoclonal antibody which reacted with linearly arrayed glomerular basement membrane antigens.

Two of these monoclonal antibodies, both of the IgM subclass and code-named PH7 and SC5, produced a heavy granular glomerular staining pattern when examined by indirect immunofluorescence microscopy. Neither monoclonal antibody was kidney specific, with reactivity being demonstrated with a number of non-renal tissues.

When administered intravenously to normal Lewis rats both SC5 and PH7 induced a mild proteinuric lesion. The proteinuria was not associated with histopathological changes at the light or electron microscope level.

Immunoblotting experiments revealed that SC5 reacted predominantly with a protein band of 96 kDa present in detergent extracts of isolated glomeruli and glomerular plasma membranes. PH7 was shown to react with three low molecular weight proteins of 14, 13 and 11 kDa.

The findings of this study demonstrate the potential for a nephritogenic response to occur following the in situ formation of immune complexes between circulating anti-kidney antibodies and discontinuously arrayed non-glomerular basement membrane glomerular capillary wall antigens, characterised by granular immunofluorescence patterns, in animal models of glomerulonephritis.

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Chapter one.

INTRODUCTION TO THE IMMUNE SYSTEM AND IMMUNOLOGICALLY-MEDIATED RENAL DISEASE.

1.1 Aim of project.

The project described in this PhD dissertation, which was initiated in 1984, and therefore in concept predates much recent published literature, constitutes a directed effort to determine the role of fixed glomerular antigens in experimental glomerulonephritis (GN) in the rat using monoclonal antibody technology. The specific aim was to generate hybridomas which would secrete monoclonal antibodies reactive with rat glomerular capillary wall (GCW) antigens that were distinct from those residing in the glomerular basement membrane (GBM). We were particularly interested in generating monoclonal antibodies which would react with antigens represented in a discontinuous array along the GCW and which would demonstrate a granular pattern of binding when examined by IF microscopy. Moreover, if monoclonal antibodies could be generated with the desired binding characteristics, then they would be used in vitro to determine the biochemical identity and tissue distribution of their target antigen, and in vivo to determine their nephritogenicity.

1.2 The immune system.

As multicellular organisms evolved, a need arose for a defence system that would protect against invasive foreign organisms. The basic requirements for such a system were the ability to recognise and distinguish self from non-self and to selectively inactivate or destroy foreign cells or organisms. As cells in these multicellular organisms differentiated, they acquired cell surface molecules that were specific and unique to their own cell type. It is likely that these cell specific molecules provided the first means of cell-cell recognition necessary for the organisation of cells and the division of labour within the organism. These cell specific surface molecules may also be involved in early growth regulation by contact inhibition.

The earliest multicellular organisms evolved a defence system that was based on the phagocyte. These cells were able to recognise and differentiate self from non-self through cell surface molecules, and to selectively phagocytose non-self. In contrast to the invertebrate phagocytic system, vertebrates evolved an adaptive immune system that is based on the lymphocyte, and is much more sophisticated than that of the invertebrate. Although centered on the lymphocyte, two humoral systems are pivotal to the operation of the vertebrate immune response, these are the complement system and the immunoglobulins.

The immune system of vertebrates is highly structured and subject to a network of integrated control which is as yet not fully understood. Present knowledge indicates that the mechanism of control involves a complex interaction between distinct subsets of lymphocytes, secreted products of lymphocytes, such as immunoglobulins, lymphokines and monokines, complement proteins and antigen.

Central to the harmonious functioning of the immune system is the ability to recognise and to selectively respond to specific regulatory self or autologous antigens. The immune system must maintain a state of self-tolerance. The failure of the host to mount a detectable pathological immune response to general autologous antigens is one of the cornerstones of immunology. Self-tolerance is accomplished by a number of mechanisms that affect B cell responses, antigen presentation by macrophages or dendritic cells, and the activity of T cell subsets (Scott, 1985).

1.3 Autoimmunity.

Autoimmunity, a state that develops when the control mechanisms mentioned above are lost, has been defined as a state in which the unresponsiveness, or tolerance, to autologous antigens terminates, resulting in the production of antibodies or T cells which react with autologous antigen (Theofilopolous and Dixon, 1982). The possibility of an autoimmune response precipitating an immunologically-induced insult to the host was recognised at the turn of the century by Ehrlich (1900) who applied the term 'horror autoxicus' to this potentially catastrophic event. Not all autoimmune responses are harmful. The control of the immune response depends on the ability of the cells of the immune system to recognise and respond to cell surface antigen encoded by the major histocompatibility complex, and therefore, by definition, is an autoimmune response. Autoantibodies are found in normal healthy individuals; as many as 10-

30% of B lymphocytes may be producing autoantibody (Cohen and Cooke, 1986). The presence of autoantibodies could be important in the suppression of the immune response to autologous antigen. This can be illustrated by considering the mechanism underlying the suppression of anti-Rhesus (Rh) factor antibody production in Rh negative mothers who have given birth to Rh positive children. Anti-Rh antibody production is suppressed by administration of preformed anti-Rh antibodies. Cohen and Cooke (1986) hypothesise that natural autoantibodies may in fact play an important role in 'blinding' the immune system by binding to autologous antigens without activating either the complement system or cellular immune responses. Natural autoantibodies might therefore act as a buffer to ensure that an immune response is not evoked against these autologous antigens. A complicating feature is that more than half of the autoantibodies found in normal individuals react with natural antibodies present. This anti-idiotypic interaction could inhibit the binding of autoantibody to its autologous antigen (Holmberg and Coutinho, 1985). Although autoantibodies may, under some conditions, fulfil an important regulatory role, the development of autoantibodies which are not regulated or suppressed, has the potential to lead to a pathological state.

1.4 Autoimmune disease.

An autoimmune pathogenesis has been established for a number of diseases hitherto designated idiopathic, including myasthenia gravis, Grave's disease, Hashimoto's disease, juvenile onset insulin-dependent diabetes mellitus, and Goodpasture's syndrome. In all these conditions, circulating autoantibodies can be demonstrated in the serum of affected persons (Theofilopolous and Dixon, 1982). Goodpasture's syndrome is a condition occurring in humans and characterised by pulmonary haemorrhage and severe progressive glomerulonephritis. In some cases, autoantibodies present in the serum of affected people react with antigens present in both the alveolar and glomerular basement membranes, and in these persons can be considered an anti-GBM antibody-initiated disease. However, there are other pathogenic mechanisms that can produce Goodpasture's syndrome, for example immune complex disease.

1.5 Imunnolgically-mediated renal disease.

End stage renal disease is the fourth leading cause of death amongst young adults in

the United States of America. Sixty per cent of these cases are the result of immunologically-mediated GN. Of this sixty per cent, however, anti-GBM antibody antibody-mediated Goodpasture's syndrome is responsible for only approximately five per cent (Adler and Couser, 1985), the underlying immunopathogenesis of the remaining 55 per cent being uncertain, although the deposition of circulating immune-complexes is clearly of some importance in many.

Much of our current understanding of the pathogenesis of imunologically-mediated GN has been gleaned from the study of experimental animal models.

1.5.1 Nephrotoxic serum nephritis (NTN).

An immunological pathogenesis of GN was first demonstrated by Lindemann in 1900 who induced a proteinuric renal lesion in rabbits with a heterologous (guinea pig) anti-rabbit kidney serum. This first experimental animal model of GN was termed nephrotoxic serum nephritis (NTN) and the results of similar experiments were subsequently published on the induction of NTN in species other than the rabbit. Masugi, using a rat model of NTN, did much to enhance the understanding of the lesion, and was the first to suggest a pathophysiological correlation between the experimental nephritic lesion and human GN (Masugi, 1934).

It was postulated that the pathological mechanism of NTN involved a reaction between anti-kidney antibodies present in the nephrotoxic serum and antigens in the glomeruli, and that it was this reaction which directly induced glomerular injury. Whilst this reaction is indeed important in the pathogenesis of the lesion, it did not explain the latent period of two to six days before the development of functional nephritis, nor did it explain the accelerated onset of nephritis observed in animals exposed to a second dose of nephrotoxic serum. This was addressed by Kay (1940a, 1940b) who, in an elegant series of experiments, demonstrated that rabbits receiving duck anti-rabbit kidney serum developed a nephritic lesion in response to the nephrotoxic serum, and that the development of the lesion could be inhibited by prior exposure of the rabbits to a sublethal dose of total body irradiation. Kay also noted the positive correlation between the onset of nephritis and the appearance of autologous anti-duck antibodies in the non-irradiated animals, a result attributed to the rabbits mounting an immune response to the 'foreign' duck proteins. From these results, Kay concluded that the nephritic lesion in rabbits was the result of a secondary

autologous immune response directed against immune complexes formed in situ between the heterologous duck anti-rabbit kidney antibody and rabbit glomerular antigens. The site of binding of the nephrotoxic antibody and the site of glomerular injury were determined to be the GBM (Krakower and Greenspon, 1951). Immunofluorescence (IF) microscopy revealed a uniform linear distribution of antibody along the glomerular loops, and electron microscopy demonstrated nephrotoxic antibody reactivity within the endothelial aspect of the GBM, as evidenced by the presence of electron dense deposits at this site in treated animals (Unanue and Dixon 1967). These early studies indicated that only rarely were deposits observed in the epithelial side of the GBM, and these were always associated with severe disease (Movat et al., 1961).

The heterologous phase of NTN is associated with polymorphonuclear leucocyte (PMN) accumulation in the glomerular capillary loops, a frequent finding being PMN infiltration of capillary fenestrations and complete displacement of the endothelial cell from the basement membrane (Cochrane et al., 1965). Depletion of PMNs with either mechlorethamine in rabbits, or anti-rat PMN serum in rats, prevented the development of proteinuria in the heterologous phase but not the autologous phase; neither did it affect the deposition of nephrotoxic serum as demonstrated by IF microscopy. However, depletion of complement with heat-aggregated gamma globulin to attain CH_{50} levels less than 20% of controls, resulted in a decrease in both IF-detectable complement deposition and PMN accumulation in glomeruli, and a consequent decrease in urine protein excretion to normal levels. In rabbits, selective depletion of macrophages with an anti-macrophage serum has no effect on the PMN-dependent heterologous phase but produces a marked reduction in the severity of the autologous phase (Holdsworth et al., 1981).

Efforts to identify the pathogenic antigen of anti-GBM GN in humans have been undertaken by a number of investigators (Marquardt et al., 1973; Mahieu and Winaud, 1970; Mahieu, 1972; Wieslander et al., 1984a, 1984b; Wieslander and Heinegard, 1985). Experimental evidence to date indicates that the antigen is present in the non-collagenous globular domain of type IV collagen (Wieslander et al., 1984a) and is not unique to the GBM (Wieslander and Heinegard, 1985). The reactivity of sera from patients with Goodpasture's syndrome in immunoblotting experiments is with the monomeric and dimeric forms of a collagenase-extracted protein of the GBM having molecular weights corresponding to 26 and 54 kDa respectively (Kefalides, 1987;

Pusey et al., 1987).

1.5.2 Immune complex glomerulonephritis.

Experimental models of GN known as acute and chronic serum sickness nephritis, were established in rabbits and subsequently studied extensively (Masugi and Sato, 1934; Rich and Gregory, 1943; Gregory and Rich, 1946; Germuth, 1953; Dixon et al., 1958). Von Pirquet (1911) had recognised the relationship between the immune response and serum sickness in human subjects undergoing serum therapy, noting that symptoms developed concomitantly with the appearance of circulating anti-serum antibodies. Although Von Pirquet had postulated that the disease was a result of antibody combining with foreign serum proteins to form 'toxic compounds', it was not until the 1950's that these were to be identified as being immune complexes (Dixon et al., 1958; Germuth and McKinnon, 1957).

An epic study of a model of chronic serum sickness nephritis was undertaken by Dixon et al. (1961). Rabbits were injected daily with one of a series of heterologous serum proteins, bovine serum albumin (BSA), human serum albumin (HSA), bovine gamma globulin (BGG) or human gamma globulin (HGG) in doses varying from 0.5 to 200 mg per day. The study established a relationship between circulating immune complexes and disease, concluding that interaction between the heterologous serum antigens, present in slight excess, and the host's antibody resulted in GN and the disease was not the result of a direct toxic property of the injected antigen. When the kidneys of the diseased animals were examined by IF microscopy a heavy granular pattern of host (autologous) immunoglobulin deposition was observed.

Later studies of a model of acute serum sickness nephritis in rabbits revealed that neither the depletion of complement (Henson and Cochrane, 1971; Holdsworth et al., 1981) nor PMNs (Knicker and Cochrane, 1965) afforded any protection to the development of glomerulonephritis. However, depletion of macrophages produced a profound reduction in both the histological changes and the severity of the proteinuria (Holdsworth et al., 1981).

From these observations it was clear that acute serum sickness GN differed markedly in its immunopathogenesis from NTN.

1.6 Heymann nephritis.

An animal model of GN known as Heymann nephritis is the most widely studied model of GN which can be induced with anti-serum raised against renal antigens. This animal model of GN is characterised by granular deposits of immunoglobulin in the glomeruli and bears a morphological similarity to membranous GN in humans. Although initially thought to arise as a result of the deposition of immune complexes in the glomeruli, later data suggested that the in situ formation of immune complexes may play an important role in the aetiology of the disease.

In 1959 Heymann et al. reported on the production of a severe progressive proteinuric renal lesion in Sprague Dawley rats following repeated intraperitoneal injection of a blood-free kidney suspension in complete Freund's adjuvant. Extracts obtained by similar processes from muscle or lung failed to induce proteinuria whilst liver extract induced a mild proteinuria in three out of 21 rats. The renal lesion produced in this experimental animal model of GN, now known as active Heymann nephritis (HN), bears a close morphological resemblance to human membranous glomerulonephritis, a condition characterised by granular deposition of immunoglobulin and complement in the glomerular capillary loops, subepithelial electron dense deposits and thickening of the GBM. Serum from HN rats was examined by Ouchterlony immunodiffusion against extracts of kidney, liver, muscle and lung. Precipitin lines were observed with kidney and liver extracts only, implying that the liver and kidney shared a common antigen (Hunter et al., 1960).

Using IF microscopy, a granular deposition of autologous gamma globulin was demonstrated in the glomeruli of rats with active HN by Okuda et al. (1965), supporting the original observations by Heymann et al. (1959). This granular pattern of immunoglobulin deposition in HN was strikingly dissimilar to that observed in NTN, a finding which suggested that different antigens may be involved in the pathogenesis of the two models. Both Heymann et al. and Okuda et al. considered the autologous antibody found in the diseased kidneys was the result of a direct reaction of these antibodies with a fixed kidney antigen, rather than a component of deposited circulating immune complexes. This hypothesis was later to be substantially supported nearly twenty years later by the discovery of gp 330 as the probable pathogenic antigen of HN (Kerjaschki and Farquhar, 1982, 1983).

Edgington et al. (1967a), however, considered the possibility that this form of experimental GN might be a variation of serum sickness nephritis, in which the autologous antibody response to the immunising foreign protein results in the deposition of circulating immune complexes in the glomeruli. If indeed this mechanism prevailed and autologous antigen was present in immune complexes, then these researchers considered the disease could be a true 'autologous immune complex disease'. To examine this question, rats were immunised with either rat or human tubular antigens. The kidneys were then examined to determine the constituents of the immune complexes by indirect IF microscopy, using antisera rendered specific by a series of immunoabsorptions. From their results, Edgington et al. concluded the disease was mediated by circulating immune complexes and that the antigenic component of the immune complexes was of autologous origin. They also stated ... 'the host produces antibody capable of reacting with an autologous antigen unrelated anatomically or immunologically to the site of injury'.

In the original work, Heymann et al. (1959) had used an homogenate of rat kidney as the immunogen. The nephritogenic component was later localised to a particulate preparation of a tubule-rich kidney fraction (Glassock and Watson, 1966) which was later named fraction 1A (Fx1A) (Edgington et al., 1967a, 1967b). An extensive and rigorous series of experiments were carried out by Edgington et al. (1967a, 1967b, 1968) to isolate and characterise the pathogenic antigen of HN. Protein solubilised from Fx1A by 1 per cent deoxycholate was analysed by electrophoresis, gel and ion exchange chromatography, analytical ultracentrifugation and immunoelectrophoresis. Immunoelectrophoresis, utilising a rabbit anti-rat Fx1A serum, identified a single precipitation line as a lipoprotein having a sedimentation coefficient of 28.6 S and a Stokes radius of 100 Å. The fraction containing this lipoprotein was termed RTEα5, and the lipoprotein itself was thought to be an integral plasma membrane protein of the proximal tubular cell brush border. The nephritogenicity of RTEα5 was confirmed in vivo by the induction of a proteinuric glomerular lesion following two injections of 3 μg of purified RTEα5 (Edgington et al., 1968). The anti-rat Fx1A and the anti-RTEα5 sera used in the above experiments reacted with normal rat proximal tubule structures but were not considered to react with glomeruli. In subsequent studies using the same classical reagents originally prepared by Edgington, reactivity with glomeruli was demonstrated by several methods including indirect IF microscopy (Neale et al., 1982).

An initial report by Alousi et al. (1967), and later a paper by Barabas and Lannigan (1968), had established a morphological similarity between HN in rats and idiopathic membranous glomerulonephritis in man. These reports were followed by histological studies at the light and electron microscopic level which revealed an increase in mesangial and epithelial cell size, an increase in osmiophilic membrane-limited droplets, fusion of the epithelial cell foot processes and large non-uniform electron dense deposits at a subepithelial site. Complement and immunoglobulin were detected by IF microscopy. PMN and macrophage recruitment in to the glomeruli was not increased, and fibrin deposits were not observed (Alousi et al., 1969).

In 1973 a paper was published which documented the first successful transfer of HN by serum from Heymann nephritic rats to normal rats (Sugisaki et al., 1973). Although Hess et al. (1962) had been unable to transfer the disease with serum from HN rats, Sugisaki et al. used serum from rats which had been immunised with kidney suspension in Freund's adjuvant which had been supplemented with pertussis vaccine. The inclusion of pertussis vaccine in the immunising suspension had previously been reported to induce a particularly severe disease (Klassen et al. 1971). In addition to whole serum, the authors also administered serum which had been fractionated by ultracentrifugation and column chromatography to yield preparations containing proteins with sedimentation coefficients of 7S and 19S. Granular deposits of immunoglobulin typical of HN were detected by IF microscopy in the kidneys of the rats receiving the 7S fraction. Electron dense deposits were also evident on electronmicrographs of glomerular sections from these rats. The 19S fraction was much less efficient at transferring the disease; no electron dense deposits were detected in the glomeruli of 19S treated rats and only faint granular immunoglobulin deposits were detected by IF microscopy. The 7S fraction of the serum from HN rats contained IgG. As IgM has a sedimentation coefficient of 19S and the anti-rat immunoglobulin serum used in the IF studies was not subclass specific, the immunoglobulin deposits in the 19S treated group could be accounted for by the presence of IgM anti-kidney antibody in the 19S fraction. However, it is possible that this fraction could contain immune complexes. The salient observation of this paper was that the study suggested that the HN lesion was not transferred by immune complexes, a possibility that could not be ruled out by the parabiotic studies of Heymann et al. (1962), but did not exclude the possibility of the formation of immune complexes containing autologous antigen and the administered anti-Fx1A serum.

A year following Sugisaki's paper, Barabas and Lannigan (1974), and later Feenstra et al. (1975), published studies on the transfer of HN with serum. The morphology of the lesion produced by these passive transfer experiments was identical to that observed in rats immunised with kidney suspensions or Fx1A. The disease produced by immunisation is now known as active HN and that induced by anti-kidney serum as passive HN.

In another paper which examined the early phase of passive HN, Barabas and Lannigan (1975) postulated that the heterologous antisera released a nephritogenic antigen from the convoluted proximal tubule. Autoantibodies were then formed against this 'released' antigen which in turn formed immune complexes that were subsequently deposited in the glomeruli.

A major contribution to the study of passive HN was made by the paper of Van Damme et al. (1978) which led to a questioning of the credibility of the circulating immune complex hypothesis of the immunopathogenesis of HN. Van Damme prepared a rabbit anti-rat Fx1A antibody and perfused rat kidneys ex vivo, (i.e. native circulation reinstated following perfusion of the kidney with anti-Fx1A antibody via the renal artery), and in vitro. A granular pattern of rabbit immunoglobulin deposition was detected by IF microscopy in the glomerular capillary loops of both ex vivo and in vitro perfused kidneys. In addition peroxidase immunoelectron microscopy revealed reaction product at a subepithelial site in kidneys perfused by either method. The site of reaction product was identical to the electron dense deposits observed in active HN. Perfusion of performed immune complexes (40 mg Fx1A with 5 or 10 mgs anti-Fx1A) resulted in foci of rabbit immunoglobulin in an occasional glomerulus. These foci were limited to the glomerular mesangium. Binding of the anti-Fx1A serum was abolished by prior absorption with Fx1A. It was significant that these authors demonstrated by IF microscopy that heterologous anti-rat Fx1A serum reacted with normal glomeruli in addition to proximal tubule. This introduced the hypothesis that the heterologous immunoglobulin found in passive HN kidney was the result of an immune complex formed in situ between anti-Fx1A antibody and a fixed glomerular antigen.

Further credence to the fixed glomerular antigen hypothesis was given by studies of heterologous anti-Fx1A serum within isolated perfused kidneys (Couser et al., 1978).

Perfusion experiments were conducted at controlled temperature, perfusion pressure, flow rate and pH, and, to avoid the possibility of tubular antigen material entering the perfusion medium, a single-pass system was employed. All the anti-Fx1A perfused kidneys showed diffuse finely granular glomerular deposits of immunoglobulin and electron dense deposits localised to the subepithelial space. Control kidneys perfused with non-immune rabbit serum did not exhibit any of these findings.

Recovery of autologous anti-Fx1A antibody by acid elution from the kidneys of rats with active Heymann nephritis was reported by Neale and Wilson (1982). The antibody eluted from the diseased kidneys reacted with normal kidney tubule brush border and, in a discrete granular pattern, with glomerular capillary walls. The eluted antibody was cross-reactive with rabbit and human tubular brush border but not with the glomeruli of either species. Neuraminidase pretreatment of sections studied by IF microscopy enhanced both tubular and glomerular staining, confirming an observation previously reported by Van Damme et al. (1978). If sections were pretreated with either anti-Fx1A or anti-RTEα5 serum, both tubular and glomerular reactivity of the eluted antibody was blocked. Glomerular reactivity of the eluates was abolished by absorption with isolated glomeruli, although a weak tubular reactivity persisted. Using a paired radiolabel technique, Neale and Wilson (1982) demonstrated specific binding of eluted antibody and of a globulin fraction prepared from the pooled sera of rats with active HN. The specific uptake of the eluates, expressed as μg/g kidney, was considerably greater than that for serum. Uptake of eluate by isolated glomeruli was 0.22 per cent of the total eluate offered at incubation; this compared with a 0.13 per cent specific uptake of eluate offered in vivo. In a separate study, eluates from Heymann nephritic kidneys were perfused through an isolated kidney preparation. Specific glomerular binding of eluate in this system ranged from 9.4 per cent to 32.3 per cent of total perfused protein (Neale et al., 1982).

The above studies unequivocally established that anti-Fx1A sera and derivatives contained antibodies that were specific for an antigenic determinant present as a structural component of the glomerular capillary wall. The granular pattern of immunoglobulin deposition observed by IF microscopy and the discrete, discontinuous distribution of electron dense deposits in electron micrographs, indicated that the antigen probably was uniformly but not linearly distributed throughout the glomerular capillary wall.

1.6.1 Identification of the Heymann nephritogenic antigen.

A major contribution was made in 1982 when Kerjaschki and Farquhar identified a glycoprotein of 330 kDa, present in both the glomerulus and proximal tubule brush border, as the possible nephritogenic Heymann antigen. These authors iodinated membrane proteins that had been extracted by detergent solubilisation of isolated proximal tubule brush border membranes. Serum from rats with active HN precipitated a number of small proteins from the protein extract; however, immunoglobulin eluted from the glomeruli of HN kidneys precipitated a single 330 kDa glycoprotein (gp 330). Rats immunised with purified gp 330 developed anti-brush border antibodies and a lesion functionally and morphologically identical to active HN. Rats immunised with brush borders that had been depleted of gp 330 developed anti-brush border antibodies, but glomerular deposits of immunoglobulin could not be detected. In a follow-up study, Kerjaschki and Farquhar (1983), using immunocytochemical staining, were able to localise gp 330 to glomerular epithelial cells and proximal tubule brush border. Although small amounts of gp 330 were associated with intracellular structures including Golgi bodies, rough endoplasmic reticulum and multivesicular bodies, the highest concentration of gp 330 was associated with clathrin-coated invaginations of the epithelial cell membrane. The presence of gp 330 in glomeruli was confirmed by the ability of anti-gp 330 antibodies to immunoprecipitate a glycoprotein of similar molecular weight from a Triton X-100 extract of radiolabelled glomeruli.

In another study, three out of seven rats immunised with a 600 kDa glycoprotein (gp 600) isolated from a sodium deoxycholate extract of Lewis rat Fx1A by lectin chromatography developed a proteinuric renal lesion (Makker and Singh, 1984). All seven rats developed granular deposits of immunoglobulin and complement typical of HN along the glomerular capillary loops. By IF microscopy a rabbit anti-gp 600 serum stained the brush border of the proximal tubules but did not stain the glomeruli, however, rats given this anti-gp 600 serum intravenously developed granular deposits of rabbit immunoglobulin along the glomerular capillary loops. Faint staining for complement was also observed at the same site. When subjected to polyacrylamide gel electrophoresis (PAGE) under reducing conditions, gp 600 was resolved into five bands corresponding to a molecular weight range of 70 kDa to 330 kDa. These authors concluded that the gp 330 antigen described by Kerjaschki and

Farquhar (1982, 1983) was in fact a subunit of the larger gp 600 molecule.

Purified rabbit anti-gp 600 serum was shown to form immune complexes in vitro with normal rat serum. These complexes had a mean molecular weight of 1.1 x 10^6. In immunoblotting experiments normal rat serum was electrophoresed under non-reducing conditions, transferred to nitrocellulose and then probed with anti-gp 600. The results indicated that anti-gp 600 reacted with four protein bands in the molecular weight range of 66 kDa to 80 kDa. When the same antiserum was employed in a radioimmunoassay, additional antigens of 110 kDa and 150 kDa were identified from serum (Singh and Makker, 1985). A competitive radioimmunoassay for gp 600 in solubilised membrane fractions of non-renal tissue indicated the antigen was present in a number of tissues, particularly those tissues having a secretory or absorptive function. Specific binding, however, was greatest in lung and pancreas. In immunoprecipitation experiments anti-gp 600 precipitated proteins ranging from 66 kDa to 330 kDa, including a 90 kDa protein common to all tissues (Singh and Makker, 1985).

Another putative Heymann antigen has been described by Natori et al. (1986). A heterologous antiserum raised against a glycoprotein isolated from a Triton X-100 solubilised Fx1A preparation induced a severe proteinuria within two days, whereas immunisation of rats with the glycoprotein failed to induce any lesion. This glycoprotein is believed to be a dimer or trimer of homologous subunits of 108 kDa (gp 108). Using a rabbit anti-Fx1A serum, gp 108 was identified by immunoblotting as a major protein band of Fx1A, distinct from gp 330. The indirect IF studies with anti-gp 108 serum revealed staining of proximal tubule brush borders in a granular pattern typical of that observed with anti-Fx1A serum. A sandwich microELISA developed for the detection of gp 108 in extracts of various rat tissues indicated the highest reactivity within the kidney, with gp 108 constituting 0.6 per cent of the total protein in the kidney extract (Natori et al., 1987). Reactivity was also detected in a number of tissues ranging from 26 per cent of kidney activity for intestine, to 0.35 per cent for heart. Reactivity was 28 times greater in isolated lymphocytes than in whole serum.

Kawai et al., (1986) analysed a pronase digest of a tubular antigen preparation by gel filtration, DEAE chromatography with a stepwise NaCl gradient, and PAGE. Antisera were raised against the six protein peaks eluted from the DEAE column and

administered intraperitoneally to normal rats. The eluted protein peaks were also used to immunise rats in an attempt to produce active HN. A rabbit antiserum raised against the 0.05 M NaCl protein peak, which did not produce any pathological changes in vivo, stained normal rat kidney when examined by indirect IF microscopy. The pattern was reported to be a diffuse granular staining of the GBM, in addition to tubular brush border, but immunoperoxidase electron microscopy revealed the reactivity to be with epithelial cell membranes and the foot processes. A second antiserum raised against the 0.3M NaCl protein peak which produced a Heymann type nephritis in vivo reacted strongly in vitro with proximal tubules but not with glomeruli when administered intraperitoneally to normal rats. Both antisera produced granular glomerular deposits of immunoglobulin, although the temporal sequence of deposit formation differed for the two antisera. Examination of the PAGE profiles of these two protein peaks indicated single protein bands corresponding to molecular sizes of 90 kDa for the 0.3M peak protein and greater than 200 kD for the 0.05M peak protein.

The importance of a 90 kDa protein to glomerular reactivity of anti-Fx1A serum has been highlighted in experiments undertaken in Hoedemaeker's laboratory in The Netherlands. Anti-Fx1A serum has been shown to contain antibodies with anti-T cell specificity (Bakker et al. 1979, 1981). Absorption of anti-Fx1A serum with rat heart tissue selectively removes anti-T cell activity but does not affect the anti-brush border titre of the antiserum. Immunofluorescence and immunoperoxidase electron microscopy demonstrated that the glomerular reactivity of the anti-Fx1A serum was abolished by the absorption procedure. Immunoblotting experiments indicated that anti-Fx1A serum reacted with two major protein bands of 330 kDa and 90 kDa and several minor ones. Following the absorption procedure, reactivity with the 90 kDa protein band was selectively removed.

All the studies which have attempted to identify the pathogenic Heymann antigen discussed to date have been conducted with polyclonal antisera. There are however problems with specificity and inconsistency of affinity and avidity associated with polyclonal antisera which necessitate a cautious approach to the interpretation of data obtained with these reagents. The development of hybridoma technology enabling the production of monoclonal antibodies has done much to alleviate some, but not all, of these problems associated with polyclonal antisera.

Chapter Two.

INTRODUCTION TO MONOCLONAL ANTIBODY TECHNOLOGY.

2.1 Historical aspects.

In the application of immunological techniques to biological research, cross-reactivity and non-specific binding may be encountered, especially when using conventional polyclonal antisera. A single purified antigen may have a number of antigenic sites or epitopes, each of which may stimulate the production of individual specific antibodies and which together contribute a polyclonal response.

The initial conception of monoclonal antibodies occurred when Sir Frank Macfarlane Burnet proposed the idea of monoclonality with the publication of his theory of clonal selection on the generation of antibody diversity (Burnet, 1957). Burnet stated that each immunoglobulin of a given specificity is the product of a single line, or clone, of a B lymphocyte or its terminally differentiated progeny, the plasma cell. This one cell-one antibody hypothesis was substantiated by Nossal and Lederberg (1958) who showed that the product of a single antibody secreting cell taken from a rat immunised with two different strains of bacteria, could inhibit the activity of one, but not both, of the immunising bacterial strains.

The first report of the production of a monoclonal antibody with a predetermined antigen specificity came from Kohler and Milstein working in Cambridge, England, who produced a monoclonal mouse IgM molecule with specificity for a sheep red blood cell antigen (Kohler and Milstein, 1975). The significance of this development was that virtually unlimited amounts of homogeneous antibody of a single specificity could be produced, thereby solving two of the potential major problems of polyclonal antisera, namely, specificity and batch to batch reproducibility.

Milstein's laboratory was studying how somatic cells diversify in culture, and, using myeloma cells, was examining the rate of mutation and how such mutations would modify the specificity of the secreted antibody. They were working with a tissue culture-adapted, mineral oil-induced plasmacytoma (MOPC) cell line named MOPC-

21. This myeloma cell line, derived from BALB/cJ mice by Potter and Boyce (1962), secreted an antibody of unknown specificity. It was their lack of success in identifying an antigen to which the MOPC-21 antibody would bind, together with myeloma cell hybridisation experiments, that led to the development of monoclonal antibody secreting hybridomas. Milstein was later to write '.... a lucky circumstance led us to the hybrid-myeloma technique' (Milstein, 1980).

The advent of monoclonal antibodies was made possible by a series of prior scientific discoveries in seemingly unrelated fields of research. A British doctor, Henry Bence Jones was the first to describe proteins excreted in the urine of patients suffering from the disease now known as multiple myeloma. A general practitioner, Dr. Thomas Watson, had written to Jones describing some unusual properties of urine samples from one of his patients. Watson sent Jones the urine samples and Jones conducted, and later published, a series of chemical analyses (Jones, 1847, 1848). It was, however, to be more than a century later before these proteins, which now bear his name, were to be identified as the kappa and lambda light chains of immunoglobulin molecules (Korngold and Lipari, 1956; Edelman and Gally, 1962). The patient, from whom the urine samples which Watson had sent to Jones were obtained, was suffering from the disease now known as multiple myeloma. This disease results from the malignant transformation of a single antibody-secreting plasma cell. The malignant cell type is known as a plasmacytoma. As the malignant cells arose from a single cell, the progeny were therefore a clone, all cells secreting antibody molecules of the same class and specificity, i.e. a monoclonal antibody. The diagnosis of multiple myeloma requires the demonstration of plasmacytomas in the bone marrow, characteristic lytic bone lesions and monoclonal proteins in the serum or urine (Wells and Ries, 1980). The study of these Bence Jones proteins has advanced our understanding of antibody structure and synthesis considerably (Potter, 1972). The induction of plasma-cell neoplasms (i.e. plasmacytomas) in BALB/cJ mice by intraperitoneal injection of the mineral oils 'Primol D' and 'Bayol F' (Potter and Boyce, 1962) was exploited for scientific study by the work of Horibata and Harris (1970) who were able to develop a plasmacytoma cell line adapted to continuous growth in culture. This cell line continued to synthesize and secrete immunoglobulin. A number of these tissue culture-adapted cell lines have been established and are now more commonly referred to as myeloma cells.

Cell fusion catalysed by inactivated Sendai virus was reported by Okada (1958).

Three years later Barski et al. (1961) demonstrated the occurrence of spontaneous fusion between cultured mouse cells. This latter phenomenon was later utilised by Gershon and Sachs (1963) to produce hybrid cells using parent mouse cell lines which differed in their histocompatibility antigen repertoire. Although unknown at the time, a major step in the progression to monoclonal antibody production came with the demonstration that specific hybrid cells could be selected from cell fusions between two mouse fibroblast cell lines. Each of the parent cell lines were deficient in either thymidine kinase or hypoxanthine guanine phosphoribosyl transferase (HGPRT). As a consequence they were resistant to growth in 5-bromo-2-deoxyuridine or 8-azaguanine, respectively, and would therefore not survive if grown in medium containing the folic acid antagonist aminopterin. Cells which had undergone spontaneous fusion contained and expressed the genome for both enzymes, and therefore survived the presence of aminopterin in medium supplemented with hypoxanthine and thymidine (HAT medium, Littlefield, 1964). Subsequently, in a study to examine the basis of allelic exclusion, Cotton and Milstein (1973) constructed hybrid cells by using inactivated Sendai virus to fuse a 5-bromo-2-deoxyuridine resistant mouse myeloma cell secreting immunoglobulin heavy and light chains, with an 8-azaguanine-resistant rat tumour cell line which secreted the rat kappa light chain only. The resulting hybrid cells differed in gross morphology from both parent cells and co-dominantly expressed the antibody chains of both parental cells.

Further reports appeared in the literature documenting the production of hybrid cells of *mouse x human* parentage which secreted both murine and human immunoglobulin (Schwaber and Cohen, 1974). The fusion of two transformed human lymphocyte lines, one of which secreted IgM, the other IgG, resulted in the production of hybridomas which secreted both isotypes (Bloom and Nakamura, 1974).

These experiments set the stage for the production of hybrid cells which would secrete antibody of a predetermined specificity. In a letter to Nature published on the 7th August, 1975, Georges Kohler and Cesar Milstein (1975) reported the production of a monoclonal anti-sheep red blood cell-secreting hybridoma following the fusion of splenocytes, from a mouse immunised with sheep red blood cells, with an 8-azaguanine-resistant mouse myeloma cell line. Kohler and Milstein stated 'The manufacture of predetermined specific antibodies by means of permanent tissue culture cell lines is of general interest'. It is unlikely that they envisaged the profound effect this experiment would have on modern immunology, or the multimillion dollar

industry that would develop over the ensuing decade to service such diverse areas as medical laboratory diagnostics and horticultural research.

2.2 Myeloma Cell lines.

Currently, the basic procedure to generate a hybridoma, that secretes a monoclonal antibody directed against a selected antigen, differs little from that first employed by Kohler and Milstein, although a number of modifications have been incorporated to enhance the chances of success. In the original experiments undertaken by Kohler and Milstein (1975, 1976) both the immune donor splenocytes and myeloma cells were of mouse BALB/cJ strain origin. The majority of monoclonal antibodies produced today still employ the murine system. Rat, human and other hybridoma systems have also been developed, each possessing advantages and disadvantages when compared with the murine system. These adaptations are briefly discussed before returning to the subject of mouse monoclonal antibody production.

The rat system is reported to have a number of benefits. In a *rat x rat* fusion, over ninety per cent of the hybrids generated secrete immunoglobulin characteristic of the donor splenocytes, compared with only sixty per cent in a *mouse x mouse* fusion (Clark et al., 1983). Loss of immunoglobulin expression from mouse hybridomas has been well documented (Kohler et al., 1978; Kohler, 1980; Goding, 1980) but is considered a rare phenomenon by others (Campbell, 1984). Loss of secretion does not appear to be a problem with rat hybridomas which are reported to have marked stability against immunoglobulin chain loss (Clark et al., 1984). Another advantage of rat hybridomas is the large volume of monoclonal antibody-containing ascitic fluid which can be obtained from a single animal (Goding, 1980). However, the number of rat myeloma cell lines suitable for fusion is limited and problems have been encountered in keeping the hybrid cells alive in culture for any length of time following fusion (Goding, 1983).

The availability of tissue culture-adapted, HAT sensitive, human myeloma cell lines is also currently limited (Kozbor et al., 1986; Samoilovich et al., 1987). Lymphoblastoid cell lines have been used as a fusion partner in *human x human* fusions, as have Epstein Barr virus-transformed lymphocytes (Kozbor and Roder, 1983; Wasserman et al., 1986; Roder et al.,1986). Fusion frequency between human cells is extremely low (Westerwoudt, 1986). It is not possible to grow human hybridomas

in laboratory animals to establish peritoneal tumours, and monoclonal antibody yield in cell culture is extremely poor (less than 0.5 μg/ml), as is long term stability in culture (Kozbor and Roder, 1983). A more recent report (Albright et al., 1987) has indicated that the stability of immunoglobulin expression and production of monoclonal antibody from human and mouse hybridomas are similar. A number of *mouse x human* fusions have been undertaken with mouse myeloma cells and human peripheral blood lymphocytes (Nowinski et al., 1980; Schlom et al., 1980; Croce, et al., 1981; Lane et al., 1982) but fusion frequency is low (Westerwoudt, 1985). The long-term stability of these hybridomas is poor due to preferential loss of chromosome 2 which carries the gene for the human kappa light chain (Ostberg, 1986). Other authors, however, have reported that the stability of these hybridomas is comparable to *mouse x mouse* hybridomas (Albright, et al., 1987). Irrespective of the lineage of the immortal parent cell, difficulties have been encountered and much remains to be overcome before human monoclonal antibody production may be seen as a routine procedure.

Efforts have also been made to establish monoclonal antibodies from other species. Splenocytes of bovine, porcine, rabbit, ovine, chimpanzee and Rhesus monkey origin have been fused with mouse myeloma cells but to date results have been disappointing (Samoilovich, et al., 1987).

The murine system is certainly the best-characterised, and currently most of the monoclonal antibodies produced for research, or commercially available for diagnostic and immunohistological procedures, are mouse antibodies. This to some extent limits their use as therapeutic agents in humans due to the immune response they provoke as a result of their xenogenous nature. For example, human anti-mouse antibodies have been detected in eighty per cent of cadaveric renal transplant recipients who had undergone treatment with the mouse anti-lymphocyte T3 receptor monoclonal antibody, OKT3 (Ortho Trial, 1985).

The first fusions reported by Kohler and Milstein (1975) were between immune splenocytes and an 8-azaguanine resistant IgG1(k) secreting myeloma cell derived from MOPC-21 (P3-x63Ag8) or a similarly derived 5-bromo-2-deoxyuridine resistant IgG2a(k) secreting cell line (P1 Bu1). Under conditions where the fusion of myeloma cells with splenocytes is random, there would be a total of sixteen heavy chain-light chain combinations, therefore only 1 in 16 of the hybridomas generated

would secrete the specific antibody of donor splenocyte phenotype (Shulman et al., 1978). Clearly a HAT sensitive, non-synthesising, non-secreting myeloma cell line would be the ideal fusion partner. Using cloning and selection techniques, improved myeloma cell lines meeting some or all of these criteria were soon available (Kohler et al., 1978; Shulman et al., 1978; Kearney et al., 1978; Fazekas de St Groth and Scheidegger, 1980). All were of MOPC-21 origin.

Two variant clones of P3-x63 Ag8 have been reported. These were, NSI/1 Ag 4.1 which synthesises, but does not secrete, kappa light chain (Kohler et al.,1978), and X63-Ag8.653, a non-synthesising, non-secreting myeloma cell line (Kearney et al., 1979). These two myeloma cell lines are currently widely used for the production of monoclonal antibody secreting hybridomas. The myeloma cell line SP2/0-Ag-14 reported by Shulman et al.(1978), which neither synthesises nor secretes heavy or light chains, is also widely used. There have, however, been reports that this line has fastidious growth characteristics, is intolerant of alkaline medium and high density, has a low fusion frequency and shows hybridoma instability (Fazekas de St Groth and Scheidegger, 1980; Goding, 1983). Sendai virus activated fusion of SP2/0-Ag-14 cells resulted in the production of a rapidly proliferating, stable hybrid myeloma named FO. In initial fusion experiments this myeloma gave encouraging results (Fazekas de St Groth and Scheidegger, 1980) but it has not become widely used.

2.3 Hybridoma Methodology.

The procedure for the generation of monoclonal antibody-secreting hybridomas may be divided broadly into five major areas: immunisation, fusion, post-fusion culture, screening, and cloning.

2.3.1 Immunisation.

A number of mouse myeloma cell lines are available, either commercially, or from the laboratory which originally developed the cell line. All are of BALB/cJ origin, are 8-azaguanine resistant, and the majority do not secrete immunoglobulin. Considering the above, the most obvious choice of mouse strain for monoclonal antibody production is the BALB/cJ mouse. It is important that there is no histocompatibility barrier to hinder the growth of the resulting hybridomas as peritoneal tumours if they are grown in normal BALB/cJ mice. Different mouse strains do, however, exhibit

considerable variation in their immune response to a given antigen (Festing, 1979), and for this reason an alternative strain of mouse may have to be employed as the splenocyte donor. For example, a CBA mouse may be found to be a better responder to the immunising antigen than a BALB/cJ, and CBA splenocytes are then fused with a myeloma cell line of BALB/cJ origin. To obtain monoclonal antibody-enriched ascitic fluid, the hybridomas must be grown as peritoneal tumours in first cross (F1) *BALB/cJ x CBA* mice.

The serum titre of antibody in the immunised mouse is directly related to the number of primed and activated plasma cells secreting specific antibody, and therefore relevant to the anticipated success of hybridoma generation. Recently activated, rapidly proliferating, antibody-secreting plasmablasts, at a stage of differentiation similar to the myeloma partner, have been shown to preferentially undergo fusion with myeloma cells (Andersson and Melchers, 1978; Stahli et al., 1980; Campbell, 1984). Although a number of immunisation protocols may prove to be successful, the salient objective is to generate the largest possible number of antigen-specific plasmablasts present in the spleen at the time of fusion, thereby increasing the possible yield of specific hybridomas. Best results are reported to be achieved when a booster immunisation is given three to four days prior to fusion (Oi et al., 1978; Oi and Herzenberg, 1980; Stahli et al., 1980).

Generally, an intraperitoneal regimen will be successful for most particulate antigens, or alternatively the intramuscular or subcutaneous route may be employed (Goding, 1980). No two antigens will elicit the same quantitative immune response, and it may be necessary to explore different routes of immunisation to determine which is optimal for the chosen antigen.

Cell surface antigens are highly immunogenic when presented to the host in their native form on intact cells (Goding, 1980). An excellent immune response, with a subsequent high yield of specific hybridomas, may therefore be expected when an intraperitoneal or intravenous immunisation protocol is employed for cell surface antigens (Damjanov and Knowles, 1983; Kearney, 1984; Westerwoudt, 1985). However, small soluble antigens may not be sufficiently immunogenic in standard immunisation regimens (Stahli, 1980; Goding, 1983). To overcome the problem encountred with molecules which provoke a poor immune response, a number of approaches have been explored. These include hapten-carrier conjugates (Van Ness

et al., 1984; Wasserman et al.,1986), in vitro immunisation of B lymphocytes (Hengartner et al., 1978; Pardue et al., 1983; Reading, 1982, 1986; Boss, 1986), direct immunisation of lymph nodes or spleen (Nilsson et al., 1983; Spitz et al., 1984; Thorpe et al., 1984; Raymond and Suh, 1986), or a prolonged immunisation procedure using antigen emulsified in complete Freund's adjuvant (Stahli et al., 1980). If using hapten conjugates, however, the major immune response may be directed against the carrier. There is some evidence that immunisation protocols which include Freund's adjuvant may influence the class of antibody which is produced (Goding, 1983). Nevertheless, Freund's adjuvant is extremely effective in potentiating the immune response to the immunogen (Stahli et al., 1980; Roitt et al., 1985), and in a number of laboratories, the immunisation protocols employed routinely incorporate Freund's adjuvant. An improved fusion result also may be obtained by the selection of a mouse strain which has been identified as a high responder (Boumsell and Bernard, 1980).

The technique of in vitro immunisation of spleen cells first reported by Hengartner et al. (1978) is being increasingly utilised for both weakly immunogenic antigens and for antigens which are available only in limited amounts. Incubation of human peripheral blood lymphocytes with specific antigen and polyclonal B cell activators, is a development which has had a degree of success (Chiorazzi et al., 1982; Pardue et al., 1983; Strike et al., 1984; Van Ness et al., 1984; Wasserman et al., 1986). Successful hybridoma generation has followed the in vitro immunisation of B lymphocytes in culture with picomolar amounts of partially purified rat gonadotrophin releasing factor (Luben et al., 1982), and the maximum number of hybridomas secreting anti-calmodulin monoclonal antibody occurred when spleen cells in culture were exposed to 500 ng of calmodulin (Pardue et al., 1983). Although a useful technique, in vitro immunisation does carry an increased risk of bacterial contamination of cultures.

Intrasplenic immunisation, first reported by Nilsson et al. (1983), and later by others (Spitz et al., 1984; Thorpe et al., 1984), is a useful technique when time and/or antigen availability is a limiting factor. As little as 20 μg of protein administered as a single intrasplenic injection is sufficient to elicit an immune response within a few days, fusion of splenocytes obtained fom mice immunised by this method results in subsequent hybridoma generation and secretion of specific monoclonal antibody (Spitz et al., 1984). If a soluble protein is conjugated to an insoluble support, the amount of protein required for an adequate immune response has been reported to be in the

nanogram range (Nilsson et al., 1987).

Using an intralymph node immunisation technique, Raymond and Suh (1986) have described the successful generation of a panel of monoclonal antibodies against a purified 35 kD polypeptide. The immunisation regimen required only 32 μg of antigen.

A review of the current literature indicates that intraperitoneal immunisation, in a variety of regimens, is the preferred first choice of immunisation. Alternative methods are generally employed in specific cases of limited antigen availability or when the immune response to the antigen of choice is poor.

2.3.2 Fusion.

A number of modifications have been incorporated into the procedure described by Kohler and Milstein (1975). The use of polyethylene glycol (PEG) as a fusogen, first reported by Pontecorvo (1975) and adapted for hybridoma fusion by Gefter et al. (1977) resulted in a 100-3000 fold increase over Sendai virus in the frequency of hybridoma generation. PEG has replaced Sendai virus in virtually all cell fusion experiments and is the fusogen of choice (Goding, 1980; Wojcieszyn et al., 1983; Samoilovich et al., 1987). The mechanism by which PEG promotes cell fusion is poorly understood (Wersterwoudt, 1986), but for optimum efficiency the cells must be closely apposed (Wojcieszyn et al., 1983). Recent communications report the use of the avidin-biotin reaction to bridge splenocytes to myeloma cells. The specific antigen, labelled with either biotin or avidin, is added to a suspension of isolated splenocytes. The myeloma cells, labelled with the complementary molecule, are then added (Wojchowski and Sutkowski, 1986; Reason et al., 1987). In addition to bringing myeloma cells and splenocytes into close proximity, this technique also favours the generation of an increased percentage of antigen-specific hybridomas.

PEG induces cell agglutination and a redistribution and segregation of intramembrane particles, making available areas of lipid-rich membrane as suitable sites for fusion with an adjacent cell (Knutton, 1979; Wojcieszyn et al., 1983). It is cell-cell contact, at these areas of membrane denuded of intramembrane particles at the time when when the PEG is diluted or removed from the medium, that results in cell fusion (Knutton, 1979; Knutton and Pasternak, 1979).

The effect of pH on PEG-promoted cell fusion is not clear from the literature. White and Helenius (1980) demonstrated that the rate of spontaneous fusion of Semliki Forest virus with liposomes was pH dependent, the greatest number of fusion events being observed at a pH of less than 6.0. In PEG-induced fusion for the production of hybridomas, a number of authors do not recommend adjusting the pH of the PEG solution (Oi and Herzenberg, 1980; Goding 1980; Campbell 1984). However, other reports have shown that optimum fusion frequency occurs when the pH of the PEG solution is adjusted to 8.0 - 8.2 (Sharon et al., 1980; Westerwoudt, 1985) or 7.0 (Lane et al., 1984).

The temperature at which the fusion procedure is undertaken (Goding, 1980; Fazekas de St Groth and Scheidegger, 1980; Westerwoudt, 1985) and the duration the cell suspension is in contact with the PEG (Goding, 1983; Campbell, 1984; Lane, 1985; Westerwoudt, 1985) are also parameters for which widely differing optimal conditions for the generation of hybridomas have been reported in the literature .

The fusogenic effect of PEG can be removed by recrystallisation of commercially obtained PEG preparations. Honda et al. (1981) identified tocopherol and other phenolic compounds, commonly added as antioxidants by the manufacturers, to be the major contaminants. These authors hypothesised that PEG brought the cells present in the suspension into contact by inducing cell agglutination, and fusion of the cell membranes was brought about by the action of the contaminating fat-soluble phenol compounds. The fusogenic capacity of recrystallised PEG can be restored by the addition of several different antioxidants (Wojcieszyn et al., 1983). This observation may explain the reported differences of the fusogenic ability between PEG obtained from a variety of manufacturers (Fazekas de St Groth and Scheidegger, 1980; Goding, 1980; Lane et al., 1984).

A technique of electrofusion of cells has been described by Zimmerman (1982). Close membrane contact between two cells is achieved by the application of a non-uniform alternating current or by a series of low intensity direct current pulses. Cells exposed to this electric field assume a chain formation, and aggregation of intramembrane particles occurs. Once cell-cell contact is established at these areas now denuded of intramembrane particles, a high intensity, direct current pulse applied at this point will initiate fusion. The intensity of the electrical field is such that it triggers a reversible

breakdown of the membrane of apposed cells which results in the establishment of a cytoplasmic continuity between cells, leading ultimately to complete fusion (Zimmerman, 1982). Ohinishi et al. (1987) reported that electrofusion efficiency was increased if 0.25 M glucose was the incubation medium rather than the traditionally employed 0.3 M mannitol medium. These authors also reported that there was an absolute requirement for Ca^{2+} in the medium. The effect of Ca^{2+} was enhanced, but could not be replaced by Mg^{2+}. Pretreatment of cells with proteolytic enzymes also led to an increased fusion efficiency. A number of conditions remain to be optimised in this initially promising technique. To date it has not yet gained much following, with few reports of its application to hybridoma production appearing in the literature.

2.3.3 Post-Fusion Culture.

Once the fusion step has been completed the cell preparation is resuspended in medium and aliquoted to tissue culture plates. Some protocols call for the resuspension of the newly fused cells in normal medium with the addition of aminopterin twenty four hours later (Kennett, 1980; Oi and Herzenberg, 1980; Galfre and Milstein, 1981). There does not appear to be any evidence that delayed addition of aminopterin is beneficial to the outcome of the fusion (Goding, 1983; Campbell, 1984), and immediate suspension of the fused cells in HAT medium is the method most commonly employed (Goding, 1983). This format is advised, as the cells at this stage are extremely intolerant to even slight fluctuations in either temperature or pH, even occasional opening of the incubator door being sufficient to lead to a growth retardation of about two days (Westerwoudt, 1985). Hybridomas left undisturbed for seven to ten days show better growth than those disturbed more frequently (Fazekas de St Groth and Scheidegger, 1980).

Some authors recommend plating the cell suspension at relatively high cell numbers per well in 1 ml or 2 ml capacity wells (Galfre and Milstein, 1981); others recommend smaller cell numbers in a larger number of 200 μl wells (Oi and Herzenberg, 1980; Kennett, 1980). Lymphoid cells in general, and particularly newly fused cells, are intolerant of high dilution (Goding, 1980, 1983). This vulnerability is safeguarded against by the former method at the expense of the possibility of multiple hybridomas growing in each well. The second method increases the likelihood of only one hybridoma colony growing in each well, but has the disadvantage that only a small amount of tissue culture supernatant is available for screening (Bastin et al., 1982).

The use of 'feeder' cells has been shown to be beneficial to the growth and survival of hybridomas post-fusion. The term 'feeder' implies that these cells furnish some necessary nutrients or growth factors, although the precise mechanisms by which they exert their influence is unknown (Goding, 1983). Feeder cells may be peritoneal macrophages (Fazekas de St Groth and Scheidegger, 1980) or thymocytes (Oi and Herzenberg, 1980). Peritoneal macrophages are more commonly used, probably because they are more easily obtainable. The use of feeder cells does, however, increase the risk of bacterial contamination of cultures.

2.3.4 Screening.

The screening procedure adopted for the selection of hybridoma colonies is one that requires careful consideration in monoclonal antibody production. An ill-designed, inappropriate or insensitive assay will result in many hours of unnecessary labour and could result in failure to detect the products of desired hybridomas. Factors to be considered when selecting a screening assay are its reliability, speed, and cost of performance and the labour input required (Goding, 1983). Commonly used screening methods are microELISA, radioimmunoassay, IF microscopy and immunocytochemical assay; ultimately the method employed will most likely be dictated by the antigen against which the monoclonal antibodies are directed. Screening is performed when hybrid colonies are well established, that is, usually 14 to 28 days post-fusion (Oi and Herzenberg, 1980). At this time the concentration of monoclonal antibody in the hybridoma culture supernatants may not be high, and, if 200 μl wells are used post-fusion, the amount of supernatant for the assay will be limited. For these reasons it is advantageous for the assay to have a high degree of sensitivity. Importantly, the assay which is ultimately chosen must be established as sufficiently sensitive and reliable prior to the generation of the hybridomas. Little time will be available to correct deficiencies in the chosen assay which may become apparent during its application to the screening of the hybridomas.

2.3.5 Cloning.

When a hybridoma colony which is secreting specific monoclonal antibody is detected it is essential the cells are cloned as early as possible (Goding, 1980). Cloning may be performed in soft agar (Kohler and Milstein, 1975; Goding, 1980) or by limiting dilution (Goding, 1983). Cloning by limiting dilution is somewhat simpler than in soft

agar and currently appears to be the method preferred by most laboratories. Cloning by limiting dilution is performed in tissue culture plates containing 96 microwells of 250 μl capacity. The post-fusion cell suspension is serially diluted to a final concentration of 1.5 - 2.0 cells per ml. If cells are distributed to the microwells at this low dilution,the number of wells in which hybridoma colonies appear will follow the Poisson distribution (see Goding 1980 for review). Cloning of hybridoma cells by fluorescence-activated cell sorting (Parks et al., 1979; Goding, 1983) has not been widely applied. Early post-fusion, if a well contains both an antibody-secreting and a non-secreting hybridoma there is a high risk of the culture being dominated by the non-secretor (Campbell, 1984). For efficient cloning, wells containing 'feeder' cells should be seeded with less than 0.5 cells/well, and culture supernatant from the emerging colonies should be screened as for the primary selection. To ensure monoclonality and to prevent overgrowth of the cultures with cells which have early chromosome loss, cells should be cloned at least twice. Even with the greatest of care, as many as fifty per cent of initially positive hybridomas may be lost during the post-fusion cloning period (Goding, 1980).

2.4 Application of monoclonal antibody techniques in the current study.

As discussed in Chapter One, the rat model of human membranous nephritis, Heymann nephritis has been studied extensively. Current data suggests that there is a strong case supporting the in situ formation of immune complexes in this and other spontaneous or induced GN models. However, the in situ formation of immune complexes in GN lesions which exhibit a granular deposition of immunoglobulin in the glomeruli is not universally accepted (Couser, 1982; Neale, 1983), although it is likely that the deposition of circulating immune complexes remains quantitatively the most important mechanism of immunologic glomerular injury.

The thesis upon which this project was based, was that there may be a number of potential nephritogenic antigens present in the GCW and distinct from those in the GBM. These molecules could serve as the ligand for circulating antibodies and form immune complexes in situ. Once formed these immune complexes could initiate inflammatory injury and a subsequent glomerular lesion. As the array of potential antigens in the glomerulus is great, the generation of monoclonal antibodies, specific for a single antigen was considered a suitable technique to approach the study of these molecules.

Chapter Three

PREPARATION OF AN IMMUNOGENIC GLOMERULAR MEMBRANE FRACTION

3.1 Introduction.

To produce monoclonal antibodies directed against rat renal antigens it is necessary to present the antigen in a form which stimulates the murine immune system. In previous reports of monoclonal antibodies directed against renal antigens, the immunogen has been either whole or sonicated glomeruli (Mendrick et al., 1983; Hancock and Atkins, 1983), renal carcinoma cell lines (Ueda et al., 1981; Bander et al., 1985), cell membranes isolated from renal cortex (Falkenberg et al., 1981a, 1981b), rat Fx1A (Ronco et al., 1984b) or the glycoprotein gp 330 (Kerjaschki and Farquhar, 1983) and components of the GBM (Pusey et al., 1987). The non-GBM capillary wall antigens which were of interest in the current study were likely to be cell surface molecules present on either endothelial, epithelial or mesangial cells of the glomerulus (Andres et al., 1979; Matsuo et al., 1987). An isolated whole glomerular preparation which had been disrupted by sonication was considered as a possible immunogen since this preparation contains epithelial, endothelial and mesangial cell membranes. It would, however, also contain a large amount of intracellular material and GBM. A necessary consideration in the choice of immunogen was the type of assay to be used for screening the resulting hybridomas for specific monoclonal antibody secretion. As the pattern of binding of the monoclonal antibodies produced was an important objective, screening of the hybridoma culture supernatants by IF microscopy on cryostat sections of normal Lewis rat kidney could have been employed. Using this procedure for screening, however, would be extremely time consuming with large numbers of samples. In addition, if the immunogen was a preparation of sonicated whole glomeruli, IF microscopy on kidney sections would probably detect a large number of monoclonal antibodies with specificity for unwanted intracellular and GBM antigens. To increase the probability of generating hybridomas that secrete monoclonal antibody specific for non-linear, discontinuous, irregularly-arrayed

antigens, purification of the antigen, as advocated by Campbell (1984), was considered necessary. To satisfy the objectives of the study, the isolation of a cell membrane-enriched fraction from whole glomeruli should constitute a significant purification step. If isolated glomerular membranes could be demonstrated to be sufficiently immunogenic to stimulate an immune response in the mouse and could also be utilised in a screening assay, the probability of producing and selecting monoclonal antibodies specific for non-GBM antigens would be greatly increased relative to studies employing whole glomerular preparations.

3.2 Materials and Methods.

3.2.1 Isolation of glomeruli.

Lewis rats bred by the animal facility at the Wellington School of Medicine, Otago University, were sacrificed by cervical dislocation under ether inhalation anaesthesia. Immediately upon sacrifice, a midline incision extending from the lower abdomen to the xiphisternum was made with a pair of surgical scissors. The inferior vena cava was severed and the heart exposed through the diaghragm. The rat was then perfused with ice cold, phosphate buffered saline, pH 7.2, (PBS, Appendix I) via a 21G needle inserted into the left ventricle. Perfusion was continued until the liver was seen to be blanched (usually 20-35 mls). Both kidneys were then removed, decapsulated, and placed in a beaker of PBS on ice. For a single preparation, 20 to 80 rat kidneys were used. The kidneys were cut in half longitudinally with a No. 22 scalpel blade, and the cortex was obtained by 'decoring' the medulla from the sectioned kidney with a small pair of scissors with curved blades. The cortex was then cut into pieces approximately 0.5-0.75 cm^2 and 2.3 mm thick and placed in fresh ice cold PBS. Glomeruli were isolated from these pieces of cortex by the sieving technique described by Burlington and Cronkite (1973) using sieves with mesh sizes of 90 μm, 125 μm and 63 μm (W.H. Tyler and Co., Ohio, USA). The cortical tissue was gently pressed through the 90 μm mesh with the bottom of a 50 ml glass beaker and washed through with copious amounts of ice cold PBS. Tubular material collected in the 125 μm sieve was gently washed with PBS and the glomeruli collected in a glass beaker on ice from the final 63 μm sieve by flushing with a stream of PBS. To help prevent the adherance of glomeruli to glassware, all glassware which came in contact with glomeruli was coated with silicone ('Silicote', Sigma Chem. Co., USA). Following harvesting, the glomeruli were allowed to settle to the bottom of the beaker over a

period of 10-15 minutes, and the supernatant was removed by aspiration. The glomeruli were resuspended in 30-40 mls of ice cold PBS, and the procedure repeated. A drop (10 μl) of the glomerular suspension was placed on a glass microscope slide and its purity was assessed by counting glomeruli and contaminating cellular material using a light microscope. Purity was expressed as the percent glomeruli by number.

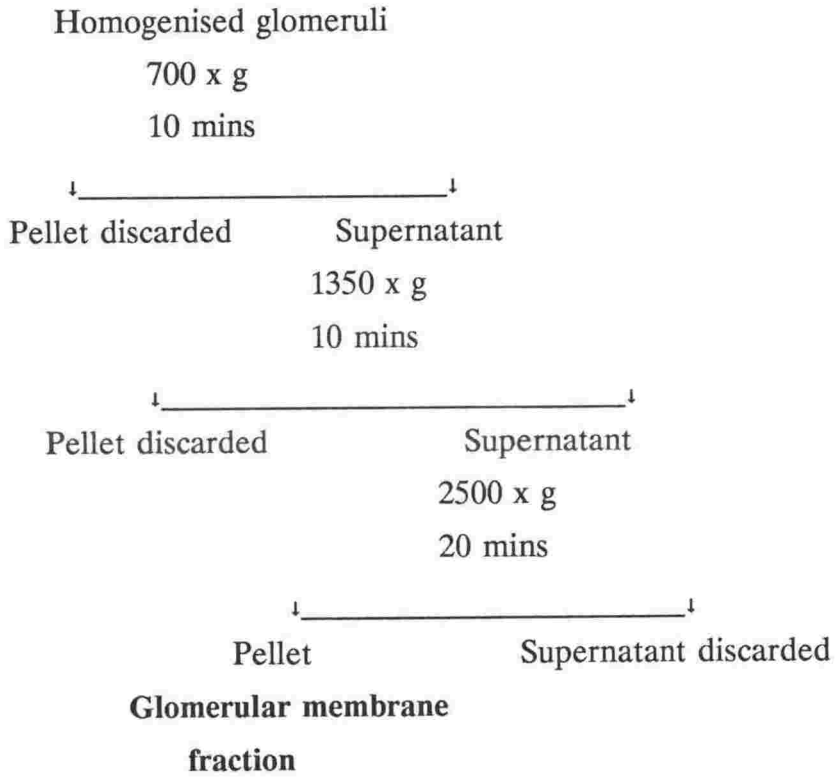
3.2.2 Preparation of a membrane fraction from isolated whole glomeruli.

The glomeruli were centrifuged at 600 x g for three minutes and resuspended in 0.25 M buffered sucrose, pH 7.2, containing protease inhibitors (homogenisation buffer, Appendix I). All subsequent procedures were performed on ice, and centrifugation was carried out at 5°C. Glomeruli were homogenised by a Willems 'Polytron' homogeniser (Kinematica, Lucerne, Switzerland) using setting No. 4. Progression of homogenisation was monitored by light microscopy. One drop of suspension was placed on a glass microscope slide with a Pasteur pipette and scanned under a light microscope for the presence of intact glomeruli. To achieve maximum disruption of glomeruli, five or six sixty second periods of homogenisation at three minute intervals were required. The homogenate was then subjected to differential centrifugation as outlined in Fig. 3.1. The final pellet containing the glomerular membrane fraction (GMF) was resuspended in 2-3 mls PBS. Protein estimation was determined by the method of Lowry et al. (1951) using a standard curve constructed with known amounts of bovine serum albumin (BSA, >99% purity, ICP, Auckland, NZ). The concentration of the albumin standard solution was determined from its absorbance at 280 nm based on an extinction coefficient of 6.6 at 280 nm for a 1% solution (w/v) in a 1 cm light path (Rutter, 1967). The absorbances of standards and samples were determined in a Zeiss PMQ II spectrophotometer (Carl Zeiss Co., West Germany). The GMF samples were stored at -20°C until used.

In attempts to further purify the glomerular membranes by density gradient centrifugation, the final pellet from the differential centrifugation step was resuspended in 7% sucrose using a Dounce homogeniser. Discontinuous density gradients were prepared as w/v sucrose solutions in 30 ml centrifugation tubes as shown in Fig. 3.2. Samples were carefully layered on top of the gradients with a Pasteur pipette and the tubes centrifuged at 95,000 x g for two hours at 5°C in a swinging bucket rotor (SW 28) in an ultracentrifuge (Model L5-65B, Beckman Instruments, Palo Alto, Calif. USA). Samples recovered from the gradient interface

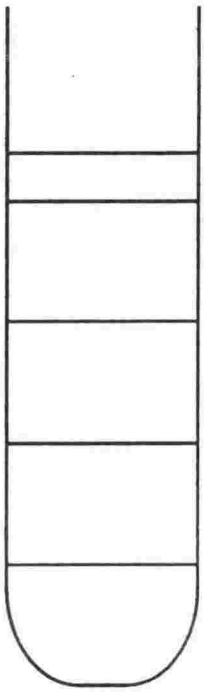
Figure 3.1.

Flow diagram of differential centrifugation of homogenised glomeruli.



The pellet obtained from the final 2500 x g centrifugation step was disrupted with a Dounce homogeniser, resuspended in homogenisation buffer, washed once by centrifugation at 2500 x g for 20 minutes, and resuspended in PBS.

Figure 3.2



7 % sucrose

38.98 % sucrose - *p* 1.148

41.62 % sucrose - *p* 1.158

44.32 % sucrose - *p* 1.168

47.06 % sucrose - *p* 1.179

Discontinuous sucrose density gradients. Solutions were carefully sequentially layered as shown. GMF samples were layered on top of the 7 % top layer and tubes centrifuged as described in the methods.

were diluted in 30 mls PBS, mixed thoroughly, and centrifuged at 75,000 x g in a fixed angle rotor (Ti60) for one hour. The final pellet was resuspended in a small volume (200-500 μl) of PBS.

3.2.3 Transmission electron microscopy (TEM).

Samples (approximately 50 μl) of material recovered from the gradient interface were transferred to microfuge tubes, fixed in 1 ml of 2.5% glutaraldehyde (Probing and Structure, Queensland, Australia) in 0.1 M sodium cacodylate, pH 7.4, for one hour and then washed twice, five minutes per wash, in cacodylate buffer. The membranes were transferred to 1% osmium tetroxide for one hour at 4° C, washed twice in distilled water, and then dehydrated in a series of ethanols, 50 to 100%, in 10% increments. Following the dehydration step the fragments were placed in polypropylene oxide for 10 minutes then transferred to 50% polypropylene oxide/50% Epon resin for one hour. Finally, the membranes were transferred to fresh resin and the resin polymerised at 60° C for 24 hours. All processing to the stage of the second resin step was performed in the microfuge tubes. Samples were sedimented in a Beckman Microfuge 11 (Beckman Instruments, Palo Alto, Calif., USA.) at setting No. 7 for five minutes. Ultrathin sections were cut on an ultratome and taken up on grids. The sections were counter-stained with 2% (w/v) uranyl acetate in 50% ethanol for four minutes and then in a lead citrate solution, prepared by the method of Reynolds (1963) for two minutes, prior to viewing in either a Zeiss 109 (Carl Zeiss,W. Germany) or a Philips 20K (Holland) transmission electron microscope.

3.2.4 Immunisation of mice with GMF.

BALB/cJ mice were obtained from the animal facility at Wellington School of Medicine, Otago University. The mice were immunised with either 50 or 100 μg of GMF in PBS mixed with an equal volume of complete Freund's adjuvant (CFA). The immunogen was emulsified in two 1 ml glass syringes that were connected via 21G hypodermic needles attached to a small bore plastic tube. The mixture of GMF and CFA was repeatedly transferred from one syringe to the other over a period of about 5 minutes to achieve complete emulsification. Mice of either sex, six to eight weeks of age, were given 50 or 100 μg of GMF in CFA intraperitoneally (IP) via a 21 G needle, followed at days 14 and 28 by a further 50 or 100 μg in PBS by the same route. Blood was obtained from the tail vein of immunised mice at days 14, 28 and

35 for assay of circulating anti-GMF antibodies by microELISA and IF microscopy. As controls, two mice were injected IP with the same volume of an emulsion of PBS and CFA without antigen or with PBS alone. One group of three mice was hyperimmunised as follows. An initial immunisation of 100 μg of GMF in CFA was followed a week later by an identical immunisation. Thereafter, this group of mice received 100 μg of GMF in PBS at weekly intervals for four weeks, six immunisations in total.

3.2.5 Enzyme-Linked Immunosorbent Assay (microELISA).

MicroELISA was performed in 96 well microtitre plates as described by Voller et al. (1975). GMF was diluted in freshly prepared carbonate/bicarbonate buffer, pH 9.6 (Appendix I) to yield a final concentration of 1-10 μg per ml. Aliquots of the diluted GMF (200 μl) were distributed to the wells of a 96 well microtitre plate (Immunoplate II, Nunc Inter Med, Denmark), incubated for two hours at room temperature (RT) and then overnight at 4°C. Plates which were not used immediately were stored at -20°C for a maximum of four weeks. Excess antigen was poured off from the wells which were then washed twice with PBS, three minutes per wash. Residual electrostatic binding sites on the microtitre wells were saturated with 1% BSA in PBS for 60 to 90 minutes, and the wells were then washed twice with PBS. Dilutions of immune and non-immune sera were made, 200 μl aliquots were then delivered to the wells and incubated for 90 minutes. The washing procedure was repeated. Bound mouse immunoglobulin was detected by a 90 minute incubation with horseradish peroxidase(HRP)-conjugated rabbit anti-mouse immunoglobulin (Dako Laboratories, Denmark) diluted 1:3000. All dilutions of first and second antibodies were made in PBS containing 0.1% (w/v) BSA and 0.05% (v/v) Tween 20 (Sigma Chemical Co., USA), and all incubations were carried out at RT. The HRP enzyme substrate was o-phenylene diamine and hydrogen peroxide in citrate buffer, pH 5 (Appendix I). The enzyme and substrate reaction was conducted in the dark and allowed to continue for 20 to 30 minutes. The reaction was stopped by the addition of 50 μl of 12.5% sulphuric acid. The absorbance of solutions in the wells was read at 492 nm in a colourimeter specifically designed for microELISA (microELISA Minireader MR590, Dynatech Laboratories, Va., USA).

3.2.6 Indirect Immunofluorescence (IF) Microscopy.

Normal Lewis rat renal cortical tissue was obtained immediately following sacrifice. Pieces of cortex, approximately 0.5 x 0.5 x 0.25 cm, were snap frozen in liquid nitrogen and embedded in OCT cryo-embedding compound (Miles Scientific, Il., USA). Frozen sections, 3-4 μm thick, were cut on a cryostat microtome (Tissue Tek, Miles Scientific, Il., USA) at -15°C. Cut sections were taken up on glass microscope slides and air dried for 90-120 minutes at RT. Sections not used immediately were stored in racks over silica gel in an air-tight box at -20°C and used within 21 days. Sections were washed in PBS prior to incubation with antisera. All dilutions of primary and secondary antibodies were made in PBS, and incubations were carried out for 40 minutes in a perspex box with a tight-fitting lid. A thin layer of water in the bottom of the incubation box maintained a high humidity which prevented evaporation of the antiserum during incubation. Sections were washed three times in PBS, three minutes per wash, following each incubation period. Secondary detecting antibodies were labelled with fluorescein (Silenus Laboratories, Australia). When staining was completed, sections were mounted under coverslips in a thin layer of 90% glycerol-10% PBS. The pH of this solution was adjusted to 9.0 with 0.1 M sodium hydroxide to reduce the amount of fading of the fluorescein emitted light. IF slides were viewed and photographed in a Zeiss photomicroscope adapted for IF photomicroscopy. All photographs were made with Ektatachrome 400 ASA colour slide film (Eastman Kodak Co., N.Y., USA) and the film processed commercially.

3.3 Results.

3.3.1 Isolation of glomeruli.

Glomeruli were isolated in high purity by the sieving technique described (Table 3.1). The major contaminants of the glomeruli were segments of tubules and unidentifiable aggregated debris. The mean purity of the isolated glomeruli from the five experiments was 93.6 % (SD 5.2%) by number.

Table 3.1.

Assessment of the purity of isolated glomeruli by light microscopy.

Experiment number	No. of Kidneys	% purity (by number)
1	16	95.0
2	40	85.0
3	28	93.5
4	80	96.3
5	44	98.4

3.3.2 Preparation of a glomerular membrane fraction (GMF).

The glomeruli proved to be fairly resistant to the sheer forces of the 'Polytron'. At least five one minute periods of homogenisation were required at setting No. 4 to achieve disruption of all glomeruli. Homogenisation was considered complete when no intact glomeruli could be seen.

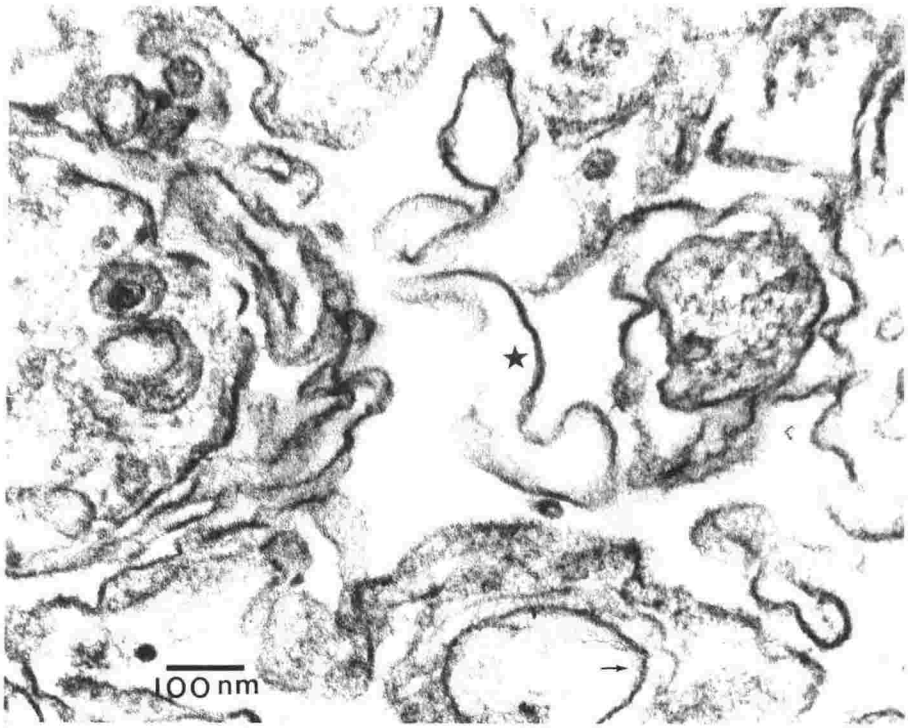
Following homogenisation and differential centrifugation of the suspension, a visible grey/cream pellet was obtained from the final centrifugation step. Electron microscopy of this pellet showed it to consist predominantly of membrane fragments together with other subcellular structures (Fig. 3.3). The total protein content of the membrane pellets is given in Table 3.2. The mean yield of GMF per kidney was 81.4 μg (SD 7.3 μg). Protein estimations were not performed on GMF fractions prepared in experiments one and two.

An attempt was made to further purify the membrane fraction obtained from experiments 2 and 3 by a sucrose density gradient technique used for the isolation of renal tubular basolateral membranes (Ebel et al., 1976). The final pellets obtained following differential centrifugation in experiments 2 and 3 were broken up in a Dounce homogeniser and subjected to density gradient ultracentrifugation. In experiment 2, a small amount of material was recovered from the *p* 1.158 - *p* 1.168 interface. A much greater amount of sample was recovered from the *p* 1.168- *p* 1.179 interface. The pellet which resulted from the ultracentrifugation step was large and appeared to contain most of the applied sample. TEM revealed the material at the *p* 1.168- *p* 1.179 and the *p* 1.158 - *p* 1.168 interface to be predominantly membranes (Fig. 3.4). In experiment 3 the total protein content of the final pellet obtained following differential centrifugation was determined to be 2.5 mgs. On density gradient ultracentrifugation, only a faint band of material was discernible at the *p* 1.168- *p* 1.179 interface and at the *p* 1.158- *p* 1.168 interface. As the amount of material recovered from both interfaces was so small, the samples were pooled and the material pelleted by ultracentrifugation. Total protein content of the recovered pellet was 45 μg, representing only 2% of the starting material; the majority of the applied sample was sedimented through all density layers and pelleted at the bottom of the tube.

Although the purity of the crude membrane preparation was presumably enhanced

Figure 3.3.

Transmission electronmicrograph of the membrane pellet obtained following differential centrifugation of homogenised glomeruli.



Plasma membranes are present in both sheet (*) and vesicle (→) forms. The section has been stained with lead citrate and uranyl acetate.

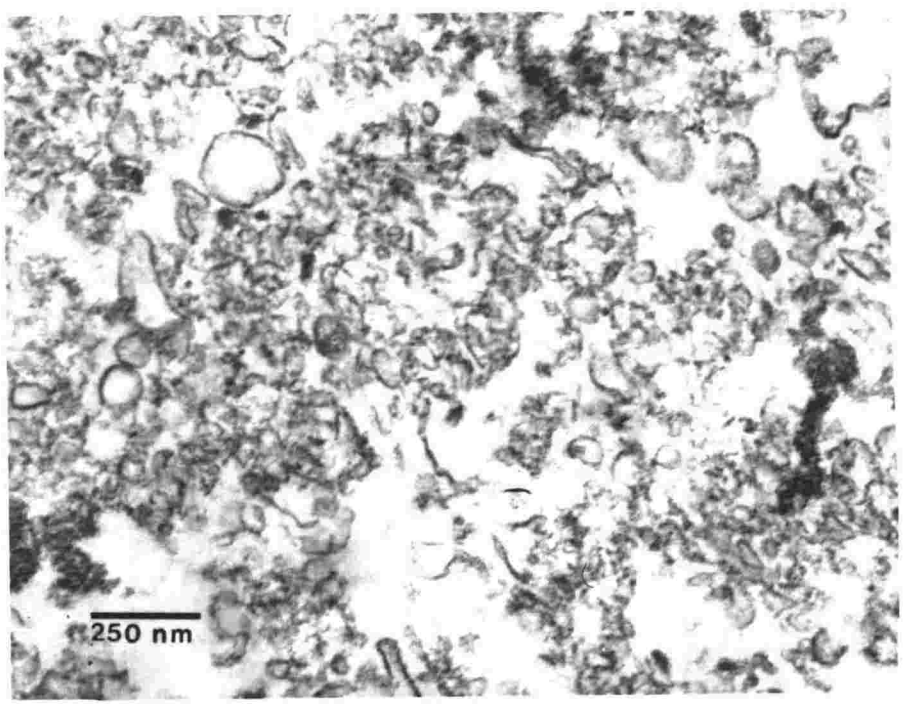
Table 3.2.

GMF protein yield.

Experiment number	No. of Kidneys	Total GMF protein (mg)	GMF Yield μg/kidney
3	28	2.5	89.3
4	40	3.2	80.0
5	44	3.3	75.0

Figure 3.4.

Transmission electronmicrograph of glomerular membrane sample obtained following density gradient centrifugation.



Plasma membranes recovered from the p 1.68 - p 1.179 interface. The section has been stained with lead citrate and uranyl acetate.

by density gradient ultracentrifugation, the decrease in yield was unacceptable. Consequently, a decision was made to progress to immunisation of mice for the production of monoclonal antibodies using the crude membrane preparation GMF obtained by simple differential centrifugation.

3.3.3 Immunisation of mice with GMF.

Sera obtained from immunised mice were examined by indirect IF microscopy for the presence of kidney-fixing antibodies. Anti-rat kidney antibodies were detected in the sera of GMF-immunised mice 14 days post-immunisation, but were not detected in the sera of control mice immunised with PBS and CFA alone or with PBS alone (Table 3.3). The intensity and patterns of staining were similar in the low dose (50 μg), high dose (100 μg) and hyperimmunised mice. The pattern of staining was unusual and was not identifiable as either linear or granular, being best described as pan-glomerular (Fig. 3.5).

3.3.4 Detection of circulating mouse anti-GMF antibodies by microELISA.

It was not known if GMF could function as a suitable solid phase in a microELISA, nor was it known if the polyclonal mouse anti-rat kidney antibodies would bind to GMF under microELISA conditions. The GMF was diluted in carbonate/bicarbonate buffer, pH 9.6, to give final concentrations of 5, 10 or 20 μg/ml. Aliquots (200 μl) of these diluted GMF solutions were distributed to microtitre wells, giving 1, 2 or 4 μg GMF per well. The microELISA assay was performed to determine the presence of anti-GMF antibodies at day 14 in the serum of a mouse which had been immunised with 100 μg of GMF IP (mouse no. 4). Two controls were conducted, one group of wells received serum from a mouse immunised with CFA in PBS without GMF as the primary antibody. The second control was a group of wells not coated with GMF and which received carbonate/bicarbonate buffer only in place of primary antibody. These two sets of wells would reflect non-specific background absorbances. Indirect IF microscopy had indicated that serum from mouse 4 reacted with normal rat kidney at a dilution of 1:200, whereas the control serum did not react with normal rat kidney. Results obtained using microELISA indicated that anti-GMF activity of mouse 4 serum could clearly be measured by this method (Fig. 3.6). Background absorbance, measured in those wells incubated with buffer only (no antigen), was less than 0.150 absorbance units for both immune and non-immune sera. Non-immune control serum

Table 3.3.

Indirect IF microscopy of anti-GMF immune sera on normal rat kidney frozen sections.

Mouse number	Immunisation regimen (μg GMF)	IF Microscopy day 14	day 28	day 35
1	50	++	++	++
2	50	++	++	+++
3	50	++	++	++
4	100	++	++	+++
5	100	++	++	++
6	100 (H)	++	++	++
7	100 (H)	++	+++	+++
8	100 (H)	+++	++	++
9	CFA/PBS	-	-	-
10	CFA/PBS	-	-	-
11	PBS	-	-	-
12	PBS	-	-	-

H - Hyperimmunisation regimen.

CFA - Complete Freund's adjuvant.

PBS - Phosphate buffered saline.

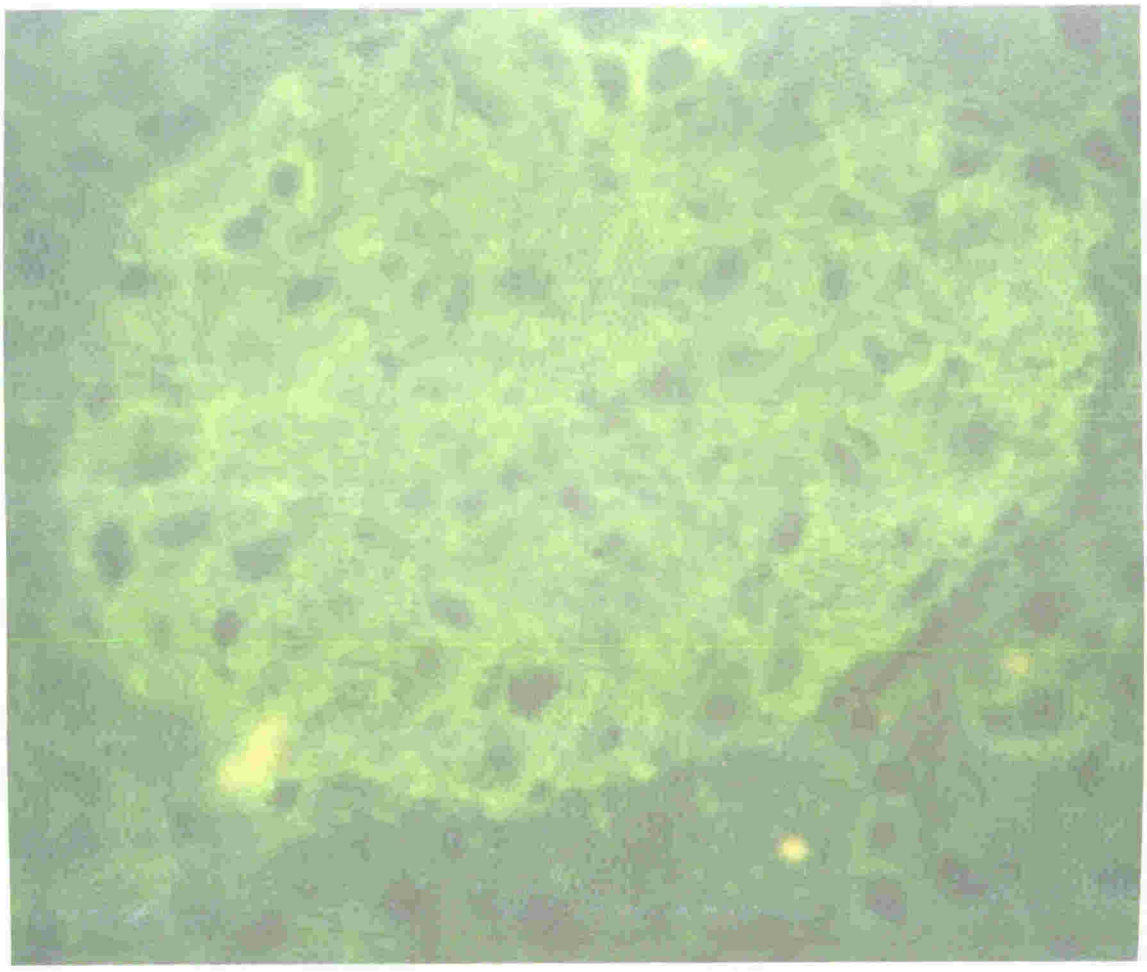
\+ - Weak staining.

++ - Moderate staining.

+++ - Heavy staining.

Figure 3.5.

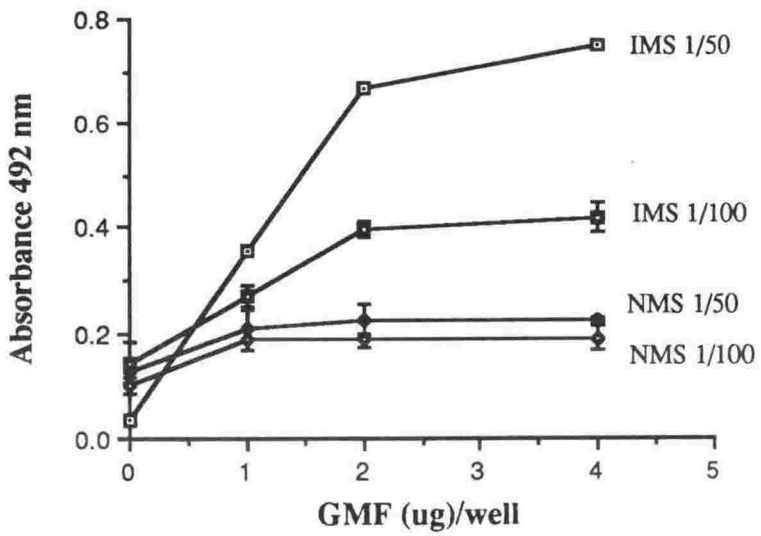
Indirect Immunofluorescence staining pattern of anti-GMF immune mouse serum on normal Lewis rat kidney.



A cryostat section of normal Lewis rat kidney was incubated with a 1/100 dilution of serum obtained from a mouse immunised with 100 μg of GMF in CFA. Bound mouse immunoglobulin was detected with a polyclonal FITC anti-mouse immunoglobulin serum. Original magnification x 400.

Figure 3.6.

Effect of GMF concentration on microELISA absorbance values.



Microtitre plates were coated with GMF as indicated on the horizontal axis. MicroELISA was performed using immune mouse serum (IMS) from mouse 4 or non-immune mouse serum (NMS) as described in the methods. The values are the mean of quadruplicate samples; error bars indicate the standard deviation.

gave absorbances which were slightly greater in GMF-coated wells than in wells which received no antigen. There was, however, no significant difference between control serum absorbance on wells containing 1, 2 or 4 μg GMF/well. Using immune serum, the increased binding was linear with GMF amounts between 0 and 2ug/well, but between 2 and 4 μg/well, at a dilution of 1:50, the increase in absorbance was 0.082 units, and at a dilution of 1:100, was only 0.022 absorbance units. From the data presented in Fig.3.6, it was concluded that 2 μg GMF/well, (200μl of a 10μg/ml solution), was the optimum amount for the detection of anti-GMF antibodies as well as conserving GMF. All subsequent microELISA experiments were therefore conducted with a GMF concentration of 2 μg/well.

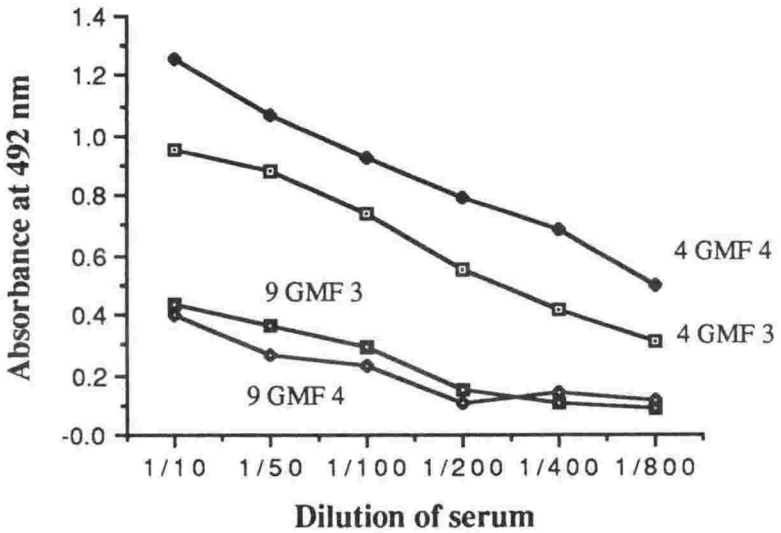
GMF was to be used for both the immunisation of mouse splenocyte donors and the screening of hybridoma culture supernatants. It could not be guaranteed that the GMF used for screening of hybridoma culture supernatants would be from the same GMF preparation that was used as the immunogen. To examine batch to batch antigenic variability of GMF, microELISA was performed using two GMF preparations prepared several weeks apart. Sera from two mice (numbers 4 and 5) which had been immunised with GMF preparation number three (GMF 3) were used as the immune sera. Control serum was obtained from a mouse which had been immunised with PBS-CFA only. MicroELISA was performed in microtitre plates which had been coated with either GMF 3 or a later GMF preparation, GMF 4, as the immobile antigen. Results from this experiment revealed that immune sera from the mice reacted with both the immunogen, GMF 3, and with GMF 4 to which the mice had no prior exposure (Figs. 3.7 and 3.8). Serum from mouse 4 gave higher absorbance values with GMF 4 than with GMF 3. The reverse was the case with mouse 5 serum. Control serum from mouse 9 gave marginally higher absorbance values with GMF 3 than with GMF 4, but this was evident at low dilutions only. These results suggested that the different batches of GMF could be considered approximately antigenically equivalent.

3.3.5 Immunisation regimens.

Circulating anti-GMF antibodies were detected by microELISA at day 28 in all mice immunised with GMF. A comparison of the three immunisation regimens is given in Tables 3.4 - 3.6. Mice which were hyperimmunised had, by day 14, received two immunisations and by day 28 four immunisations. The 50 μg and 100 μg groups had

Figure 3.7.

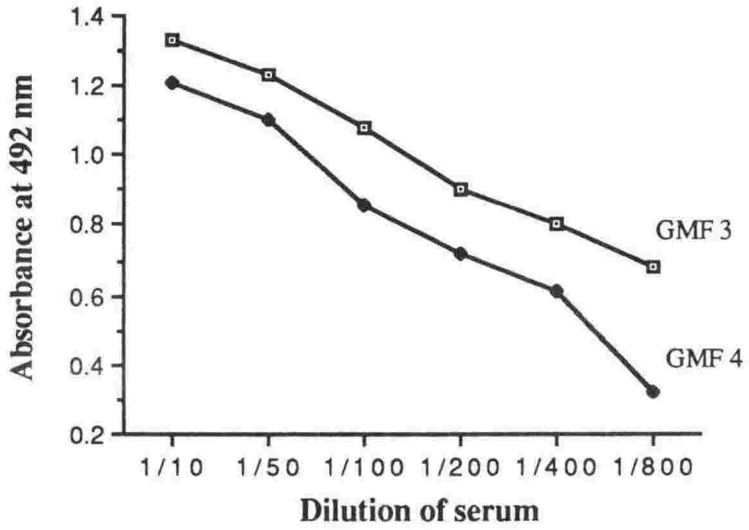
Comparison of microELISA absorbance values obtained with immune and non-immune mouse serum on separately prepared GMF preparations.



Microtitre plates were coated with either GMF 3 or GMF 4 at 2 μg GMF/well. MicroELISA was performed with GMF immune serum obtained from mouse 4 which had been immunised with GMF 3 and non-immune mouse serum obtained from mouse 9 which had been immunised with CFA in PBS only. Values are the average of duplicate samples.

Figure 3.8.

Comparison of microELISA absorbance values obtained with separately prepared GMF preparations.



Microtitre plates were coated with either GMF 3 or GMF 4 at 2 µg GMF/well. MicroELISA was performed with GMF immune serum obtained from mouse 5 which had been immunised with GMF 3. Values are the average of duplicate samples.

Table 3.4.

MicroELISA absorbance values of anti-GMF immune sera obtained from mice immunised with 50 μg of GMF in CFA.

Day	Dilution of mouse serum					
	1/10	1/50	1/100	1/200	1/400	1/800
14	1.2	0.97	0.75	0.64	0.54	0.45
n=3	(.12)	(.17)	(.02)	(.04)	(.06)	(.04)
28	1.09	1.00	0.79	0.61	0.44	0.34
n=3	(.15)	(.19)	(.13)	(.06)	(.03)	(.02)
35	1.14	0.92	0.81	0.61	0.48	0.35
n=2	(.27)	(.06)	(.04)	(.06)	(.06)	(.06)

The value n is the number of mice, immunised with 50 μg of GMF in CFA via the IP route, from whom serum samples were obtained. Samples were assayed in duplicate by microELISA as described in the methods, and the results recorded as the average absorbance of the duplicate samples. The values shown in the table are the mean values from the two or three animals. The figures in parentheses are the standard deviations of the means.

Table 3.5

MicroELISA absorbance values of anti-GMF immune sera obtained from mice immunised with 100 μg of GMF in CFA.

Day	Dilution of mouse serum					
	1/10	1/50	1/100	1/200	1/400	1/800
14	1.14	1.06	0.91	0.73	0.61	0.50
n=3	(.17)	(.25)	(.14)	(.05)	(.07)	(.06)
28	1.02	0.92	0.83	0.69	0.61	0.46
n=3	(.04)	(.02)	(.05)	(.05)	(.06)	(.03)

The value n is the number of mice immunised with 100 μg of GMF in CFA via the IP route, from whom serum samples were obtained. Samples were assayed in duplicate by microELISA as described in the methods, and the results recorded as the average absorbance of the duplicate samples. The values shown in the table are the mean values from the two or three animals. The figures in parentheses are the standard deviations of the means.

Table 3.6

MicroELISA absorbance values of anti-GMF immune sera obtained from mice hyperimmunised with 100 μg of GMF in CFA.

Day	Dilution of mouse serum					
	1/10	1/50	1/100	1/200	1/400	1/800
14	1.40	1.15	1.01	0.88	0.76	0.61
n=3	(.19)	(.17)	(.14)	(.12)	(.11)	(.08)
28	1.58	1.33	1.10	0.93	0.78	0.63
n=3	(.20)	(.05)	(.09)	(0.0)	(.03)	(.07)

The value n is the number of mice immunised with 100 μg of GMF in CFA via the IP route, from whom serum samples were obtained. Samples were assayed in duplicate by microELISA as described in the methods, and the results recorded as the average absorbance of the duplicate samples. The values shown in the table are the mean values from the two or three animals. The figures in parentheses are the standard deviations of the means.

received only one immunisation when serum samples were obtained at day 14 and two by day 28. As expected, absorbance readings obtained with hyperimmune serum were greater at all dilutions than those of the 50 μg and 100 μg group at both day 14 and 28.

3.4 Discussion.

The sieving technique used in this study resulted in the isolation of rat glomeruli in high purity. Using a similar sieving technique Misra (1972) reported the isolation of mammalian glomeruli with a purity of 95-98%. The mean purity of the glomeruli in the present study was 93.6%. This value would have been greater were it not for a single experiment which returned a low purity of only 85%. Three out of five experiments resulted in purities of 95% or greater.

A number of techniques have been employed to disrupt intact cells with the subsequent production of a heterogeneous mixture of subcellular organelles. Methods commonly employed include freeze-thaw cycles, sonication, and shear force generators, such as the hand operated Dounce homogeniser or the electrically operated Polytron homogeniser. Differential centrifugation of homogenised tissues or cells is a relatively quick, simple and inexpensive method for the isolation of plasma membranes from a heterogeneous homogenate (Evans, 1978). The differential centrifugation method adopted in the present study resulted in the recovery of a glomerular plasma membrane enriched fraction as assessed by transmission electron microscopy. Further purification of the crude membrane fraction by ultracentrifugation through sucrose density gradients resulted in an unacceptable decrease in yield.

The results reported in this chapter indicated that the GMF preparation was sufficiently immunogenic, at the doses and regimens used, to provoke a satisfactory humoral immune response in BALB/cJ mice. Goding (1983) has stated that the greater the phylogenetic distance between the antigen and recipient, the more vigorous the immune response. Goding also states that proteins present on cell surfaces or membrane fragments are particularly immunogenic. The immune response generated in BALB/cJ mice immunised with rat GMF could be measured as serum anti-GMF activity and titrated using microELISA with GMF as the immobile antigen. Additionally, the anti-GMF mouse serum reacted with normal Lewis rat kidney by

indirect IF microscopy. These results indicated that the epitopes present on the GMF, against which the mouse antibodies were directed, were accessible on frozen sections of normal rat kidney to react with their respective antibodies. It was likely that any subsequent screening of hybridomas generated by fusion of splenocytes obtained from mice immunised with GMF would be performed with a different GMF preparation from that used in the initial immunisation of the mouse. For this reason it was necessary to establish whether the antigenicity of different GMF preparations was reproducible. Results obtained by microELISA with mouse serum against GMF to which the mice had not had prior exposure indicated that the epitope specificity of the two preparations was similar.

From the results of the work detailed in this chapter, a decision was made to proceed with the fusion of splenocytes, from mice immunised with 50 μg of GMF IP, with mouse myeloma cells to generate hybridomas that would produce anti-GMF monoclonal antibody. Screening of the hybridomas post-fusion would be performed by microELISA.

Chapter Four

GENERATION OF ANTI-GMF MONOCLONAL ANTIBODY-SECRETING HYBRIDOMAS

4.1 Introduction.

The application of monoclonal antibody technology to the investigation of experimental animal models of GN has been reported from only a few laboratories (Ronco et al., 1984a, 1984b, 1986a; Mendrick et al., 1983; Mendrick and Rennke, 1986; Nishi et al., 1984). The immunogens employed in these studies have varied from homogenised kidney cortex to Fx1A antigen. The aim of the present study was to produce monoclonal antibody probes specific for discontinuously represented structural antigens of the rat GCW. Such antigens may, as stated in Chapter Three, be associated with either glomerular epithelial, endothelial or mesangial cells and distinct from antigens of the GBM. The use of GMF as the immunogen could therefore result in the production of monoclonal antibodies with the desired specifications, since GMF presumably contains plasma membranes from all of these cell types, but lacks a major GBM component.

The method used to generate hybridomas in this work is based on that of Claflin and Williams (1978), which is in turn a modification of the method of Gefter et al. (1977). A number of additional modifications have been incorporated in the present study based on more recent monoclonal antibody production methods reported in the literature.

4.2 Materials and Methods

4.2.1 Immunisation regimens.

BALB/cJ mice of either sex were immunised with GMF by one of two routes.

a. Intraperitoneal (IP) - Mice six to eight weeks of age were immunised as described in Chapter Three with 100 μg of GMF. Two to six weeks later, and four days prior to cell fusion, the mice were boosted with 400 μg of GMF in PBS IP.

b. Intrasplenic (IS) - Four days prior to cell fusion mice 35-40 days old were immunised IS following the procedure described by Spitz et al. (1984). Anaesthesia was induced with IP pentobarbitone ('Sagatal', May and Baker, England) 60 mg/kg body weight. The anaesthetised mouse was placed on a wooden procedure board and held by elastic bands placed around the limbs which were then secured to the board. Fur was clipped from the left side of the abdomen and the skin prepared with 70% ethanol. A 1.5 cm skin incision was made 0.5 cm below the left lower costal margin. The abdominal muscles were incised and the spleen exposed. 50 μg of GMF in PBS was injected into the lower pole of the spleen through a 27 G needle. The muscle wall and skin were closed separately with interrupted silk sutures. The mice were returned to their cages and allowed food and water ad libitum. Four days later and immediately prior to fusion, the spleen was removed under sterile conditions for the preparation of splenocytes.

4.2.2 Mouse Myeloma Cell Lines.

NSI/1 Ag 4.1 (NSI) cells were kindly donated by Dr M.V. Berridge, Malaghan Institute of Medical Research, Wellington and Dr F. Austin, MRC Virus Research Unit, Dunedin, N.Z. Sp 2/0 cells were obtained from Dr Berridge and also from CSL Laboratories, Australia. All myeloma cell lines and resulting hybridomas were grown in Rosewall Park Memorial Institute medium 1640 (RPMI, Appendix II). An exception was the NSI cell line donated by Dr Berridge which were initially grown in Dulbeccos modified Eagles medium (DMEM, Appendix II) but later adapted to growth in RPMI medium. Cells were grown in 75 cm^2 cell culture flasks (Nunc, Denmark) containing RPMI supplemented with 10% foetal calf serum (FCS). All new stocks of FCS were heated at 56° C for one hour as a precautionary step against possible mycoplasma contamination of the serum. To ensure the cultures were not contaminated with 8-azaguanine-resistant revertant cells, all myeloma cells were cultured in RPMI-10% FCS containing 2 x 10^{-5} M azaguanine three weeks prior to fusion. Growth in this medium was continued for one week following which the 8-azaguanine was diluted out over a period of two to four days. Growth was monitored

daily, and cell density was determined by counting in a Levy-Hausser haemocytometer (Hausser Scientific, USA) using a light microscope. Only cells which appeared healthy and were in an exponential growth phase were used in cell fusion experiments.

4.2.3 Use of peritoneal macrophages.

Microculture plate wells were seeded with mouse peritoneal macrophages on the day prior to the fusion experiment. A BALB/cJ mouse was sacrificed by cervical dislocation, the carcass was immersed in 70% ethanol for 10-15 seconds and then removed to absorbent tissue. A midline abdominal skin incision was made and the skin layer dissected from the abdominal wall. Under sterile conditions four to five mls of RPMI-10% FCS was injected IP, the abdomen was gently agitated over 30-45 seconds and the culture medium then recovered. The cell suspension was diluted to 40 mls in a sterile glass beaker, transferred to a multichannel pipette reservoir and 200 μl aliquots distributed to all wells of two microculture plates (Nunc, Denmark). Lids were replaced on the microculture plates which were then placed in a humidified incubator maintained at 5% CO_2 and 37°C. The following day, immediately prior to aliquoting of the hybridized cells, the medium in the wells was aspirated by suction and the wells washed once with RPMI.

4.2.4 Preparation of spleen cells.

Mice were sacrificed by cervical dislocation and immersed in 70% ethanol for 10-15 seconds as described in 4.2.3 above. Under sterile conditions, the spleen was removed through a midline abdominal incision and placed in a petri dish containing RPMI. The spleen was cut transversely at its midpoint, a 27 G needle was inserted into the uncut ends and four to five mls of RPMI was injected into each half to flush out the splenocytes. Following this, the spleen was finely diced and repeatedly drawn into and expelled from a glass Pasteur pipette. The cell suspension was transferred to a 50 ml conical test tube and the tissue debris allowed to settle to the bottom of the tube. The supernatant containing the spleen cells was removed to a clean conical 50 ml tube and centrifuged at 500 g for 5 minutes. The supernatant was discarded. Erythrocytes were lysed by resuspending the cell pellet in 0.17 M ammonium chloride, (pH adjusted to 7.2), and incubating at 37°C for 5 minutes. The cell suspension was centrifuged (100 x g for 5 minutes), the supernatant discarded, and the spleen cell pellet resuspended in serum-free RPMI. Cell density was determined by counting the

cells on a Levy-Hausser haemocytometer. Splenocyte and myeloma cell viability was determined by the ethidium bromide-acridine orange fluorescence method described by Lee et al. (1975). Briefly, 5 μl of a 1/50,000 solution of ethidium bromide was added to 50 μl aliquots of each of the cell suspensions. Thirty seconds later 5 μl of a 1/50,000 solution of acridine orange was also added. A 10 μl sample of the stained cells was then examined in a fluorescence microscope. Viable live cells were stained light green whilst dead cells were stained red.

4.2.5 Preparation of polyethylene glycol (PEG) solutions.

Samples of PEG were weighed in 'Pyrex' glass test tubes graduated to a 1 ml volume. Each test tube was plugged with cotton wool, the mouth covered with aluminium foil and sealed with autoclave indicator tape. The sealed test tubes were then steam-autoclaved at 120°C for 40 minutes. Immediately prior to use, the tubes were warmed in a water bath at 37°C and serum-free RPMI added under sterile conditions to 1 ml.

4.2.6 Fusion protocol.

Myeloma cells were harvested from cell culture flasks, centrifuged (500 x g for 5 minutes), and resuspended in approximately 50 mls of serum-free RPMI. Cell density and viability were determined as for splenocytes. Myeloma and splenocytes were mixed at a ratio of 10:1 and centrifuged at 500 x g for 5 minutes. The supernatant was removed and the cell pellet gently broken up by tapping the bottom of the centrifuge tube on the bench top. The PEG solution was prepared at 37°C, a timer was started, and 0.5 ml was added dropwise from a Pasteur pipette gently down the side of the tube containing the mixed cell suspension over the next two minutes. The cells were mixed after the PEG had been added by flicking the bottom of the tube once or twice. The cell suspension was then centrifuged at 100 x g for 5 minutes. After a total PEG exposure time of ten minutes, 5 mls of serum-free RPMI was added over two minutes, initially slowly, one drop every three to four seconds, and gradually increasing the rate of addition. The tube was tapped on the bench top and swirled by hand over the next two to three minutes to disrupt the cell pellet. The resulting suspension was centrifuged (100 x g for 5 minutes), the cell pellet was resuspended and washed twice in approximately 50 mls of serum-free RPMI. The final pellet was

resuspended in 39 mls of RPMI medium containing hypoxanthine, aminopterin and thymidine (HAT medium, Appendix II) supplemented with 20% FCS and 1 IU/ml soluble insulin (Actrapid, Novo Ind., Denmark) as advocated by Feit, et al. (1984). The cell suspension was distributed in 200 μl aliquots to the two microculture plates previously seeded with peritoneal macrophages. Lids were placed on the microculture plates which were then placed in an incubator (5% CO_2, 37° C) and left undisturbed for 48 hours. Cultures were fed with one drop of HAT medium on days four and seven. Fusion frequency was expressed as the number of wells supporting hybridoma growth at post-fusion day 10 per 10^7 splenocytes.

4.2.7 Screening of hybridoma culture supernatants.

When hybridoma colonies had grown to occupy more than 50% of the total area of the well, 100 μl of supernatant was removed under sterile conditions with an automated pipette. The supernatant was diluted 1:3 with PBS and 200 μl aliquots added to the test (GMF-coated) and control (no GMF) wells of a microELISA plate. GMF immune mouse serum was used as a positive control. Additional supernatants taken at random from wells that were negative for hybridoma growth were also screened. MicroELISA was then performed as described in Chapter Three.

Those colonies whose supernatant recorded a test absorbance at least three times greater than their respective control wells were scored as positive. These colonies were transferred to 2 ml culture wells on a 24 well cell culture plate (Nunclon, Inter Med, Denmark) in a 50-50 mixture of HAT medium and RPMI-10% FCS. Cells were fed and maintained approximately every second or third day by resuspending the cells which had attached to the surface of the plate by blowing medium over the surface of the plate with a pipette. Two-thirds of the suspension was removed and replaced with RPMI-10% FCS. Supernatant samples, 200 μl, were removed from the wells after five to seven days and examined for anti-GMF antibody reactivity by indirect IF microscopy on normal Lewis rat kidney as described in Chapter Three. Those hybridomas whose supernatant gave a positive result by indirect IF were then cloned twice as described in section 4.2.8 below. These anti-GMF antibody-secreting hybridomas were designated a code number which was related to the original microwell in which they arose. Aliquots of these hybridomas were frozen and stored under liquid nitrogen as described below (4.2.9).

4.2.8 Cloning of hybridomas.

All hybridoma supernatants registering a positive microELISA result were immediately cloned by limiting dilution. Hybridomas were grown in 2 ml wells, the cells were suspended in the medium and the cell density determined. Dilutions of the cell suspension were made in cloning medium (Appendix II) containing 5 x 10^{-5} M 2-mercaptoethanol to obtain densities of 10, 3 and 0.5 cells/well. Each dilution was distributed in 200 μl aliquots to 32 wells of a microculture plate which had been seeded with peritoneal macrophages 24 hrs earlier. Cultures were incubated undisturbed for four to eight days. At ten days post-cloning a count of hybrid colonies was made. The criterion for a successful cloning was that greater than 37 per cent of the wells seeded with less than 0.5 cells/well should be negative for hybridoma growth (Goding, 1980). Supernatants from wells seeded with less than one cell/well and those from wells seeded with 3 cells/well but where only a single hybridoma colony was visible were screened by indirect IF microscopy on normal Lewis rat kidney. Those hybridomas whose supernatants produced the most intense staining were expanded to 2 ml culture wells and recloned 5-10 days later. The strongly positive hybridomas from the second cloning were expanded first to 2 ml wells and then to 75 cm^2 cell culture flasks, and stocks were stored frozen.

4.2.9 Freezing and thawing of cell lines.

Myeloma and hybridoma cell lines were grown in 75 cm^2 cell culture flasks in RPMI-10% FCS. During exponential growth, the cells were harvested by centrifugation and resuspended (to a density of 0.5-1.0 x 10^6 cells/well) in freezing medium (Appendix II) containing 10% dimethyl sulphoxide and 20% FCS. One ml aliquots were transferred to cryovials (Nunc, Inter Med, Denmark), wrapped in cotton wool and placed in a thick-walled (2-3 cm) polystyrene container. The container was refrigerated at 4°C for 30-60 minutes and at -20°C for one hour, and then removed to a -80°C freezer overnight, or for a period not exceeding 72 hours. The cryovials were then stored under liquid nitrogen in a cryopreservation dewar (MVE, Mn., USA). When required, vials were removed from the liquid nitrogen and immediately placed in a water bath at 37°C. The vials were constantly agitated by hand until the cell suspension was completely thawed. The contents of the vial were drawn up once through a Pasteur pipette and layered on top of 10 mls of serum free RPMI in a conical centrifuge tube. The tubes were centrifuged (100 x g for 4 minutes), the

supernatant discarded, and the pellet resuspended in 20-30 mls of RPMI-10% FCS and cultured in 75 cm^2 cell culture flasks. Aliquots (25 μl) of the resuspended cell suspension were removed for cell viability assessment by ethidium bromide - acridine orange staining.

4.3 Results.

4.3.1 Fusion.

Splenocyte yield from immunised mice was 3×10^7 - 8×10^7 cells per spleen. Following lysis of erythrocytes, splenocyte viability exceeded 90% in all experiments. Initial attempts to produce viable hybrid cells were not successful. In the first two fusion experiments the NS1 cells were grown in DMEM; post-fusion, the HAT medium contained DMEM. After 21 days incubation post-fusion with feeding, no hybrid colonies were visible in any of the wells from either fusion. The NS1 cells appeared to be healthy; doubling time was determined to be 17-18 hours, and pre-fusion cell viability of both splenocytes and NS1 cells exceeded 90%. The cause of the failure to produce viable hybrids was unknown. The NS1 cells were adapted over a period of two to three weeks to growth in RPMI medium. This medium is used extensively in other laboratories undertaking hybridoma work. The cells were also cultured for seven days in 8-azaguanine to ensure that the NS1 cell line was indeed HGPRT deficient. Two further fusion experiments were undertaken in RPMI. In fusion experiment three, a small colony of cells was observed in one well at day seven. This colony did not continue to grow and by day 10 all cells were dead. No further colonies were visible after 21 days of incubation in either fusion three or four. One further fusion experiment, number five, was performed using a different myeloma cell line, Sp 2/0, as the fusion partner. Again no hybrid cell colonies were visible after 21 days of culture. Splenocytes used in all of these unsuccessful fusion experiments were from mice immunised via the IP route, with a booster immunisation being given 4 days prior to fusion.

One possibility for the failure of the fusions was that the myeloma cell lines were contaminated with mycoplasma. Mycoplasma infection has been cited in the literature as a cause of failed fusion (Bastin et al., 1982; Foster, 1982; Kearney, 1984). To

investigate this possibility, samples of NS1 and Sp 2/0 cells were sent to the National Health Institute, Porirua, NZ., for mycoplasma isolation and culture. Mycoplasma colonies were cultured from both cell lines. On receipt of this result, all NS1 and Sp 2/0 cells were discarded, as was all liquid medium and stock solutions used in the culture of the cells. The sterile work cabinet and incubator were cleaned thoroughly with a chlorine-based disinfectant. Fresh stocks of NS1 cells were obtained from Dr F. Austin, MRC Virus Research Unit, University of Otago, Dunedin. These new NSI cells were passaged in RPMI medium (to which they were already adapted) for five days before a further seven day period of culture in 2 x 10^{-5}M 8-azaguanine. A sample of these cells submitted for mycoplasma screening returned a negative result. Aliquots of these NS1 cells were stored frozen for future use.

The first fusion experiment undertaken with mycoplasma-free NSI cells (fusion 6) yielded 66 wells supporting hybridoma colonies at day 10 out of a total of 192 wells seeded, a fusion frequency of 19 hybridomas per 10^7 splenocytes (Table 4.1). A further three fusion experiments (fusions 7-9) were undertaken which gave even better fusion frequencies ranging from 18 - 22. The majority of colonies were formed at the edge of the wells, possibly due to differential settling of the cells. In 10-20% of wells supporting hybridoma growth, more than one colony was observed; in most cases the colonies were of unequal size. In some cases the colonies were separated by a distance equal to or greater than the radius of the well, in others the colonies were in close proximity. It was not possible to determine whether these colonies were indeed individual clones or if they were subcolonies originating from one or more hybrid cells migrating from the original colony.

Splenocyte partners in all fusion experiments, with one exception (fusion eight), were obtained from mice immunised IP. The fusion frequency obtained in fusion eight was identical to that obtained in fusion seven splenocytes from an IP immunised mouse. All hybridoma colonies generated attained a cell density of 50% of the total well area by day 10-14 post-fusion. By day 28 no late-appearing colonies had developed in wells

TABLE 4.1

Details of fusion experiments.

Fusion number	Immunisation Regimen	No of wells with hybrid colonies at day 10	Spleen cell input ($x10^7$)	Fusion frequency
1 - 5	IP	0	-	-
6	IP	66	3.4	19
7	IP	84	4.8	18
8	IS	84	3.8	22
9	IP	100	5.1	20

IP - Intraperitoneal
IS - Intrasplenic

The first five fusion experiments were unsuccessful. Fusion frequency is expressed as the number of wells with hybridoma colonies per 10^7 splenocytes.

positive supernatants of fusion 6 are shown in Table 4.2. One of the control supernatants taken from a fusion 6 well negative for hybridoma colony growth returned a positive result (test 0.12, control 0.01, data not shown). As a result of this, supernatant was taken from 63 wells negative for hybridoma growth and screened by microELISA for anti-GMF activity. Eight of these supernatants recorded a positive result. MicroELISA screening of supernatants from fusions seven, eight and nine included only RPMI medium as negative primary antibody controls. The results of microELISA screening of supernatants from fusions six to nine are summarised in Table 4.3.

The hybridoma colonies whose supernatants returned a positive microELISA result were transferred to 2 ml culture wells in 24 well plates, and HAT medium was slowly diluted out. Following a period of 5-10 days culture in the 2 ml wells, supernatant was removed and screened for kidney-fixing antibodies by indirect IF microscopy. Results are shown in Tables 4.4 to 4.7.

a. Fusion six (Table 4.4).

The splenocytes used in this fusion were from a mouse immunised by the IP regimen. Eleven wells recorded a positive microELISA result, representing 17% of all wells supporting hybridoma growth. Of these eleven wells only three supernatants reacted with normal rat kidney by indirect IF microscopy, representing only 5% of all hybridomas (Table 4.8). Unfortunately one of the IF positive colonies, designated 1-D11, grew very slowly. After three weeks of culture in 2 ml wells, this colony ceased to proliferate and all cells died. The remaining two surviving colonies designated 2-C12 and 2-E12 were cloned twice by limiting dilution. The resultant clones returning the highest absorbance values on microELISA were cultured and aliquots of the cells stored under liquid nitrogen.

b. Fusion seven (Table 4.5).

This fusion was also undertaken with splenocytes from an IP immunised mouse. Of the 192 wells seeded with cell suspension post-fusion, 6% were scored as positive for anti-GMF activity by microELISA, and 4% returned a positive staining of glomeruli by IF microscopy (Table 4.8). Sixty percent of the microELISA positive colonies in fusion seven produced an IF positive supernatant, whereas in fusion six only 27% of

TABLE 4.2.

MicroELISA absorbance values of anti-GMF positive colonies from fusion 6.

Colony code	Test	Control
1-93	.40	.04
1-B6	.27	.03
1-C8	.40	.16
1-D11	.51	.02
1-E11	.16	.01
1-F11	.19	.03
2-H6	.12	<0
2-G8	.23	.06
2-E12	.27	.08
2-C12	.43	<0
2-F12	.20	.03
Negative control		
RPMI medium	.08	.06
Positive control		
IMS 1/50	.62	.14

IMS - Immune mouse serum, obtained from a GMF immunised mouse. This serum was previously shown to be both microELISA and IF positive.
RPMI medium contained 10% FCS.

TABLE 4.3

Summary of MicroELISA absorbance values from positive wells, fusions 6 to 9.

Fusion number	Number of supernatants	Number of supernatants microELISA positive	Mean absorbance	Range
6	66	11	0.29 (±0.13)	0.12-0.51
7	84	5	0.32 (±0.29)	0.12-0.82
8	84	7	0.24 (±0.10)	0.10-0.36
9	100	8	0.20 (±0.10)	0.10-0.42

The values in parenthesis are the standard deviation of the absorbances.

TABLE 4.4.

Screening of microELISA-positive hybridoma supernatants from fusion 6 by indirect IF microscopy on nomal rat kidney.

Hybridoma clone	MicroELISA	Indirect IF Microscopy	
		Glomeruli	Tubules
1-G3	+	-	-
1-B6	+	-	-
1-C8	+	-	-
1-D11	+	+	-
1-E11	+	-	-
1-F11	+	-	-
2-H6	+	-	-
2-G8	+	-	-
2-C12	+	+ +	±
2-E12	+	+	-
2-F12	+	-	-

+	weak staining
+ +	moderate staining
+ + +	strong staining
±	occasional/inconsistent staining
-	negative

TABLE 4.5.

Screening of microELISA-positive hybridoma supernatants from fusion 7 by indirect IF microscopy on nomal rat kidney.

Hybridoma clone	MicroELISA	Indirect IF Microscopy	
		Glomeruli	Tubules
PG6	+	-	-
PH7	+	+ +	+
APE10	+	+ +	+ +
PF1	+	-	±
BPC4	+	+ +	+ +

+	weak staining
+ +	moderate staining
+ + +	strong staining
±	occasional/inconsistent staining
-	negative

the microELISA positive colonies produced positive IF staining. The three IF positive colonies were cloned twice by limiting dilution and grown in culture until sufficient numbers of cells were present to prepare frozen stocks. These cell lines were designated PH7, BPC4 and APE10.

c. Fusion eight (Table 4.6).

This fusion was undertaken with splenocytes from an IS immunised mouse. The IS immunisation procedure produced no obvious ill effects, and the mouse looked healthy when sacrificed prior to fusion. MicroELISA and IF positive colonies were 8% and 6% respectively of all colonies (Table 4.8). Over 70% of microELISA positive wells were IF positive. One IF positive colony produced in this fusion, BSG12, failed to grow beyond one week when transferred to a 2 ml well. The remaining colonies were cloned twice and frozen stocks of the cell lines maintained. These cell lines were designated ASE10, SC5, ASD5 and BSG11.

d. Fusion nine (Table 4.7).

This fusion performed with splenocytes from an IP immunised mouse yielded the highest number of hybrid colonies and the highest percentage of IF positive hybridomas. MicroELISA positive colonies represented 8% of all colonies and IF positive colonies represented 7% (Table 4.8). Over 80% of microELISA positive colonies were IF positive. The IF positive cell lines were designated 9-F4, 9-E8, 9-F10, 9-D11, 9-B4, 9-C11 and 9-C9. In addition to the above anti-GMF positive cell lines, an IgM producing (see Chapter Five) colony which had no specificity for kidney antigens (i.e. was negative by microELISA and IF microscopy) was cloned twice and stocks frozen. This cell line, named G6, was to be used to produce monoclonal antibody for use as a negative control in studies performed in vitro and in vivo with the anti-GMF monoclonal antibodies.

4.4 Discussion.

The cause of the failure to produce viable hybridomas in the first five fusion attempts was uncertain. The demonstration of mycoplasma infection of the myeloma cell lines, however, provides a likely explanation for the failed fusions. Mycoplasma infection

TABLE 4.6.

Screening of microELISA-positive hybridoma supernatants from fusion 8 by indirect IF microscopy on nomal rat kidney.

Hybridoma clone	MicroELISA	Indirect IF Microscopy	
		Glomeruli	Tubules
BSG12	+	+ + +	±
BSC3	+	-	-
BSG11	+	+ +	-
ASE10	+	+ + +	-
SC5	+	+ +	-
ASD5	+	+ + +	±
ASG10	+	-	-

+	weak staining
+ +	moderate staining
+ + +	strong staining
±	occasional/inconsistent staining
-	negative

TABLE 4.7.

Screening of microELISA-positive hybridoma supernatants from fusion 9 by indirect IF microscopy on nomal rat kidney.

Hybridoma clone	MicroELISA	Indirect IF Microscopy	
		Glomeruli	Tubules
9-F4	+	+	-
9-E8	+	+	++
9-F10	+	+	-
9-B11	+	-	-
9-B4	+	±	-
9-C11	+	±	++
9-C9	+	++	-
9-D11	+	+	-
G6	-	-	-

+	weak staining
++	moderate staining
+++	strong staining
±	occasional/inconsistent staining
-	negative

TABLE 4.8.

MicroELISA and IF-positive colonies
from fusions 6 to 9.

Fusion	No. of wells positive for hybridoma growth	microELISA positive (%)	IF positive (%)
6	66	16.6	4.5
7	84	5.9	3.5
8	84	8.3	5.9
9	100	8.0	7.0

MicroELISA and IF positive wells from the four fusions which resulted in the successful production of hybridomas. The results are expressed as a percentage of the total number of wells seeded with cell suspension following the fusion step.

of cell cultures may result in a number of deleterious effects on cell function (Stanbridge, 1981; Bastin et al., 1982). In addition, the eradication of mycoplasma from infected cultures is difficult and not always successful (Campbell, 1984; McGarrity et al., 1984). If antibiotics are used in an attempt to eradicate the contamination, the mycoplasma rapidly acquire resistance to them (Goding, 1983). As alternative sources of uncontaminated myeloma cell lines were readily available, it was considered safer to replace the contaminated myeloma cell lines with fresh stocks.

Fusion frequency has been reported to be as low as only 1 to 100 cells per 10^7 lymphocytes; from a typical fusion using the murine system a total of 100 hybridoma clones could be expected (Campbell, 1984). Not all of these 100 hybridomas would secrete monoclonal antibody of the desired specificity. The rate of successful generation of hybridomas secreting antibody of the desired specificity is difficult to determine as it varies with the antigen used and the expertise of the operator (Bastin et al., 1982). Westerwoudt (1986) has put the chance of generating an antigen-specific hybridoma in the order of 10^{-8}. From four fusion experiments undertaken with uncontaminated myeloma cells, a total of 18 anti-GMF secreting hybridomas were generated. Two of these colonies failed to grow and were subsequently lost. Hybridomas are known to be highly unstable during the early post-fusion period (Foster, 1982; Westerwoudt, 1985). Chromosome loss is greatest during this vulnerable period and decreases with time. The chance of chromosome loss, however, is always present and may occur at any subsequent time.

The number of microELISA positive colonies producing a positive IF result varied from 28% to over 80%. In addition, nine out of 64 wells negative for hybridoma growth returned a positive microELISA result. The frequency of antibody-forming cells present in the spleen is reported to be low, being of the order of 10^{-3} to 10^{-5} (Kearney, 1984); however, it is probable that unfused splenocytes producing anti-GMF antibody could continue to secrete antibody during the first seven days post-fusion. Supernatants taken from these wells and examined by microELISA for anti-GMF activity would return a positive result. This may also be the mechanism by which the supernatants from wells containing hybridomas returned a positive microELISA result, but when transferred to 2ml wells, with fresh medium diluting the post-fusion HAT medium, the supernatant was unable to produce a positive IF staining.

Chromosome 12 carries the immunoglobulin heavy chain locus. If this chromosome was lost during the post fusion period by a hybridoma clone which had initially recorded positive anti-GMF activity, the result would be a loss of immunoglobulin secretion and therefore anti-GMF activity in the culture supernatant. Although this possibility was not investigated, it cannot be excluded and may acount for the negative results obtained by IF microscopy on supernatants taken from wells which had initially recorded a positive result by micoELISA.

The loss of chromosome six, which carries the kappa light chain locus, from hybridoma cells results in the intracellular accumulation of free heavy chains which is reported to be toxic to the cells (Kohler, 1980). Although the preferential loss of light chain expression is rare (Goding, 1983), the possibility exists that this may account for the loss of the two hybridoma colonies post-fusion.

Chapter Five

RECOVERY AND PURIFICATION OF ANTI-GMF MONOCLONAL ANTIBODIES.

5.1 Introduction.

After hybridomas have been cloned and established, the monoclonal antibody secreted by them must be recovered in a useable form. Monoclonal antibody may be purified from either hybridoma culture supernatant or from ascitic fluid recovered from mice in which the hybridomas have been established as tumours in vivo or from serum. Spent cell culture medium, recovered from cultures in which the hybridomas have been grown to a high density, contains secreted monoclonal antibody. Typically the concentration is low, 1 - 10 μg/ml (Westerwoudt, 1985) but may reach 5 - 50 μg/ml (Goding, 1983). Monoclonal antibody recovered from spent cell culture medium has an advantage when compared with antibody produced by other procedures in that it is usually the only mouse protein found in any significant concentration within the medium. However, the vast majority of protein that is present in spent cell culture medium originates from the FCS added to induce growth of the hybridomas. Theoretically, a nearly pure monoclonal antibody could be recovered from hybridomas grown in serum-free defined medium; however, no such medium that supports significant hybridoma growth is presently available. Defined medium is also unlikely to solve the problem of poor secretion by hybridomas in vitro and the resulting low concentration of monoclonal antibody in spent cell culture medium (Samoilovich, 1987).

If hybridoma cells are transferred to syngeneic mice, they undergo transformation from immature to mature antibody secreting plasma cells (Westerwoudt, 1985). Antibody secretion from these hybridomas that have become established as peritoneal tumours is reported to be greater than from hybridomas maintained in vitro (Renau-Piqueras et al., 1983) and is typically in the range of 20mg/ml in ascites (Goding,

1983). The major advantage of hybridomas grown intraperitoneally is that large quantities of ascitic fluid containing a high concentration of monoclonal antibody can be obtained from a single mouse. The disadvantage of harvesting monoclonal antibodies from ascitic fluid is that the fluid contains a heterogeneous population of proteins from which the monoclonal antibodies may not be easily separated. When mouse immunoglobulin is isolated from the ascitic fluid, the monoclonal antibody is contaminated with native irrelevant antibodies. Ascitic fluid also contains proteolytic enzymes which may degrade the monoclonal antibody if the enzymes are not removed or if protease inhibitors are not added.

A number of procedures, originally developed for the purification of immunoglobulins from serum and other complex solutions, have been applied to the purification of monoclonal antibodies. These procedures include ammonium sulphate precipitation (Goding, 1983), polyethylene glycol precipitation (Neoh et al., 1986), staphylococcal protein A binding (Kearney, 1983), and diethylaminoethyl (DEAE) chromatography (Goding, 1980). A complication in the use of these methods is that the two major classes of antibodies, IgG and IgM, behave differently under identical conditions. The purification of IgM has generally presented greater problems than has IgG purification. IgM does not have a high affinity for protein A and therefore binds poorly to it (Ey et al., 1978). IgM may also be denatured by classical euglobulin precipitation (Goding, 1983). Exclusion chromatography usually produces IgM peaks which are contaminated with α-2 macroglobulin, a protein which has a similar molecular weight to IgM.

It was intended that the monoclonal antibodies produced in this project be examined both in vitro and in vivo to determine both their renal and non-renal binding patterns. It was also intended to determine the nephritogenicity of the monoclonal antibodies in vivo. In order to characterise the monoclonal antibodies produced by the hybridomas and to examine the in vitro and in vivo binding and nephritogenicities of individual monoclonal antibodies, large quantities of pure monoclonal antibodies would be required.

This chapter describes the procedures used to produce and purify the monoclonal antibodies for use in the studies described in Chapters Six and Seven.

5.2 Materials and Methods.

5.2.1 Production of monoclonal antibody.

a. In vitro :

Hybridoma cells were grown in 75 cm^2 cell culture flasks to a density which rendered the phenol red indicator dye a pale yellow, indicating a shift towards a more acidic pH. At this point cells were removed from the cell culture medium by centrifugation at 500 x g for 5 minutes. Supernatant was transferred to a sterile 50 ml centrifuge tube which was found to be a satisfactory vessel in which to store the supernatants. Thimerosal (Sigma Chemical Co., USA) was added as a bacteriostat to a final concentration of 0.01%, and the supernatants were stored at 4°C until used.

b. In vivo :

In order to establish the hybridomas as peritoneal tumours, male BALB/cJ mice, 40 to 80 days of age, were injected IP with 0.5 mls of 2,6,10,14-tetramethyl-pentadecane ('Pristane', Sigma Chemical Co., USA) 7 to 21 days prior to the injection of hybridoma cells. In later experiments, mice also received 400 Rads total body irradiation 24 hours prior to the injection of cells to improve the number of successful tumour inductions (Kozbor et al., 1985; Weissman et al., 1985). Hybridoma cells were grown as in (a) above but were not allowed to attain a high enough density to cause a pH shift within the medium. Cell density was determined by counting in a cytometer prior to harvesting of the cells by centrifugation. The cell pellet was gently resuspended in 0.5-1.0 mls of PBS. A total of 3-5 x 10^6 cells were resuspended in 0.25 - 0.5 mls of PBS and injected IP into each mouse. When the abdomen of the mouse was noticeably distended by accumulation of ascitic fluid, a peritoneal paracentesis was performed with an 18 G needle under light ether anaesthesia. Following the first paracentesis, the procedure was performed on alternate days until the mouse died. Ascitic fluid was collected into clean, heparinised test tubes and centrifuged at 1000 x g for 10-15 minutes. Supernatant was transferred to sterile plastic containers, and either thimerosal, as for cell culture supernatant storage, or sodium azide to a final concentration 0.01 M, was added.

5.2.2 Ouchterlony radial immunodiffusion.

The isotypes of the monoclonal antibodies were determined by radial immunodiffusion in agarose gel. A 1% (w/v) solution of agarose was prepared in PBS. The solution was heated to 70°C on an electric hotplate with a magnetic stirrer until all the agarose had dissolved. The solution was layered onto glass microscope slides while still warm and allowed to cool. When set, a centre well and 5 peripheral wells were cut using a gel punch and template. Monoclonal antibody was precipitated from cell culture supernatant by ammonium sulphate precipitation (see 5.2.3 below), dissolved in PBS, and 10 μl samples of the monoclonal antibody were distributed to the central wells of the gels. Aliquots of 10 μl of anti-mouse immunoglobulin class-specific anti-sera (Sigma Chemical Co.) were distributed to the peripheral wells. The slides were placed on damp tissue paper in a plastic box with a tight-fitting lid and diffusion allowed to continue at 4°C for 48 hours. The slides were then dialysed against PBS for 48 hours. Following dialysis, the gels were stained in 0.25% Coomassie blue G 250 in 40% methanol/10% acetic acid at RT on a shaking platform for 45-60 minutes. The gels were destained in 40% methanol/10% acetic acid, usually overnight, in an airtight container and examined for the presence of precipitin lines.

5.2.3 Ammonium sulphate precipitation of monoclonal antibody.

Monoclonal antibody was precipitated from either cell culture supernatant or ascitic fluid by the method of Goding (1983). A saturated ammonium sulphate (SAS) solution was prepared by addition of solid ammonium sulphate to one litre of distilled, deionised water (ddH_2O) at 60°C with constant stirring until the solution was saturated. This was allowed to cool and was then stored as a stock solution at 4°C. Immediately prior to use, the SAS was filtered through Whatman No. 1 filter paper and the pH adjusted to 7.2 with sulphuric acid. An equal volume of SAS was then added to spent cell culture medium or ascites dropwise with constant stirring at 4°C. Stirring was continued for 10-15 minutes after all the SAS had been added. The proteins precipitated in 50% SAS were recovered by centrifugation at 10,000 x g for 10 minutes. The pellet was washed twice in 40% SAS and the final pellet was dissolved in PBS and dialysed for up to 48 hours against PBS.

5.2.4 Gel Chromatography.

a. Hydroxylapatite chromatography.

Cell culture supernatant and ascitic fluid were chromatographed on hydroxylapatite by the method described by Stanker et al. (1985). A 15 cm hydroxylapatite (HTP grade, BioRad Laboratories) column was poured into a 2.5 cm (internal diameter) glass column 20 cm high (Econocolumn, BioRad Laboratories) following the manufacturer's instructions. The column was equilibrated overnight with 0.01 M phosphate buffer pH 6.8 (Appendix I) at a constant flow rate of 1 ml/min delivered by a peristaltic pump. Samples (5-15 mls) were applied to the column via the peristaltic pump, followed by one bed volume of 0.01 M phosphate buffer. The absorbance of the column effluent at 280 nm was monitored by a Uvicord spectrometer (LKB, Sweden). The column was eluted with a 0.01 M to 0.3 M linear phosphate gradient delivered from a gradient maker (Pharmacia, Sweden). All procedures were performed at RT.

b. Agarose gel chromatography.

A 1.5 cm (internal diameter) column 50 cm high was packed with BioGel A 1.5 M, 100-200 mesh (BioRad Laboratories). This agarose gel has an exclusion limit of 1500 kDa. The column was allowed to settle for 24 hours and then equilibrated with PBS, 0.3 ml/min via a peristaltic pump. Samples of cell culture supernatant or ascitic fluid were layered on the top of the gel, and the column was then eluted with PBS at RT. Flow rate was maintained with a peristaltic pump, and effluent was monitored by UV (280 nm) absorption and fractions collected.

c. Affinity chromatography.

A 10 ml plastic syringe was adapted to function as a chromatography column, and 5 mls of an anti-mouse IgM-agarose-conjugated affinity gel (Sigma Chemical Co.) suspended in PBS was de-aired under vacuum and poured into the column. The column was equilibrated with PBS at RT for at least 15 minutes prior to the application of samples. Monoclonal IgM-containing ascitic fluid or spent cell culture supernatant was applied to the column in a peristaltic pump at 1 ml/min. Column effluent was monitored by UV absorption and the column eluted with PBS. Elution was continued for 10-15 minutes after UV absorption had peaked and returned to zero. Bound IgM was released from the column by elution with 0.1 M glycine-HC1, pH 2.8 (Appendix I) and the eluted protein peak collected. The sample was

immediately returned to neutral pH by the addition of 0.1 M sodium hydroxide. The samples were concentrated by negative pressure dialysis and then dialysed against PBS for 24-48 hours. Following elution at pH 2.8, the column was re-equilibrated with 50-60 mls PBS. When not in use all columns were stored at 4°C in buffers containing 0.01% thimerosal.

5.2.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed in polyacrylamide slab gels using the discontinuous buffer system described by Laemmli (1970). Gel casting and electrophoresis were performed in Protean II or Mini-Protean systems (BioRad Laboratories). Polyacrylamide separating gels varied from 5 to 12.5% polyacrylamide. All gels were cast with a 4% stacking gel. For composition of gels and buffers, see Appendix I. Prior to SDS-PAGE, chromatography peak fractions were concentrated to approximately 1/10 of their original volume in 'Minicon' concentrator cells (Amicon Scientific, Victoria, Australia).

For electrophoresis, 20 - 100 μl of concentrated sample was diluted in not less than two volumes of sample buffer containing glycerol and the dye bromophenol blue. For samples of dilute protein solutions, 100 μl aliquots were diluted in an equal volume of double-strength sample buffer. Before application to the gels, samples were reduced by boiling in a water bath for four minutes. Samples were applied to the gel wells with a 100 μl glass microsyringe (SGE Ltd., Australia). In the Protean II system, electrophoresis was conducted at 16 mA per gel until the dye front had traversed the stacking gel, about 60-75 minutes, and at 24 mA per gel through the main separating gel until the dye front reached the end of the gel. For samples run on the Mini-Protean system, electrophoresis was conducted at a constant voltage of 50 V through the stacking gel and 200 V through the separating gel. Following electrophoresis, the gels were stained with Coomassie blue and destained as described in 5.2.3 above for Ouchterlony gels. If protein band staining was weak, the gels were destained for a further 24 hours and then silver-stained. Silver staining was performed using a prepared kit (BioRad Laboratories) following the protocol recommended by the manufacturers. Stained gels were then equilibrated in ddH_2O for 24 hours prior to being photographed.

5.2.6 Determination of reactivity titres of samples and column fractions.

The reactivity titres of samples prior to SAS purification or column chromatography, and of column fractions, was determined by indirect IF on normal Lewis rat kidney as described in Chapter Three. Doubling dilutions of the test sample were made and applied as the first antibody. Sections were scored as 3+ for strong reactivity, 2+ medium, and 1+ weak. The end point of titration was the last dilution at which 1+ reactivity was clearly evident.

5.3 Results

5.3.1 Monoclonal antibody isotype determination.

Ouchterlony immunodiffusion revealed that the majority of the monoclonal antibodies were IgM in type (Table 5.1). Precipitin lines were often only faintly visible following diffusion but were clearly obvious after staining with Coomassie blue (Fig 5.1). SDS-PAGE of SAS-precipitated fractions, obtained from spent cell culture medium, revealed the presence of an 80 kDa band characteristic of IgM (Fig 5.2). This 80 kDa band was common to all samples obtained from spent cell culture supernatants taken from hybridoma cultures but was absent from the control sample obtained from unused RPMI medium supplemented with FCS. These data support the results obtained with Ouchterlony immunodiffusion.

5.3.2 Production of monoclonal antibody-containing ascitic fluid.

Initial attempts to produce ascites in Balb/cJ mice with SC5, ASD5 and PH7 hybridoma cells produced less than 50% success rates (Table 5.2). In subsequent experiments the mice received 400 Rads total body irradiation 24 hours prior to the injection of cells. The inclusion of this procedure in the protocol greatly improved the success of ascites formation. After adopting routine irradiation of mice prior to the administration of cells, only two out of over 100 mice failed to develop ascites.

5.3.3 Isolation of monoclonal antibody from ascitic fluid by SAS precipitation.

Following precipitation of immunoglobulins by SAS, samples of ascites and SAS-precipitates were examined by SDS-PAGE (Fig 5.3). The number of protein bands

Table 5.1

Determination of anti-GMF monoclonal antibody isotype and subclass.

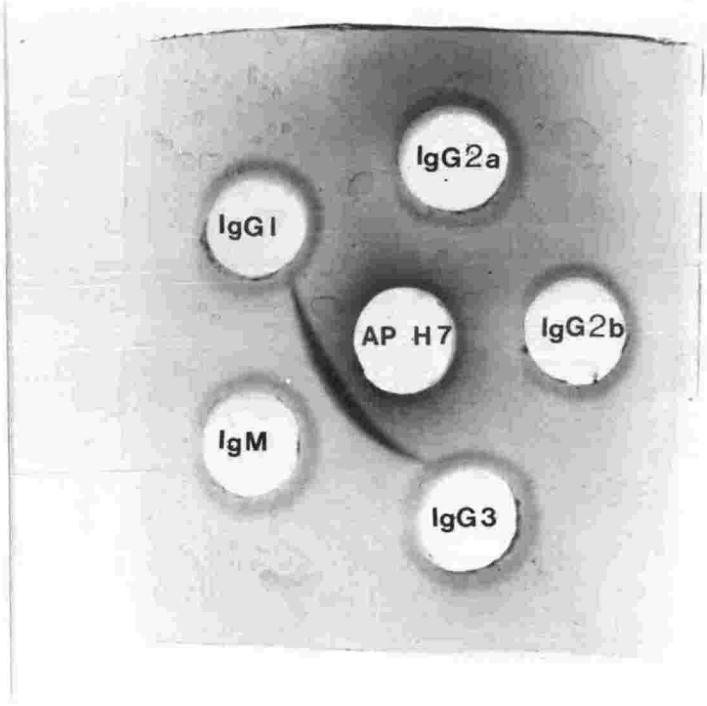
Monoclonal Antibody	Immunisation Regimen	Isotype/ Subclass
9-F4	IP	IgM
9-E8	IP	IgG1
9-F10	IP	IgM
9-D11	IP	IgG1
9-B4	IP	IgG1
9-C11	IP	IgG1
9-C9	IP	IgM
PH7	IP	IgM
APE10	IP	IgM
BPC4	IP	ND
IG12	IP	ND
IC12	IP	ND
ASE10	IS	IgM
SC5	IS	IgM
ASD5	IS	IgM
BSG11	IS	IgM

IS - Intrasplenic
IP - Intraperitoneal
ND - Not done

Monoclonal antibody isotype and subclass were determined by Ouchterlony immunodiffusion in agar gels.

Figure 5.1.

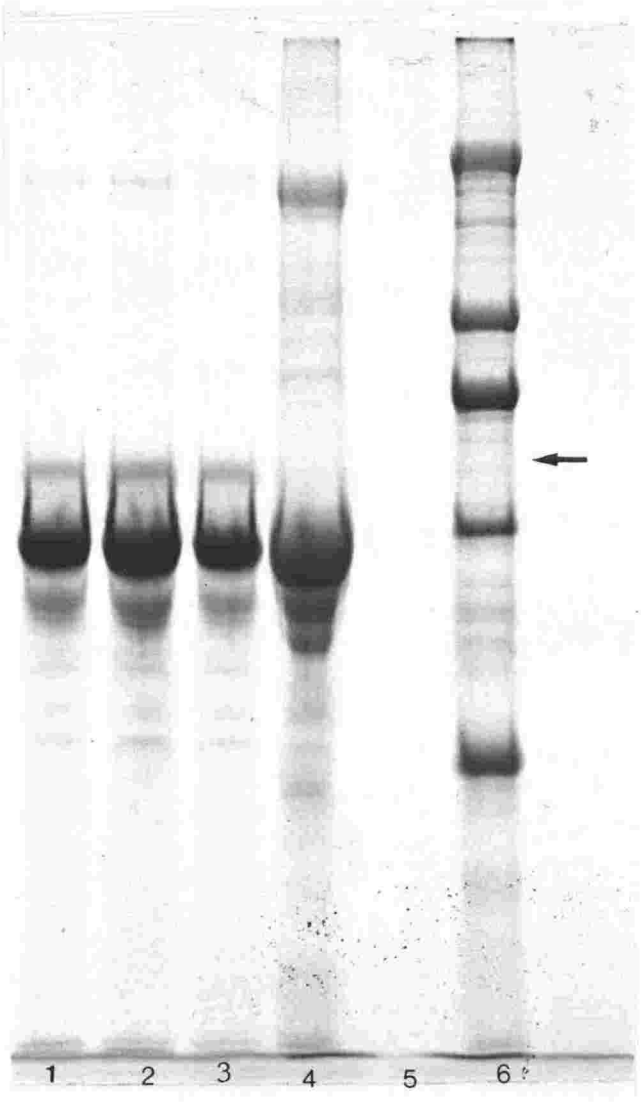
Ouchterlony Immunodiffusion of SAS-precipitated PH7 monoclonal antibody-containing cell culture supernatant against anti-mouse class-specific anti-sera.



A 10 μl sample of PH7 monoclonal antibody, precipitated from cell culture supernatant and redissolved in PBS, was placed in the central well, and anti-mouse class specific anti-sera in the peripheral wells. The gel has been stained with Coomassie blue. A single precipitin line is clearly seen with anti-mouse IgM anti-serum.

Figure 5.2.

SDS-PAGE of cell culture supernatants from hybridoma cultures.



Samples of SAS-precipitated cell culture supernatants containing monoclonal antibody were applied to the lanes of a 7.5% polyacrylamide gel.
Lane 1 - PH7; Lane 2 - G6; Lane 3 - SC5; Lane 4 - Unused RPMI; Lane 6 - High molecular weight markers. No sample was applied to lane 5. The arrow represents 80 kDa.

Table 5.2.

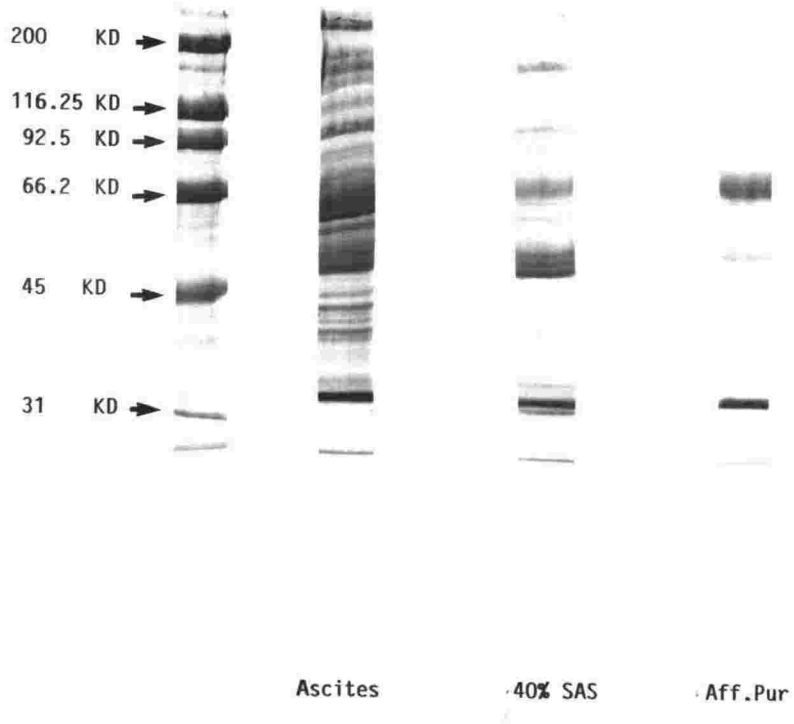
Ascites production in non-irradiated mice.

Cell line	Number of mice receiving cells	Number of mice developing ascites
PH7	8	3
SC5	9	4
ASD5	8	4

All mice were primed with 'Pristane' prior to receiving 3-5 x 10^6 cells IP. Mice were observed for a period of 28 days. If ascites had not developed at this time, the surviving mice were electively sacrificed.

Figure 5.3.

SDS-PAGE profile of monoclonal antibody-containing ascites and SAS precipitates of ascites.



Samples of monoclonal antibody-containing ascites and unfractionated ascites were applied to a 7.5% polyacrylamide gel and electrophoresed as described in the methods. The gels are stained with Coomassie blue. The protein content of the samples was similar and ranged from 40 to 50 μg.

visualised in the precipitated sample was less than the unfractionated sample. Some high molecular weight bands which were present in both samples were generally of lesser intensity in the precipitated fraction. The 79 kDa band, representing IgM heavy chains, was dominant in both samples. SAS precipitation greatly reduced the amount of contaminating proteins without affecting the reactivity titre of the monoclonal antibody. Typically, a 20 ml sample of ascites precipitated by SAS was dissolved in 10 mls of PBS. When reactivity was examined by indirect IF microscopy, the titre of the SAS precipitate was either the same or one dilution greater than the unfractionated ascites; thereore, the maximum loss of activity was only 50%.

5.3.4 Gel chromatography.

a. Hydroxylapatite chromatography.

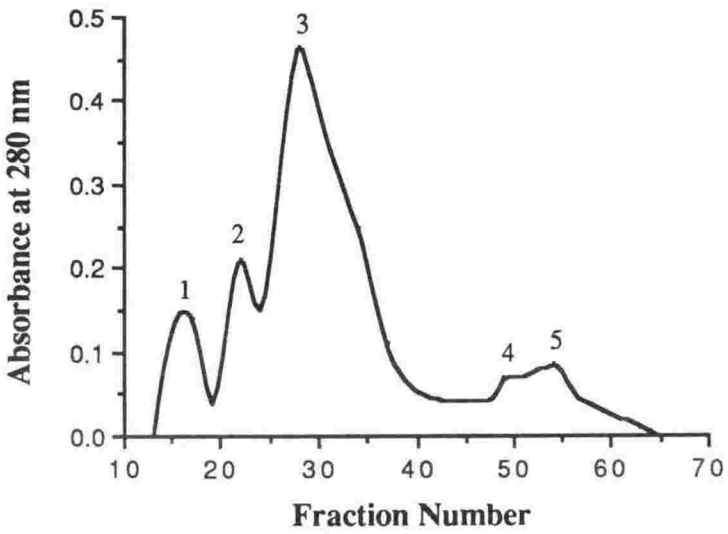
Samples of cell culture supernatant and monoclonal antibody-containing ascites were titrated for anti-GMF activity by indirect IF microscopy prior to chromatography on hydroxylapatite. The elution profile of a 10 ml sample of SC5 ascites is shown in Fig. 5.4. Three major peaks were seen at fractions 17, 22 and 29. A small shoulder occured following peak three, and two smaller peaks occurred at fractions 48 and 54. The anti-GMF titre of the SC5 ascites prior to chromatography was 1/1600. Following chromatography, column fractions with peak absorbance at 280 nm were examined by indirect IF microscopy for anti-GMF activity. None of these peaks showed anti-GMF activity.

The electrophoretic profile of the concentrated peak fractions is shown in Fig. 5.5. A major band corresponding to 29 kDa was seen in peak one together with some larger molecular weight protein. Very few low molecular weight proteins were seen in this peak. Two major bands were evidennt in peak two, corresponding to 52 and 26 kDa. A small amount of larger molecular weight material was also present in this peak. Peak three was a heterogenous fraction with protein bands occurring across a wide range of molecular weights with a major band occurring at 26 kDa. No material was resolved from peak four; a single band is resolved in peak five corresponding to 26 kDa. This 26 kDa protein is common to peaks two, three and five.

Further samples of ascites containing PH7 and ASD5 were chromatographed on hydroxylapatite. The reactivity titres of the ascites, as assessed by indirect IF

Figure 5.4.

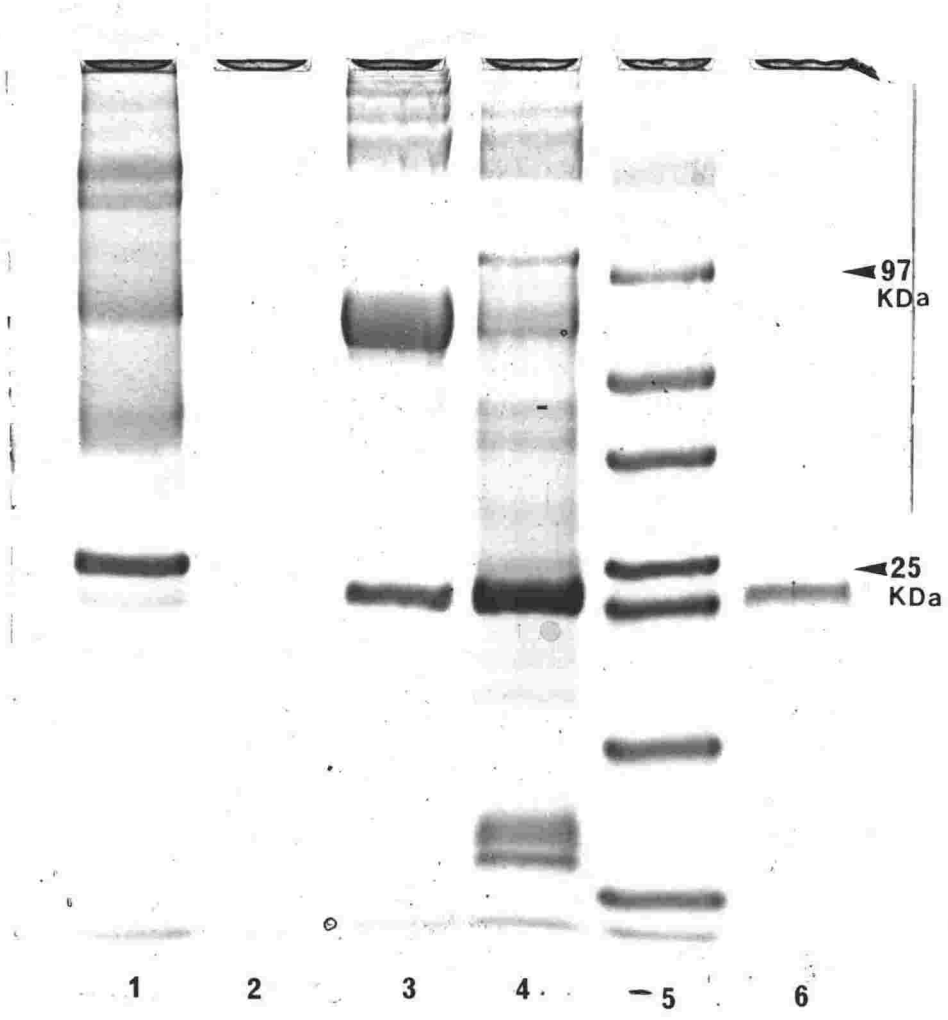
Chromatography elution profile of SC5 ascites on hydroxylapatite.



A 10 ml sample of SC5 ascites was applied via a peristaltic pump to a hydroxylapatite column at a flow rate of 0.5 ml/min. The column was eluted with a 0.1 - 0.3 M, pH 6.8, phosphate buffer as a continuous gradient. Four minute (2 ml) fractions were collected. Column eluate absorbance at 280 nm was monitored through a flow cell.

Figure 5.5.

Electrophoretic profile of peak fractions obtained from chromatography of SC5 on hydroxylapatite.



Samples were applied to the wells of a 12% polyacrylamide separating gel and electrophoresis continued until the dye front was within 1 - 2 mm of the end of the gel. The gel is stained with Coomassie blue. Lane 1 - peak 1; Lane 2 - peak 4; Lane 3 - peak 2; Lane 4 - peak 3; Lane 5 - low molecular weight standards; Lane 6 - peak 5.

microscopy prior to chromatography, were 1/1600 for APH7 and 1/3200 for ASD5. Four peaks were obtained with both preparations at similar positions to those occurring with SC5. The peak fractions were examined by indirect IF microscopy. None of the peak fractions stained normal rat kidney.

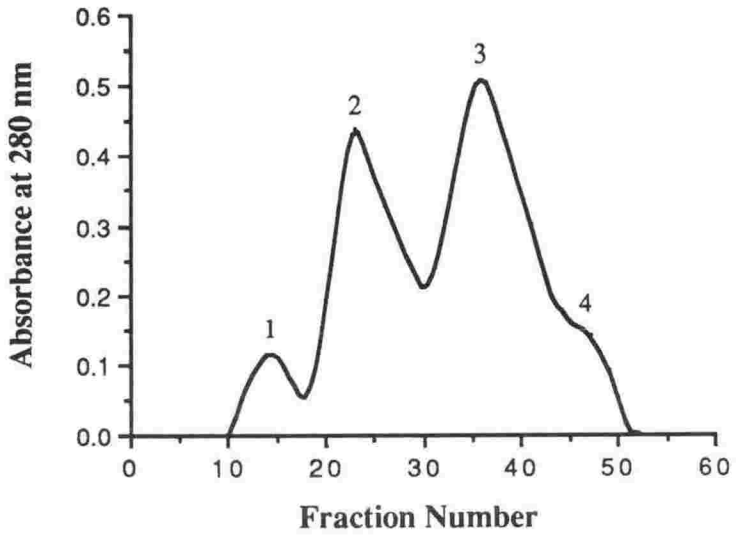
b. Agarose gel chromatography.

The elution profile obtained from chromatography of 10 mls of SC5 ascites on BioGel A 1.5 M is shown in Fig. 5.6. Three major peaks were clearly evident with a pronounced shoulder on the descending limb of the third peak. Prior to chromatography, indirect IF microscopy indicated the anti-rat kidney titre of the SC5 ascites to be greater than 1/3200 (the greatest dilution examined). Following chromatography, the reactivity was seen to reside in peaks one and two only. SDS-PAGE of the column peaks was undertaken on a 7.5% polyacrylamide gel (Fig 5.7). A major band, presumably IgM heavy chains, was seen at 80 kDa in both peaks one and two together with a less intensely stained band at 52 kDa. Both of these bands were absent from peak three. PH7 ascites was also chromatographed on BioGel A 1.5 M. The 280 nm absorbance profile (Fig.5.8) was similar to that obtained with SC5 (Fig 5.6), although a distinct fourth peak was evident with PH7, whereas only a shoulder on peak three was seen with SC5. The pre-chromatography IF reactivity titre of the PH7 ascites was 1/1600. Following chromatography, the anti-kidney reactivity was confined to peaks one and two, being 1/800 for peak one and 1/200 for peak two. The SDS-PAGE profile of the peak fractions on a 12% polyacrylamide gel is shown in Fig. 5.9. The majority of the large molecular weight protein was seen to lie in peak one, as was the majority of the albumin (66 kDa). Major bands were seen at 80 kDa and 25 kDa which correspond to the molecular weights of IgM heavy chain and light chains respectively. The 80 kDa band was also seen with less intensity in peak two. A band at 25 kDa was common to all four peaks. It appearred greatest in peaks two and three and was the only band visualised in peak four.

A sample of cell culture supernatant obtained from a culture in which PH7 hybridoma cells had been grown to a high density was also chromatographed on BioGel A 1.5 M agarose. The elution profile is shown in Fig. 5.10. The anti-kidney reactivity titre of the supernatant prior to chromatography was 1/20. Following chromatography, no reactivity was found in any of the peak fractions.

Figure 5.6.

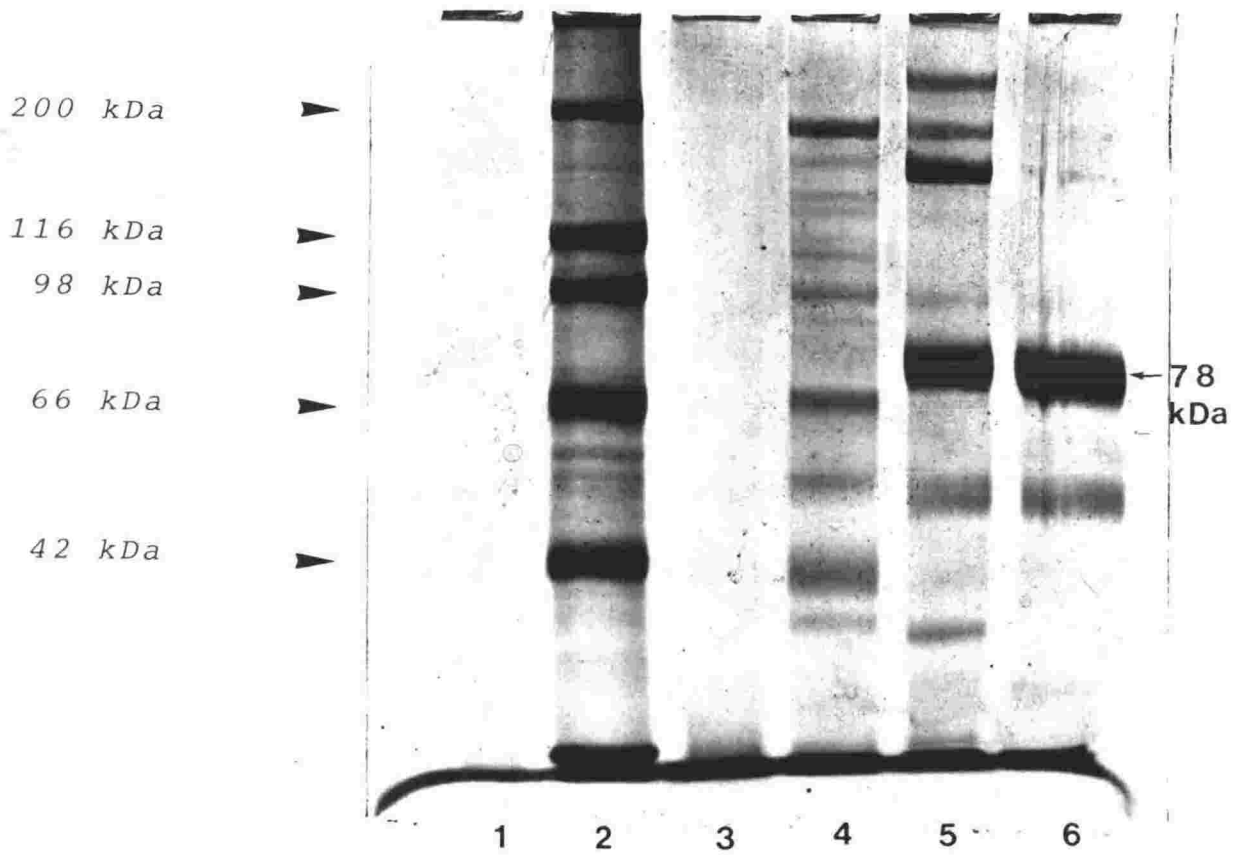
Elution profile of SC5 ascites chromatographed on BioGel A1.5 M agarose.



A 10 ml sample of SC5 ascites was applied to a 50 x 25 cm BioGel A1.5 M agarose column via a peristaltic pump. The column was eluted with PBS, pH 7.2, at a flow rate of 0.5 ml/min. Four minute (2 ml) fractions were collected. Column eluate absorbance at 280 nm was monitored through a flow cell.

Figure 5.7.

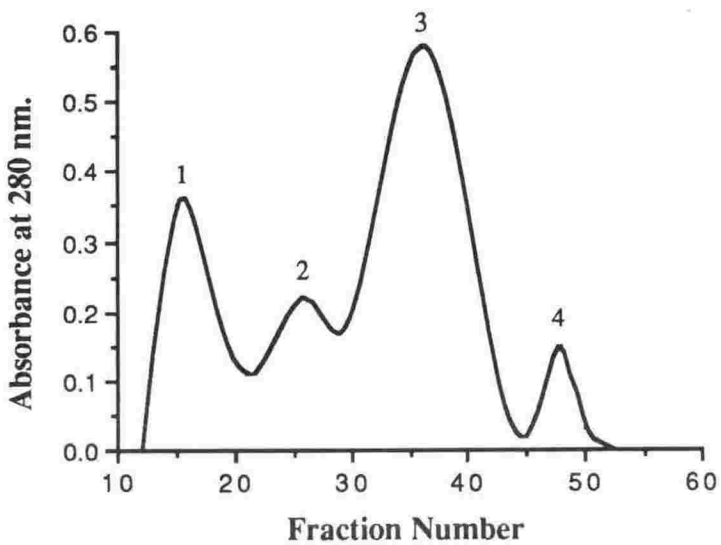
SDS-PAGE profile of peak fractions obtained from chromatography of SC5 ascites on BioGel A 1.5 M agarose.



Peak fractions from experiment of Fig 5.6 were concentrated and electrophoresed on a 7.5% polyacrylamide gel. The gel has been stained with Coomassie blue. Lane 2 - High molecular weight markers; Lane 4 - peak 3; Lane 5 - peak 2; Lane 6 - peak 1.

Figure 5.8.

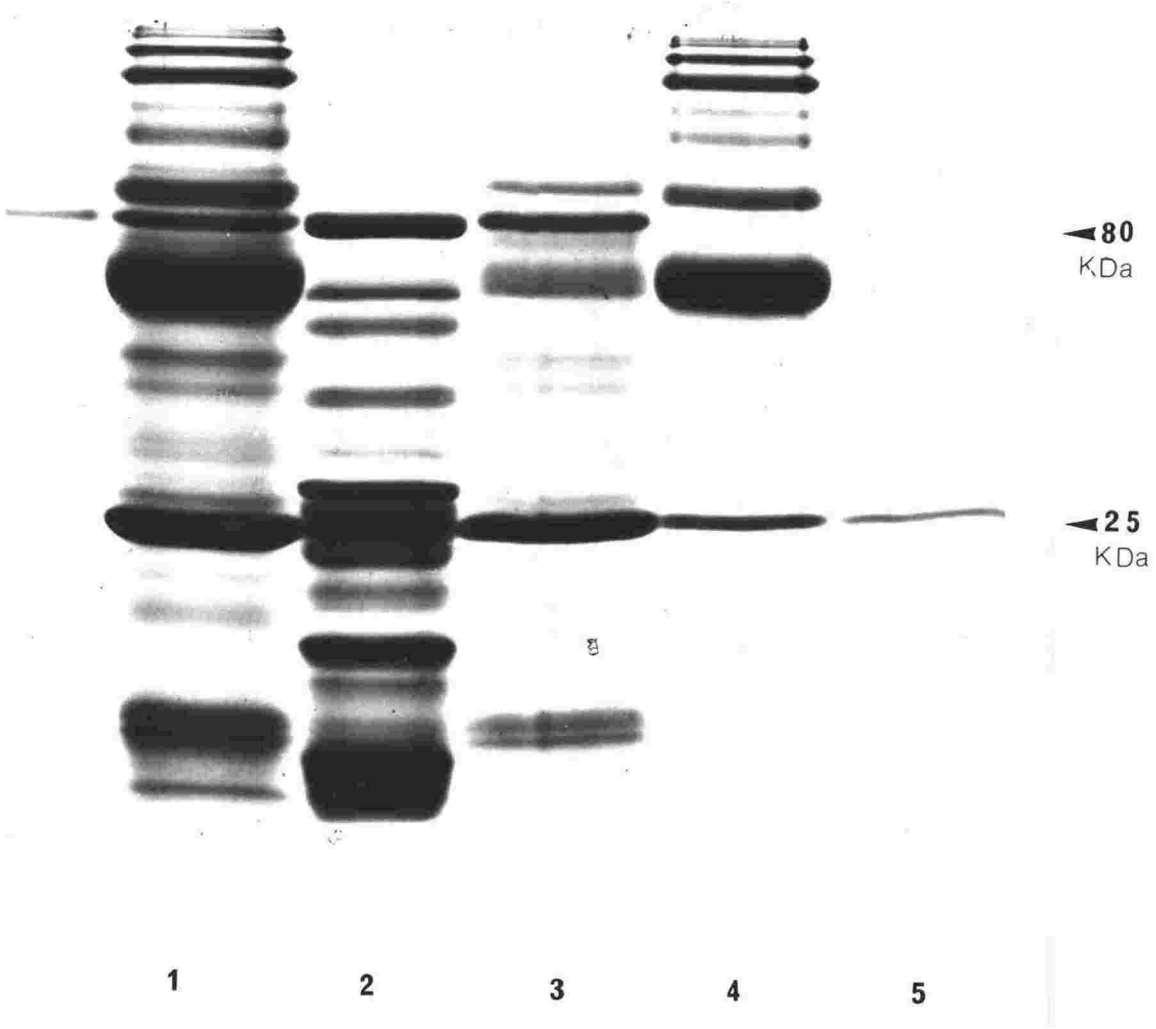
Elution profile of PH7 ascites chromatographed on BioGel A1.5 M agarose.



A 10 ml sample of PH7 ascites was applied to a 50 x 25 cm BioGel A1.5 M agarose column via a peristaltic pump. The column was eluted with PBS, pH 7.2, at a flow rate of 0.33 ml/min. Fractions were collected at six minute intervals (approx. 6 ml). Column eluate absorbance at 280 nm was monitored through a flow cell.

Figure 5.9.

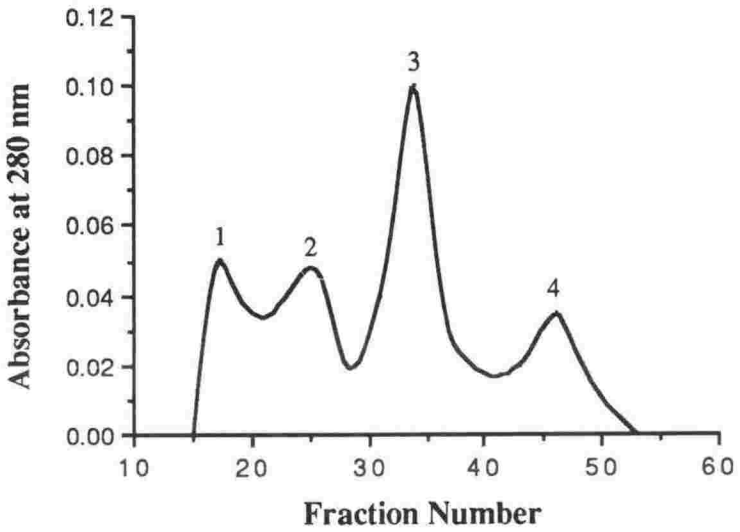
SDS-PAGE profile of column peaks obtained by chromatography of PH7 ascites on BioGel A 1.5 M agarose.



Column peak fractions of Fig. 5.8 were concentrated and electrophoresed on a 12% polyacrylamide gel. The gel has been stained with Coomassie blue. Lane 1 - PH7 ascites; Lane 2 - peak 3; Lane 3 - peak 2; Lane 4 - peak 1; Lane 5 - peak 4.

Figure 5.10.

Elution profile of PH7 cell culture supernatant chromatographed on BioGel A1.5 M agarose.



A 20 ml sample of cell culture medium in which PH7 hybridoma cells had been grown to a high density was applied to a 50 x 25 cm BioGel A1.5 M agarose column via a peristaltic pump. The column was eluted with PBS, pH 7.2, at a flow rate of 0.33 ml/min. Fractions were collected at six minute intervals (approx. 6 ml). Column eluate absorbance at 280 nm was monitored through a flow cell.

c. Affinity chromatography.

Samples of monoclonal antibody-containing ascites, cell culture supernatants, or SAS precipitates of cell culture supernatants were applied to a column of an anti-mouse IgM-agarose-conjugated gel. The elution profile of a sample of PH7 cell culture supernatant is shown in Fig 5.11. A small peak was seen to precede the main peak. The reactivity titre of the eluted monoclonal antibody in the main peak, as assessed by indirect IF microscopy, was four to five times greater than the applied sample. Reactivity of the fraction containing the small first peak was very weak and found only in the undiluted sample. An unusual elution profile was obtained with samples of ascites (Fig 5.12a and b). The reactivity of fractions corresponding to the two peaks was examined by indirect IF microscopy. Both peaks were found to contain anti-kidney activity; however, the reactivity titre of peak one was consistently two to four dilutions greater than peak two. Examination of the eluted monoclonal antibody by SDS-PAGE revealed only three protein bands, occurring at 80, 52 and 26 kDa (Fig.5.3). The 52 kDa protein was also found on SDS-PAGE of affinity-purified PH7 and ASD5.

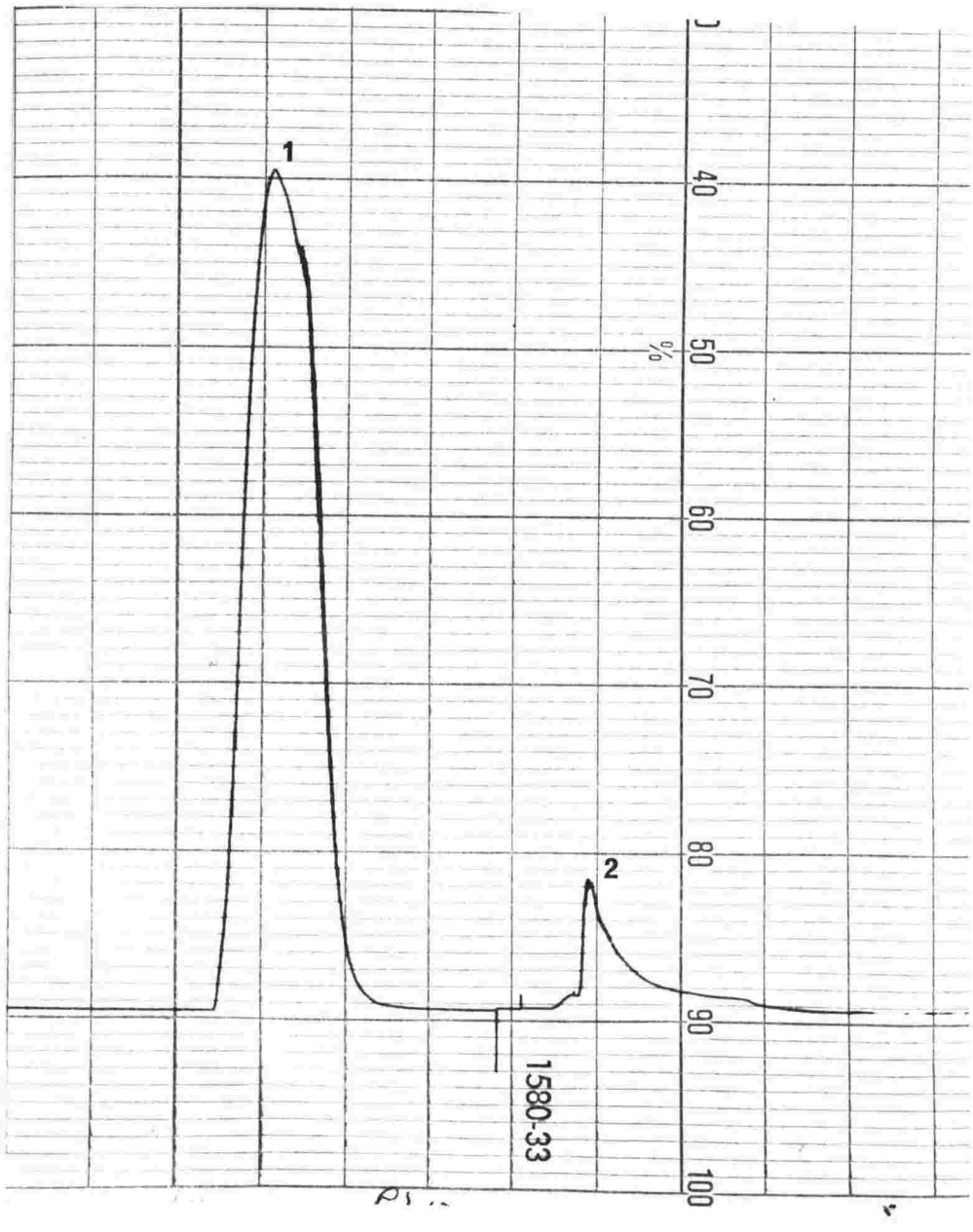
5.4 Discussion

Nine of the thirteen monoclonal antibodies examined by Ouchterlony immunodiffusion were found to be of the IgM subclass. This was not totally unexpected, particularly from those monoclonal antibodies derived from IS-immunised mouse splenocytes. Spitz et al., (1984) reported the production of both IgG and IgM secreting hybridomas from IS-immunised splenocytes; whereas, in this study only IgM-secreting hybridomas were produced. However, it has been reported that IgM may be not only the first, but the only immunoglobulin generated by the immune response to certain antigens (Teale and Klinman, 1984); and therefore this predominance may be anticipated.

All the hybridomas which were chosen and propagated in vivo were IgM secretors. The inability to produce ascites in a number of the 'Pristane'- primed mice was of concern, although it has been reported that IgM-secreting hybridomas may be difficult to establish in vivo (Campbell, 1984). The use of total body irradiation to immunosuppress mice prior to the injection of hybridoma cells has been reported previously (Goding, 1983; Stanker et al., 1985; Kozbor et al., 1985; Weissman et al., 1985). The use of total-body irradiation in this study proved to be very successful.

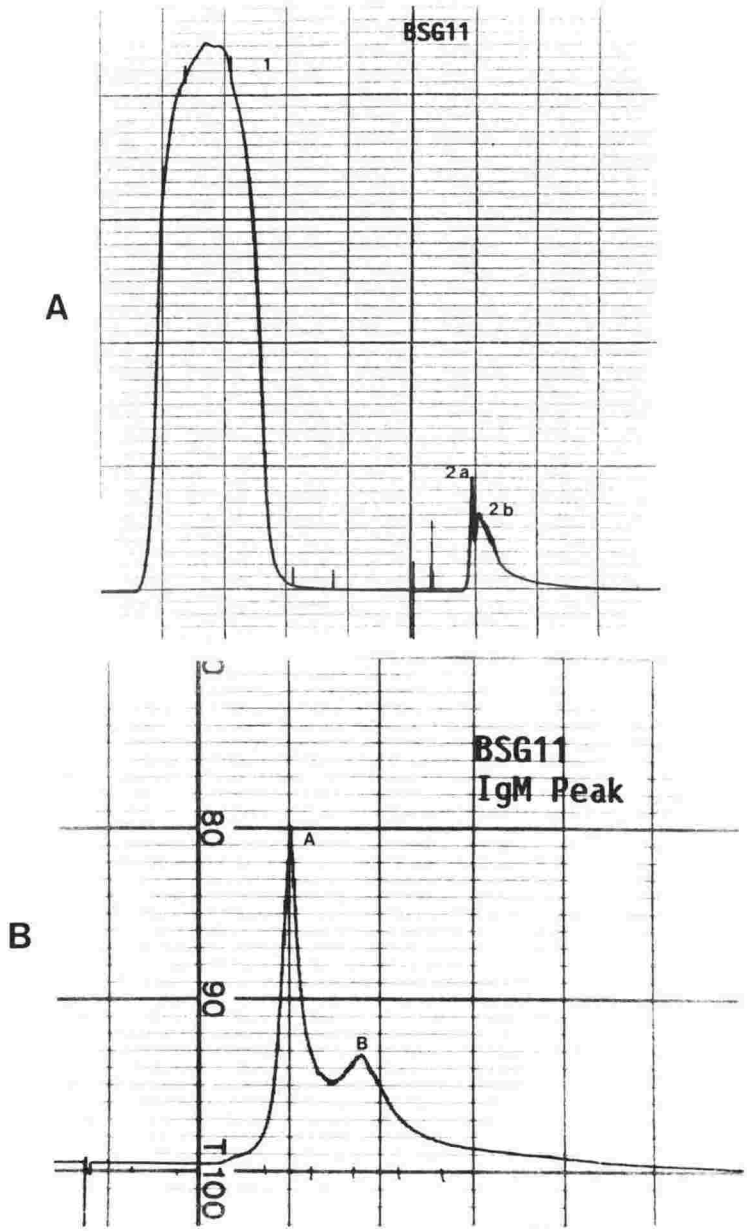
Figure 5.11.

Elution profile of PH7 cell culture supernatant
obtained by affinity chromatography.



Culture supernatant was applied to the column and eluted with 0.1M glycine-HCl, pH 2.8 as described in the methods. Peak one contains that material which did not bind to the column. Peak two is the IgM eluting with the glycine-HCl. A small peak is seen to precede the major IgM peak; the greatest anti-GMF activity resided in the main peak.

Figure 5.12.



Precipitation of monoclonal antibody from both ascitic fluid and cell culture supernatant by 40% SAS produced a monoclonal antibody preparation that retained high titre anti-GMF activity. Although the examination of the SAS-precipitated fractions revealed that there was a number of contaminating proteins remaining, they were considerably reduced compared to crude ascitic fluid.

A number of procedures have been reported in the literature detailing the purification of monoclonal antibodies from ascitic fluid or from culture supernatant using hydroxylapatite chromatography (Stanker et al., 1985; Bukovsky and Kennet, 1987). In addition, an hydroxylapatite column for high pressure liquid chromatography (HPLC) is available commercially (Bio-Rad Laboratories) for large and small scale purification of monoclonal antibodies. The purification of the monoclonal antibodies produced in this study by hydroxylapatite chromatography was disappointing. Examination of the SDS-PAGE profiles of the peaks eluted from the columns indicated that some separation of antibody was obtained, but reactivity was lost. An interesting finding was the appearance of a single protein corresponding to 26 kDa eluting in peak 5 from ASD5 ascites. This molecular weight is common to immunoglobulin light chains and could therefore represent free light chains. The first description of the use of hydroxylapatite HPLC for the purification of monoclonal antibodies (Juarez-Salinas et al., 1984) reported the resolution of two immunoglobulin light chains. The light chains appeared in the three peaks that were subsequently examined by SDS-PAGE. The authors could not determine whether the extraneous light chain was synthesised by the host mouse from which the ascites was derived, or was synthesised by the hybridoma. The myeloma cell line used to generate the hybridoma was Sp2/0, a cell line that is reported to neither secrete nor synthesise light chains (Shulman et al., 1978). It is therefore more likely that the light chain originated from the host.

The choice of BioGel A 1.5 M agarose was based on its high exclusion limit of 1500 kDa. Pentameric IgM which is in the region of 900 kDa could be expected to elute fairly soon after the bed volume. Although this gel medium was able to separate proteins with anti-GMF activity, the peaks isolated were not homogeneous. The appearance of a protein corresponding to the molecular weight of immunoglobulin light chains, in all four peaks isolated from PH7 ascites, again raises the question of free light chain secretion.

Affinity chromatography produced the best isolation of IgM from both ascites and cell culture supernatant. The identity of the 52 kDa protein is uncertain. The affinity chromatography procedure exposed the monoclonal antibody to a pH of 2.8 for a period of up to ten minutes, the most severe conditions of all the chromatography methods. Following neutralisation, the recovered monoclonal antibody retained high titre activity. It is unlikely that the two peaks observed could represent intact IgM and free light chains, since the anti-mouse IgM conjugated to the agarose was μ chain specific. The most likely reason that two peaks were present is that IgM of host origin dissociated from the column at a fractionally lower pH than did the monoclonal IgM. The major drawback to the adoption of affinity chromatography for purification of the monoclonal antibodies for use in vitro and in vivo in this study was that only small quantities can be processed at one time.

Precipitation of immunoglobulin by 40% SAS produced a reagent that retained high titre anti-GMF activity. The chromatographic procedures examined were judged not to possess significant advantages to SAS precipitation for the procedures in which the monoclonal antibodies were to be employed. The decision was therefore made to proceed with the simple but effective SAS preparations for further examination of the in vitro and in vivo reactivities of the monoclonal antibodies.

Chapter Six.

IN VITRO REACTIVITIES OF MONOCLONAL ANTI-GMF ANTIBODIES.

6.1 Introduction.

There have been a number of reports in the literature detailing the reactivities of serum from HN rats and polyclonal antibodies raised against glomerular antigens (Kerjaschki and Farquhar, 1983; Makker and Singh, 1984; Singh and Makker, 1986; Natori et al., 1986, 1987). The techniques of immunoblotting and immunoprecipitation have commonly been employed to obtain these data. Studies using these techniques have also been conducted with monoclonal antibodies directed against renal antigens (Bhan et al., 1985; Ronco et al., 1986a, 1986b; Mendrick and Rennke, 1988a, 1988b.) to determine the biochemical identity of the individual antigens with which the anti-kidney antibodies react.

MicroELISA and IF microscopy studies reported in Chapter Four, indicated that anti-GMF monoclonal antibodies reacted with glomeruli. The exact biochemical identity of the specific antigen was unknown. The monoclonal antibodies exhibited differing patterns of reactivity with the glomerulus when examined by IF microscopy, and it was assumed that there were a number of antigens present, each unique to a single monoclonal antibody. It was also likely that the antigens with which the monoclonal antibodies reacted were expressed not only on different glomerular cell types, but could also be expressed on homologous cell types outside the kidney. In other studies, monoclonal antibodies raised against kidney antigens have been reported to react with a variety of non-renal tissues (Mendrick et al., 1983; Nishi et al., 1984; Ronco et al., 1984a, 1984b; Chatelet et al., 1986b; Mendrick and Rennke, 1988a, 1988b). The fusion of splenocytes obtained from BALB/cJ mice which had been immunised with either rat kidney cortex or isolated whole glomeruli resulted in the production of a number of hybridomas secreting monoclonal antibodies that reacted with kidney but were not kidney specific (Mendrick et al., 1983). Chatelet, et al. (1986a, 1986b)

reported the wide renal and non-renal tissue distribution of a 90 kDa antigen recognised by a monoclonal antibody raised against rat Fx1A.

The following chapter details the binding sites of the anti-GMF monoclonal antibodies as assessed by IF microscopy, immunobiochemical analysis of the antigens reacting with the antibodies, the ultrastructural localisation of renal binding, and the extrarenal distribution of reactivity. To identify the reactive antigens, immunoblotting experiments were conducted with proteins solubilised by detergent extraction from either particulate GMF or from isolated whole glomeruli. The ultrastructural localisation and the extrarenal distribution of the antigens were examined by immunogold electron and IF microscopy, respectively.

6.2 Materials and Methods.

6.2.1 Examination of renal and extrarenal binding by indirect IF microscopy.

Tissue specimens were obtained immediately following sacrifice of a normal Lewis rat. Tissue was obtained from kidney, spleen, liver, small intestine, brain, aorta, stomach, skeletal muscle and the left ventricle of the heart. These were immediately snap frozen in liquid nitrogen and processed for indirect IF microscopy as described in Chapter Three. To examine interspecies reactivity of the monoclonal antibodies, renal cortical tissue was also obtained from rabbit and sheep kidney immediately following sacrifice. Human kidney tissue was obtained from an unaffected area of a nephrectomy specimen removed for renal cell carcinoma. The tissues were processed for indirect IF microscopy as described for rat kidney. The non-kidney-fixing IgM monoclonal antibody, G6, was used as a negative control in all IF microscopy experiments.

6.2.2 Determination of cross-reactivity with Heymann nephritis antigens.

To determine whether the antigens recognised by the anti-GMF monoclonal antibodies were also antigens involved in the pathogenesis of HN, experiments were carried out using polyclonal sheep anti-rat Fx1A and eluates obtained from HN kidneys in an attempt to block the binding of the monoclonal antibodies to the target. The polyclonal sheep anti-rat Fx1A and the Heymann eluates were donated by Assoc. Prof. T.J. Neale of the Wellington School of Medicine, Otago University, New

Zealand. Indirect IF microscopy was perfomed as described in Chapter Three. Sections were incubated with the monoclonal antibodies at an optimal dilution that had been determined previously. Following washing, the sections were incubated with serial dilutions of either sheep anti-rat Fx1A or Heymann eluate. Bound antibody was probed for with fluoresceinated anti-sheep or anti-rat immunoglobulins (Dako Laboratories, Denmark.). To obtain positive controls, sections were incubated with anti-rat Fx1A or Heymann eluate without prior exposure to monoclonal antibody. Negative controls were incubated with G6 as the first antibody. Sections were also incubated with anti-Fx1A and Heymann eluate and then incubated with the monoclonal antibody. Bound antibody was detected with fluoresceinated anti-mouse immunoglobulins (Silenus Laboratories, Australia.).

Immunoblotting experiments were carried out on nitrocellulose strips onto which an NP 40 detergent extract of either human or rat glomerular plasma membranes had been transferred following electrophoresis. These nitrocellulose strips were donated by Dr. D. Kerjaschki, Institute of Pathology, University of Vienna, Austria. The immunoblotting procedure was carried out using the anti-GMF monoclonal antibodies as described in 6.2.6.

6.2.3 Extraction of proteins from GMF.

Soluble proteins were obtained from GMF by extraction with one of the following detergents: 100 mM 1-0-n-octyl-ß-D-glucopyranoside (octylglucoside (OG), Boehringer-Mannheim, W. Germany); 1% deoxycholic acid (DOC, Sigma Chemical Co., USA, D-6750); 8 M urea (Mathesen, Coleman and Bell, Ohio, USA); or 10 mM (3-[cholamidopropyl)-dimethylammonio]-1-propane (CHAPS, Calbiochem, USA). OG, CHAPS and DOC were prepared as double strength solutions in PBS containing 2 mM phenylmethyl sulphonyl fluoride and 2% thimerosal. GMF was prepared as described in Chapter Three, resuspended in PBS and briefly sonicated on ice for two 15 second periods at setting Number 6 on a W375 sonicator (Heat Systems-Ultrasonics, NY, USA). Aliquots of GMF containing 3 to 6 mg of membrane protein were mixed with an equal volume of double strength detergent in buffer. Extractions were carried out at RT for 60 minutes, and samples were agitated continuously, either on a shaking platform (New Brunswick Scientific, NJ, USA) or a rotating wheel (H.I. Clements and Son, Sydney, Australia). For extractions with urea, 2 ml samples of GMF were placed in a glass beaker and stirred continuously at RT with a magnetic

stirrer. Solid urea (1.92 g) was added slowly, and the solution was made up to a final volume of 4 mls with ddH_2O. A paraffin wax film was stretched over the beaker to prevent evaporation during extraction and the beaker placed in a shaking water bath at 37°C for 24 hours.

Following extraction, all samples were centrifuged at 100,000 x g for 60 minutes. The supernatants were transferred to dialysis tubing with a high molecular weight cut-off point of 5000 kDa. The dialysis tubing was soaked in boiling ddH_2O for 5 minutes prior to use. The supernatants were dialysed against PBS at 4°C for at least 24 hours, with frequent changes of dialysis solution. The PBS dialysate was changed three times at 60 minute intervals and thereafter at three hourly intervals during the day. Following dialysis, the GMF extracts were concentrated two to five times by negative pressure dialysis, transferred to new dialysis bags, and dialysed for a further 2 hours against PBS. The final protein concentration of the extracts was determined by the method of Markwell et al., (1978). The extracts were then stored at -20°C until required.

Protein extracts were also prepared from isolated whole glomeruli. Lewis rat glomeruli, isolated by the sieving technique, were suspended in homogenisation buffer without aprotinin. The glomeruli were sonicated on ice (Model W375, Heat Systems-Ultrasonics, NY, USA) at setting number 6, with disruption monitored by light microscopy. The sonication was applied for one minute periods until no whole glomeruli were observed. The suspension was then treated with detergents and concentrated in an identical manner to that described for GMF.

6.2.4 SDS-PAGE.

Electrophoresis was performed as described in Chapter Five. A range of separating gels were used, but in most cases they consisted of a 5-15% gradient gel. This was prepared in a standard two chamber gradient maker (Pharmacia, Sweden). All gels were overlayed with a 1 cm 4% stacking gel. Aliquots of GMF protein extracts, 80-150 μl, were diluted in not less than two volumes of sample buffer, with or without dithiothreital. Samples containing 30 to 100 μg of total protein were applied to the gels with a glass microsyringe. Conditions of electrophoresis were as described in Chapter Five. Following electrophoresis, gels were either processed for transfer to nitrocellulose and subsequent immunoblotting, or stained for protein visualisation by

either Coomassie blue G250 or silver staining as described in Chapter Five.

6.2.5 Dot-blot Assay.

Dot-blot assays were performed using a 'Bio-Dot' microfiltration apparatus (Bio-Rad Laboratories, Calif. USA). A nitrocellulose sheet (BA 85, Schleicher and Schuell, W. Germany) was cut to size and prewetted in tris buffered saline (TBS, Appendix I) for 15 to 30 minutes. The nitrocellulose sheet was then positioned over the sealing gasket and the whole apparatus assembled according to the manufacturers instructions. GMF protein extracts were diluted in TBS containing 0.3% BSA (TBS-0.3% BSA) to a concentration of 10 μg/ml. All buffers containing BSA were filtered through 0.22 μm membranes prior to use. Aliquots (200 μl) of diluted GMF were dispensed to each of the 96 wells with a multichannel pipette. The antigen solution was allowed to drain from the wells by gravity, a process requiring 40 to 60 minutes for completion. When all the wells had emptied of antigen, residual binding sites on the membrane were blocked by the addition of 200 μl of TBS-3% BSA which was also allowed to drain from the wells by gravity. The wells were then washed with 200 μl of TBS/0.3% BSA. Ascitic fluid, 40% SAS-precipitated globulin fractions of ascites or cell culture supernatants, all containing anti-GMF monoclonal antibody, were filtered through 0.22 μm membranes and then diluted in TBS-0.3% BSA. Aliquots of 200 μl were then dispensed to each test well and allowed to drain by gravity. Control wells received either the non-specific G6 monoclonal antibody or TBS-0.3% BSA only. When the wells had drained, they were washed three times with TBS-0.3% BSA. The wells were emptied by vacuum between washes. Bound monoclonal antibody was detected with a horseradish peroxidase-conjugated polyclonal sheep anti-mouse immunoglobulin (Silenus Laboratories, Australia). This detecting antibody was diluted 1:1000 in TBS-0.3% BSA and distributed in 200 μl aliquots to all wells. When the diluted second antibody had drained from all wells the washing procedure was repeated. At this point the dot-blot apparatus was disassembled and the nitrocellulose sheet placed in a 2 x 15 x 20 cm glass dish containing TBS-0.3% BSA and gently agitated for 2 to 3 minutes. The buffer was then removed by vacuum and replaced by a substrate solution consisting of TBS containing 4-chloro-1-napthol (0.5 mg/ml) and 0.018% (v/v) hydrogen peroxide. The dish was then covered and placed on a shaking platform. Incubation was contined at RT until colour development was obvious. The reaction was stopped by replacing the substrate with ddH_2O and washing the nitrocellulose sheet twice in 100-150 mls ddH_2O. The nitrocellulose sheet

was air-dried prior to photography.

To examine the reactivity of the anti-GMF monoclonal antibodies with extracts of human kidney, a collagenase digest of human GBM and a DOC extract of human glomerular plasma membranes prepared by a similar method to rat GMF and named hGMF, were kindly donated by Assoc. Prof. T.J. Neale, Wellington School of Medicine, Otago University. The anti-GMF monoclonal antibodies were assayed for anti-hGMF activity in a manner identical to that used for rat GMF.

6.2.6 Immunoblotting.

Immunoblotting was performed as described by Towbin et al., (1979). Following electrophoresis, gels were equilibrated in Tris-glycine transfer buffer, pH 8.3, containing 20% (v/v) methanol (Appendix I) for 45 to 60 minutes. Using forceps and latex rubber gloves, a nitrocellulose membrane sheet (BA 85, Schleicher and Schuell, W. Germany) was cut almost to the shape of the gel, exceeding the size of the gel by 3 to 5 mm in both dimensions. This sheet was prewetted in transfer buffer, taking great care to ensure there were no air bubbles trapped in the membrane and that it was evenly wetted. The polyacrylamide gel and nitrocellulose membrane were then assembled as a sandwich between layers of Whatman No. 1 filter paper and two 'Scotchbrite' pads. Care was taken to eliminate air bubbles between the gel and the nitrocellulose sheet by gently smoothing the two components together with a 10 ml glass test tube. The assembly of the sandwich was carried out in a large dish with all components submerged in transfer buffer. Transfer was performed on a Bio-Rad Trans Blot cell (Bio-Rad Laboratories, Calif., USA), in Tris-glycine-methanol transfer buffer overnight (13-16 hours) at 0.1A without cooling. Following transfer, one lane of protein extract and molecular weight standards were cut from the nitrocellulose sheet for protein visualisation by staining with 0.5% Ponceau S in 1% acetic acid. The individual lanes were carefully cut from the sheet and incubated in TBS-3% BSA for 60 minutes. The transferred proteins were then incubated with either ascitic fluid containing monoclonal antibody, 40% SAS-precipitated globulin fractions or cell culture supernatant for two to three hours. Washing was performed in TBS-0.3% BSA; 3 washes of 5 minutes each. Bound antibody was detected either by incubation with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (as for dot-blot) diluted 1:1000 (Silenus Laboratories, Australia) or 1:500 (Dako Laboratories,

Denmark) for one to two hours, or by a biotin-streptavidin system (Amersham, UK). The procedure for the latter method was as follows. After incubation with monoclonal antibody and washing, nitrocellulose strips were incubated with a biotin labelled polyclonal anti-mouse immunoglobulin diluted 1/500 in TBS-0.3% BSA for one hour. The nitrocellulose strips were washed three times in TBS-0.3% BSA and then incubated in a streptavidin-horseradish peroxidase conjugate diluted 1/500. Following three washes in TBS-0.3% BSA, the nitrocellulose strips were incubated in 4-chloro-1-napthol 0.5 mg/ml and 0.018% hydrogen peroxide in TBS for colour development.

6.2.7 Immunogold electron microscopy (ImAu EM).

All ImAu EM studies were kindly undertaken by Ms. N.S. Fernando, Junior Research Fellow, Renal Immunopathology Division, Wellington School of Medicine of Otago University, Wellington.

a. Post-embedding ImAu EM.

Renal cortical tissue was obtained from a normal Lewis rat immediately following sacrifice by carbon dioxide inhalation. Tissue pieces approximately 1 mm^3 were fixed in 1% glutaraldehyde at RT for a minimum of 30 minutes and then washed in PBS. The tissue was reacted with 1% ammonium chloride in PBS for one hour to quench aldehyde groups (Brown and Revel, 1976), washed in PBS and then dehydrated in a progressive series of ethanols (50-100%) at -20° C. Infiltration of the tissue fragments with LR White resin (London Resin Company, UK) was carried out over a minimum period of 48 hours and the infiltrated tissue embedded in gelatin capsules. Polymerisation of the resin was achieved by the addition of LR White accelerator at -20° C. Gold to pale-gold ultrathin sections were collected on Formvar-coated (Probing and Structure Ltd., Queensland, Australia) gold or nickel grids. The sections were incubated with anti-GMF monoclonal antibodies for one to five hours at either RT or at 37° C. The sections were then washed for 15 minutes in TBS, pH 7.4, containing either 0.1% (v/v) Tween 20 (Sigma, Chemical Co., USA) or 0.1% (w/v) BSA as a blocking agent, followed by a further wash in distilled water. The sections were then incubated with a gold-conjugated goat anti-mouse IgG-IgM (Janssen Laboratories, Denmark) for one to five hours at RT or at 37° C. The gold particle diameters were 5, 15 or 30 nm. All antibodies were diluted in TBS containing 1%

Tween 20. Following the antibody incubations, the sections were counter-stained with 2% uranyl acetate in 50% ethanol for four minutes and for two minutes with lead citrate as described in Chapter Three. Sections were viewed in either a Zeiss 109 TEM (Carl Zeiss, W. Germany), or a Philips 20K TEM (Philips, Holland).

b. Pre-embedding ImAu EM.

Normal Lewis rats were injected IV with 5mg of a 40% SAS-precipitate of monoclonal antibody-containing ascites two hours prior to sacrifice by ether anaesthesia. Immediately upon sacrifice, the kidneys were perfused with 2% paraformaldehyde in PBS via the aorta for three to five minutes. The kidneys were then removed and placed in a beaker containing 2% paraformaldehyde in PBS for 30 minutes. After a ten minute wash in PBS, the kidney was snap frozen in liquid nitrogen and sections 6 μm thick were cut on a cryostat (Tissue Tek, Miles Scientific, Il, USA). The cut sections were placed in PBS prior to incubation with a gold-conjugated anti-mouse IgM-IgG antibody (Janssen Laboratories, Denmark) for one to five hours at either RT or at 37^0 C. Following incubation, the sections were washed in PBS for ten minutes and then placed in half strength Karnovsky's fixative (Appendix II) for one hour, followed by a further hour in 0.5 M sucrose-0.2 M sodium cacodylate buffer at RT. The sections were then processed as described in Chapter Three. Ultrathin sections were cut on an ultratome and viewed in one of the transmission electron microscopes described above.

6.3 Results.

6.3.1 Reactivity with renal tissue.

The renal reactivities of the anti-GMF monoclonal antibodies determined by indirect IF microscopy are shown in Table 6.1. Initial screening of the monoclonal antibodies was performed with supernatants obtained from cultures of the hybridoma cells. The IF staining produced was generally weak; however, the coarse granular patterns of IF staining produced by SC5, ASD5, BSG11 and PH7 were of interest, and these monoclonal antibodies were selected for further study.

Initial IF microscopy studies on cell culture supernatants from SC5, ASD5 and BSG11 indicated that these three monoclonal antibodies reacted only with glomeruli,

Table 6.1.

Reactivity sites of anti-GMF monoclonal antibodies as determined by indirect IF microscopy on normal rat kidney.

Monoclonal Antibody	Glomerular IF Pattern	Site of Reactivity
9-F4	Diffuse, hazy, granular.	EP membrane, vessels.
9-E8	Diffuse, granular, pan-glomerular.	Membranes and cytoplasm.
9-F10	Weak, fine granular.	Indeterminate.
9-D11	Diffuse, fine granular.	EP cell membrane.
9-C11	Granular.	Indeterminate.
9-B4	Fine granular.	Indeterminate.
9-C9	Fine granular.	EP cell membrane and cytoplasm.
PH7	Coarse granular.	EP and tubular membranes, BC.
APE10	Granular.	EP cells and vessels.
BPC4	Circular inclusions.	Nuclear membranes.
2-E12	Coarse granular.	EP membrane.
2-C12	Granular.	Indeterminate.
ASE10	Weak segmental, granular.	EP membrane.
SC5	Course granular.	glomerular and tubular EP, BB.
ASD5	Coarse granular.	Mesangium, ED, BB, pT capillaries.
BSG11	Coarse granular.	EP, pT capillaries and BB.

Cryostat sections of normal Lewis rat kidney were stained by indirect IF microscopy.

EP - epithelial cells.
ED - endothelial cells.
pT - peritubular.
BB - tubular brush border.

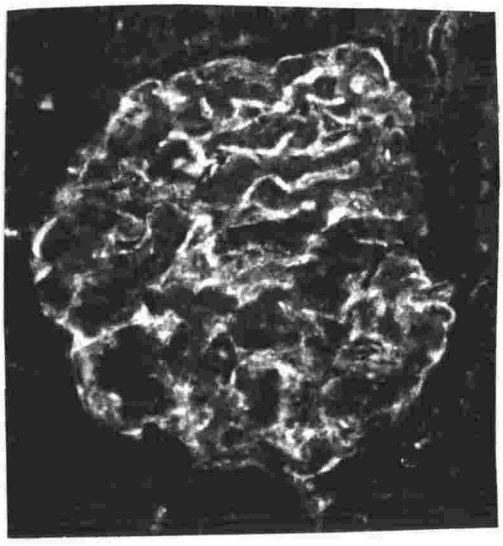
producing a coarse granular pattern of glomerular staining. In addition, PH7 also reacted strongly with glomeruli, exhibiting a similar coarse granular pattern of staining, and weakly with tubules. Subsequent indirect IF microscopy studies, performed with affinity-purified monoclonal antibody, revealed that reactivity of these monoclonal antibodies was not confined to glomerular antigens as suggested by the results using cell culture supernatants. Fig. 6.1 shows the reactivity of SC5 with normal Lewis rat kidney. The overall staining of the glomerulus is a coarse granular pattern. Staining of proximal tubular cell membranes and tubular brush border membranes is also seen. ASD5, which also appeared to stain only glomeruli in the initial screening, was shown to react with a number of other renal structures when affinity-purified antibody was used as the primary antibody (Fig. 6.2). ASD5 reacted with glomerular endothelial and mesangial cells, as well as with tubular brush borders and peritubular capillaries. It did not appear to react with epithelial cells. Affinity-purified BSG11 also reacted with a number of renal structures (Fig 6.3). The glomeruli were stained in a coarsely granular pattern, and the reactivity was most likely associated with epithelial cell membranes. There was a punctate staining of the tubular brush borders and staining also of the peritubular capillaries. The IF staining produced by PH7 was also in a coarse granular pattern. Reactivity with glomerular epithelial cell and tubular cell membranes and also with Bowman's capsule was observed. PH7 was the only monoclonal antibody that gave a positive reaction with Bowman's capsule.

6.3.2 Reactivity with non-renal tissues.

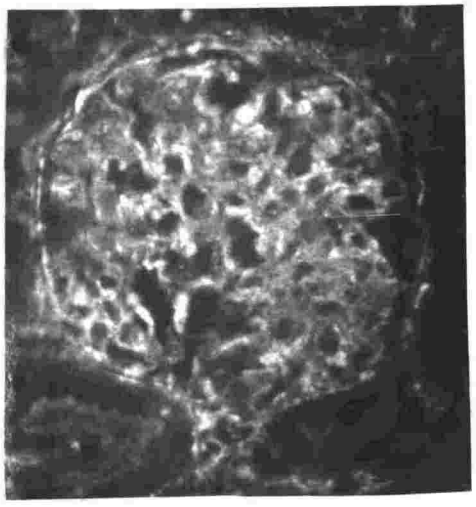
The monoclonal antibodies selected for in vivo studies, ASD5, SC5, PH7 and BSG11, were examined to determine whether the individual antigen with which each reacted was restricted to the kidney or had a wider tissue distribution. The binding of these monoclonal antibodies to extrarenal tissues is shown in Table 6.2. The monoclonal antibody G6, which did not bind to kidney, was examined for non-renal reactivity to determine its suitability as a negative control for in vivo studies; G6 did not bind to any of the tissues examined. PH7 was found to bind to all tissues, although reactivity with spleen and liver was inconsistent. The reactivity of PH7 with gut was marked as seen in the photomicrograph of Fig. 6.4. ASD5 reacted with all tissues examined, with the exception of liver, heart and brain. Like PH7, ASD5 also reacted strongly with gut (Table 6.2), and the pattern of staining was similar to that obtained with PH7 (Fig. 6.5). The reactivity of SC5 was somewhat more restricted than PH7 and ASD5. Beside its renal reactivities, SC5 reacted with cardiac muscle (Fig. 6.6) and showed

Figures 6.1 to 6.3.

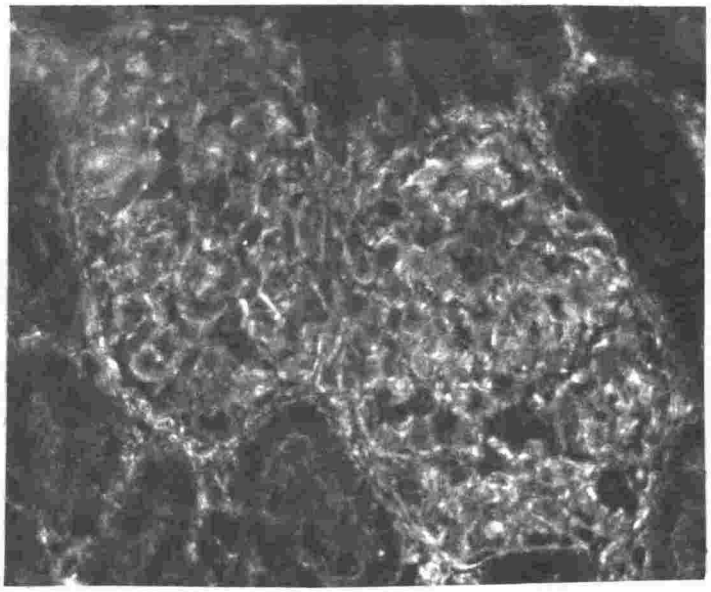
Immunofluorescence staining pattern of affinity-purified anti-GMF monoclonal antibodies.



6.1



6.2



6.3

Normal Lewis rat kidney was incubated with the respective monoclonal antibody. Bound mouse immunoglobulin was detected with an FITC polyclonal anti-mouse serum. Original magnification of all micrographs was x 160.

Fig. 6.1 - SC5; Fig. 6.2 - ASD5; Fig. 6.3 - BSG11

Table 6.2.

Reactivities of anti-GMF monoclonal antibodies
with non-renal tissues.

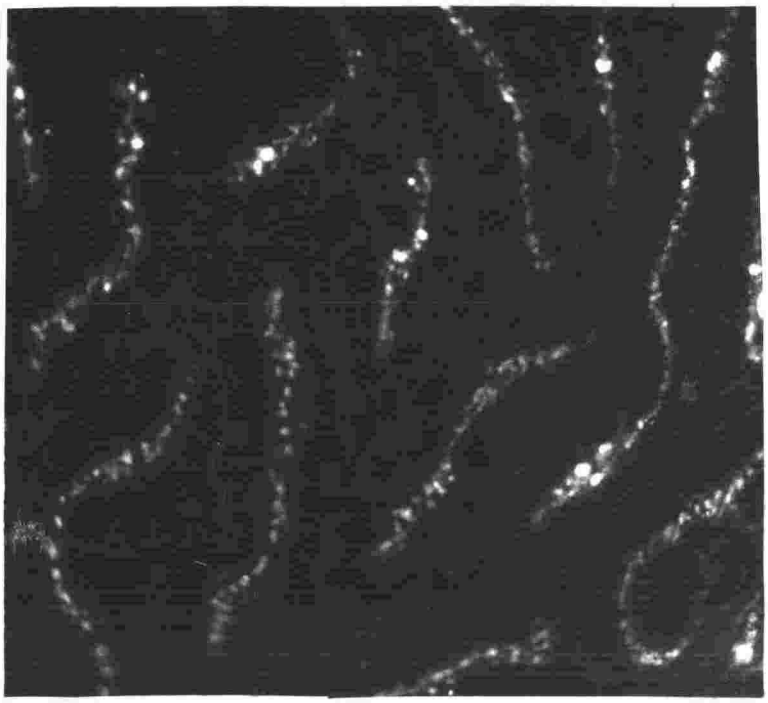
Tissue	APH7	ASD5	SC5	BSG11	G6
Spleen	±	+	±	-	-
Gut	+	+	-	-	-
Skeletal muscle.	+	+	±	-	-
Lung	+	+	±	+	-
Liver	±	-	-	+	-
Cardiac muscle.	+	-	+	-	-
Aorta	+	+	-	+	-
Brain	+	+	+	+	-

Tissues obtained from normal Lewis rats were examined by indirect IF microscopy using affinity-purified monoclonal anti-GMF as the primary antibody.

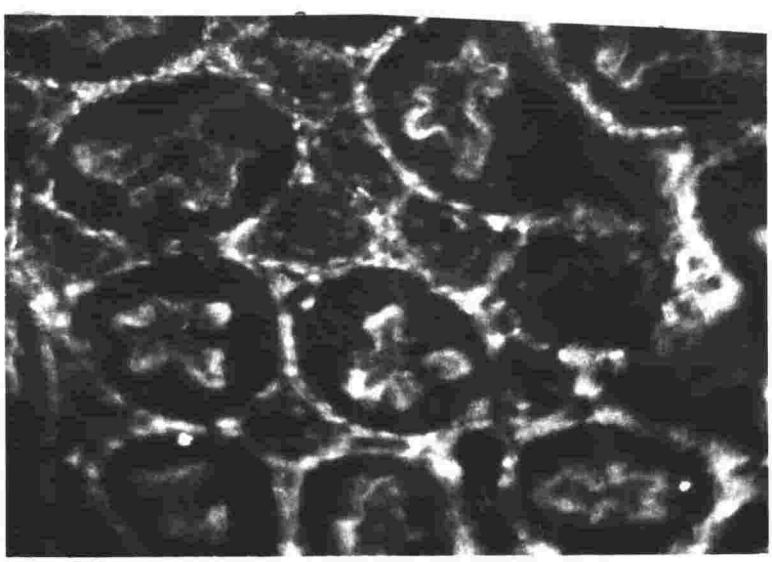
\+ Indicates a positive staining of the tissue.
\- Indicates a negative result.
± Inconsistent, irregular staining.

Figures 6.4 - 6.5.

Immunofluorescence staining patterns of the anti-GMF monoclonal antibodies PH7 and ASD5 on rat intestine.



6.4



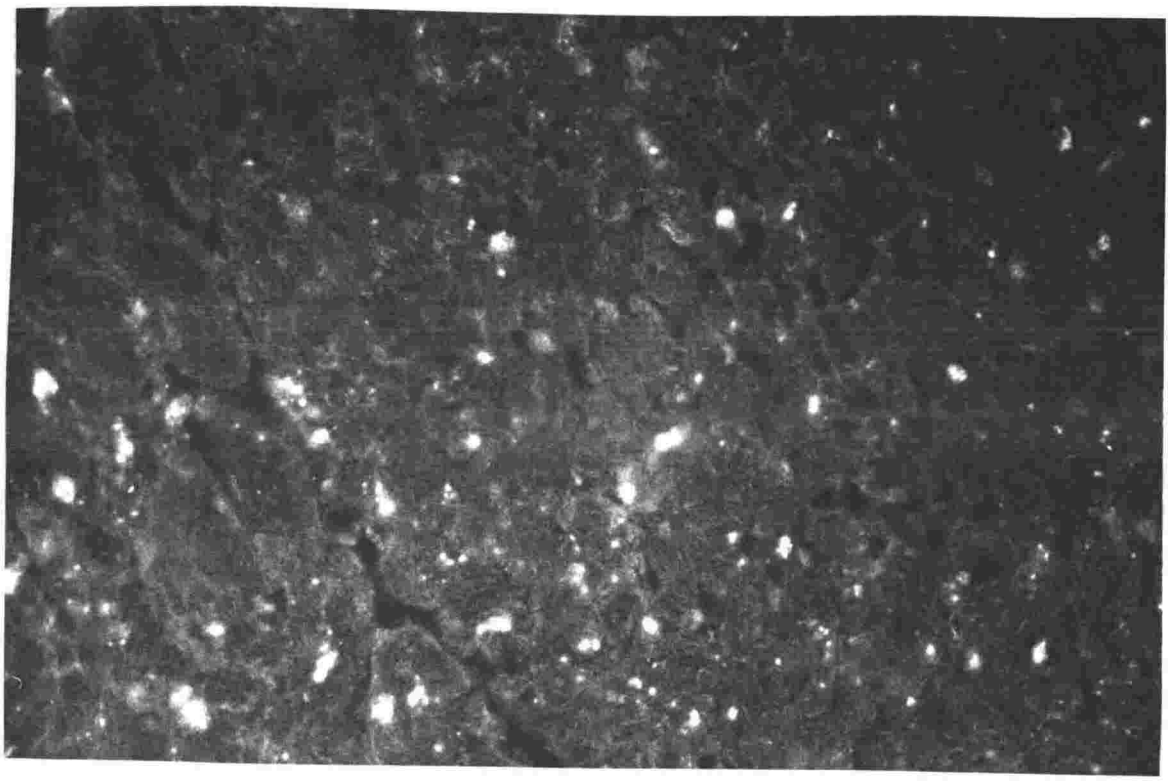
6.5

Cryostat sections of small intestine from a normal Lewis rat were incubated with the respective monoclonal antibody. Bound mouse immunoglobulin was detected with an FITC anti-mouse serum.

Fig.6.4. - PH7; Fig.6.5. - ASD5.

Figure 6.6.

Immunofluorescence staining pattern of SC5 on
rat cardiac muscle.



Cryostat sections of cardiac muscle from the left ventricle of a normal Lewis rat were incubated with SC5-containing ascites. Bound mouse immunoglobulin was detected with a polyclonal anti-mouse serum.

inconsistent patchy reactivity to three other non-renal tissues (Table 6.2). BSG11 reacted with lung, liver, aorta and brain. The staining of lung by this monoclonal antibody was particularly strong (Fig. 6.7).

6.3.3 Interspecies reactivity.

The reactivities of ASD5, PH7, SC5 and BSG11 with human, sheep and rabbit kidney are shown in Table 6.3. SC5 only reacted with the glomeruli of human kidney. The pattern of staining was granular, similar to that obtained on rat kidney. PH7 reacted with the glomeruli and tubules of all three species. It did not appear to stain the vessels. ASD5, but not BSG11, reacted with human kidney. Both of these reacted with sheep kidney, and the pattern of staining was similar to that observed on rat kidney. On rabbit kidney, ASD5 stained the glomeruli but did not stain the tubules. It did, however, react with vessels. BSG11 reacted with rabbit glomeruli and weakly with rabbit tubules.

6.3.4 Cross-reactivity with Heymann nephritis antigens.

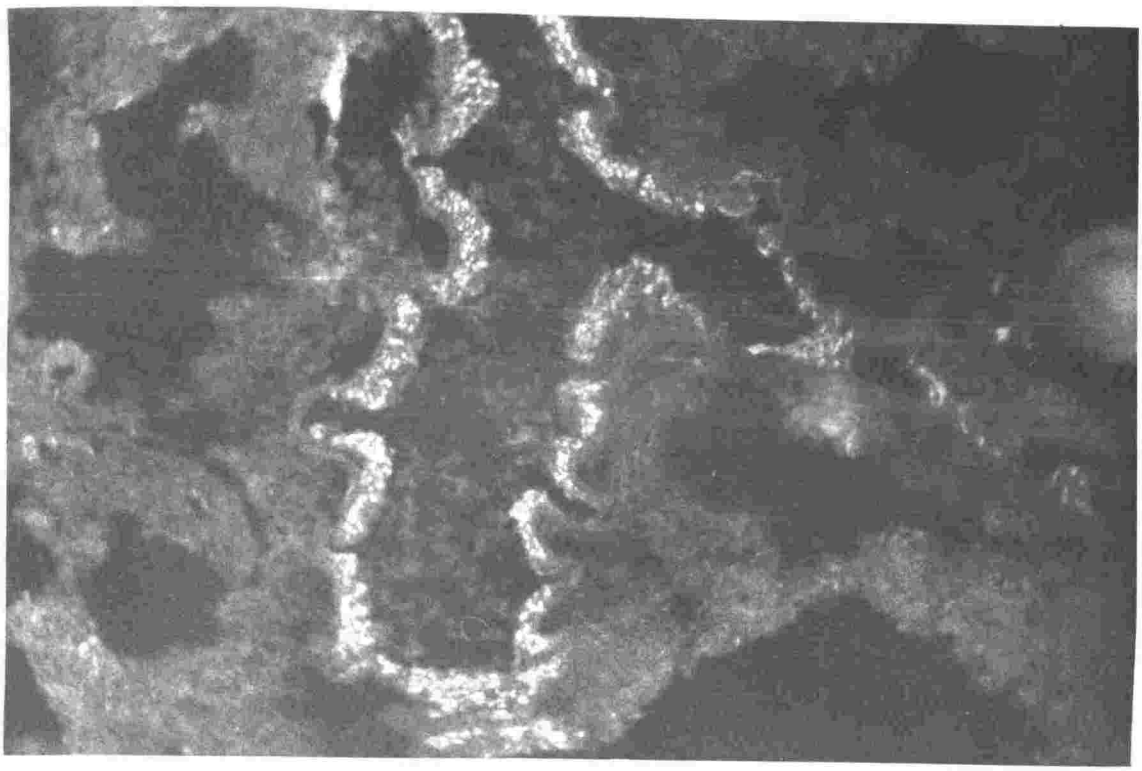
Neither polyclonal sheep anti-rat Fx1A, nor eluates from Heymann nephritic kidneys were able to block the binding of ASD5, SC5, PH7 or BSG11 to rat kidney. The staining patterns of the monoclonal antibodies, obtained after prior exposure of the kidney sections to either of the reagents, were identical to those obtained on sections incubated with the monoclonal antibodies alone. Attempts to block the binding of sheep anti-rat Fx1A or Heymann eluates by anti-GMF monoclonal antibodies were also unsuccessful. These results indicated that the epitopes with which the monoclonal antibodies were reactive were unlikely to be significant in the pathogenesis of HN.

6.3.5 SDS-PAGE profiles of GMF extracts.

In all protein extraction experiments using GMF, with the exception of urea extracts, the recovered, solubilised protein, expressed as a percentage of weight of the starting material, was 10% to 23%. The electrophoretic profile of OG, CHAPS and DOC extracts of GMF are shown in Fig. 6.8. Approximately 40 μg of OG and CHAPS protein extract and 50 μg of DOC extract were applied to a 5-15% gradient gel. The general high background silver staining and poor resolution seen with the DOC extract was typical of the behaviour of this extract when subjected to SDS-PAGE. In some

Figure 6.7.

Immunoflouorescence staining pattern of BSG11-containing ascites on normal Lewis rat lung.



A cryostat section of rat lung was incubated with BSG11 monoclonal antibody. Bound mouse immunoglobulin was detected with an FITC polyclonal anti-mouse immunoglobulin serum.

Table 6.3.

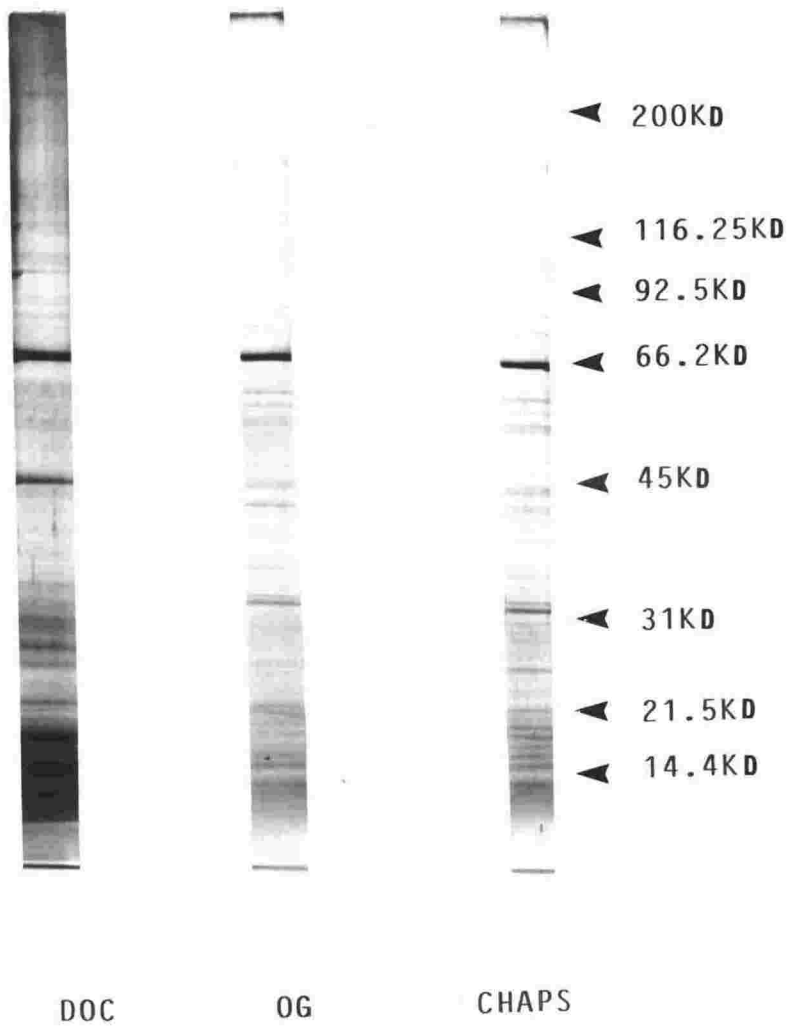
Interspecies kidney reactivities of anti-GMF monoclonal antibodies.

Monoclonal antibody	Human			Sheep			Rabbit		
	G.	T.	V.	G.	T.	V.	G.	T.	V.
SC5	+	-	-	-	-	-	-	-	-
PH7	+	+	-	+	+	-	+	+	-
ASD5	-	-	-	+	+	-	+	-	+
BSG11	-	-	-	+	+	-	+	±	-

G glomeruli.
T tubules.
V vessels.
+ denotes positive staining.
± denotes irregular, inconsistent staining.
- denotes a negative staining.

Figure 6.8.

SDS-PAGE profile of soluble proteins obtained by detergent extraction of GMF.



Samples of protein extracts were applied to the wells of a 5-15% gradient polyacrylamide gel and electrophoresed as described in the text. The gel has been silver-stained.

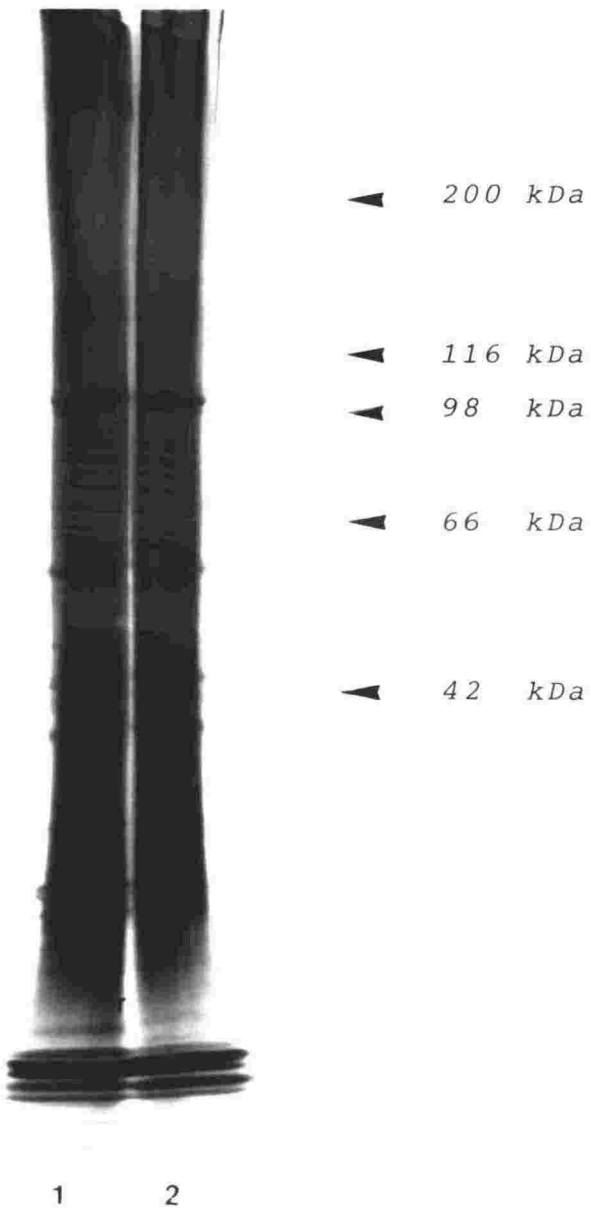
cases the resolution was such that it was impossible to clearly define the protein bands due to the 'smearing' (Fig. 6.9). Running non-reduced samples of DOC extracts on the gel and extensive dialysis of the extracts (>72 hours) did not eliminate the 'smearing'. A number of protein bands were common to all three extracts with major bands appearing at 66 and 33 kDa. Few protein bands greater than 100 kDa were observed, and the majority of the proteins extracted by these detergents were less than 66 kDa. The sensitivity of Coomassie blue staining of gels was insufficient to detect some of the proteins extracted. Fig 6.10a shows an SDS-PAGE gel stained with Coomassie blue. Few bands were evident in the DOC, urea or OG extracts, however, the molecular weight markers were clearly visualised. Following photography the gel was destained in 40% methanol/10% acetic acid for 24 hours at 37° C, and then silver stained (Fig. 6.10b). A number of bands were visualised in all three extracts which were not detected by Coomassie blue. Although the silver staining allowed visualisation of proteins present in low concentration, it made identification of the molecular weight standards more difficult. The molecular weight standards used were obtained from Bio Rad Laboratories. It was clear that there were a number of proteins present in addition to the named standards.

A number of problems were encountered with 8M urea extracts of GMF. The extracts obtained were inconsistent in their protein yields, and in some extraction experiments, no proteins were visualised after SDS-PAGE and silver staining of the gels, in spite of adding normal amounts of protein to the gels.

Proteins were also solubilised from isolated whole glomeruli (Fig. 6.11). The extract obtained from whole glomeruli contained numerous proteins larger than 100 kDa which were not present in extracts obtained from GMF. The 66 kDa protein common to OG and CHAPS extracts of GMF was also present in extracts from whole glomeruli. The 33 kDa band which is common to both OG and CHAPS extracts of GMF was not found in extracts from whole glomeruli. As could be expected, there was overall a greater number of proteins appearing in the extract obtained from isolated whole glomeruli than in the extract obtained from GMF.

Figure 6.9.

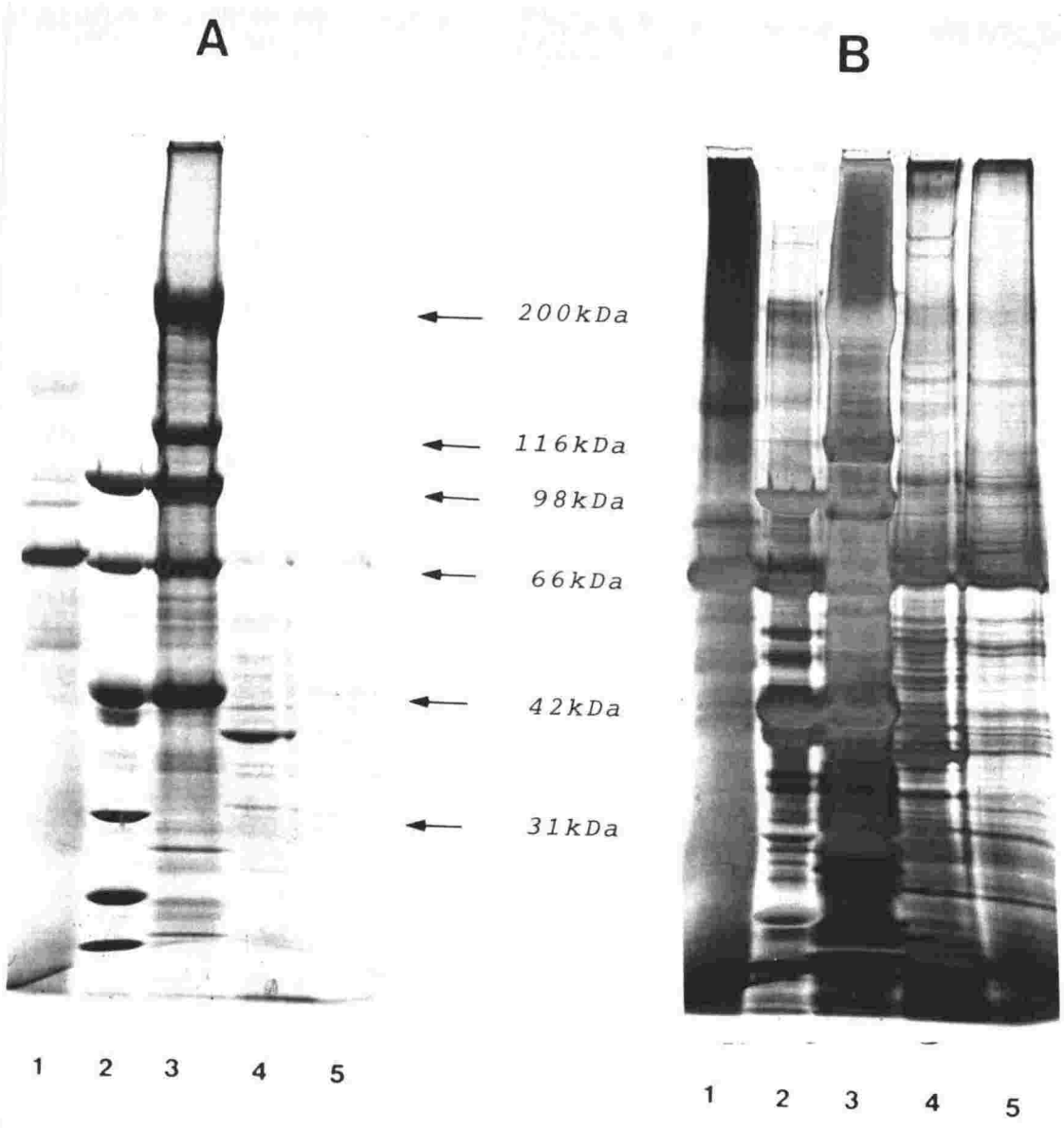
SDS-PAGE profile of samples of DOC extracts of GMF



Samples (60μg) of DOC extracts of GMF were reduced by dithiothreitol, applied to the lanes of a 10% polyacrylamide gel and electrophoresed as described in the text. The gel has been silver-stained. Lanes one and two are duplicate samples.

Figure 6.10.

Comparison of Coomassie blue and silver for the staining of SDS-PAGE gels.



Samples were applied to a 5-15% polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie blue (A) and then silver (B).

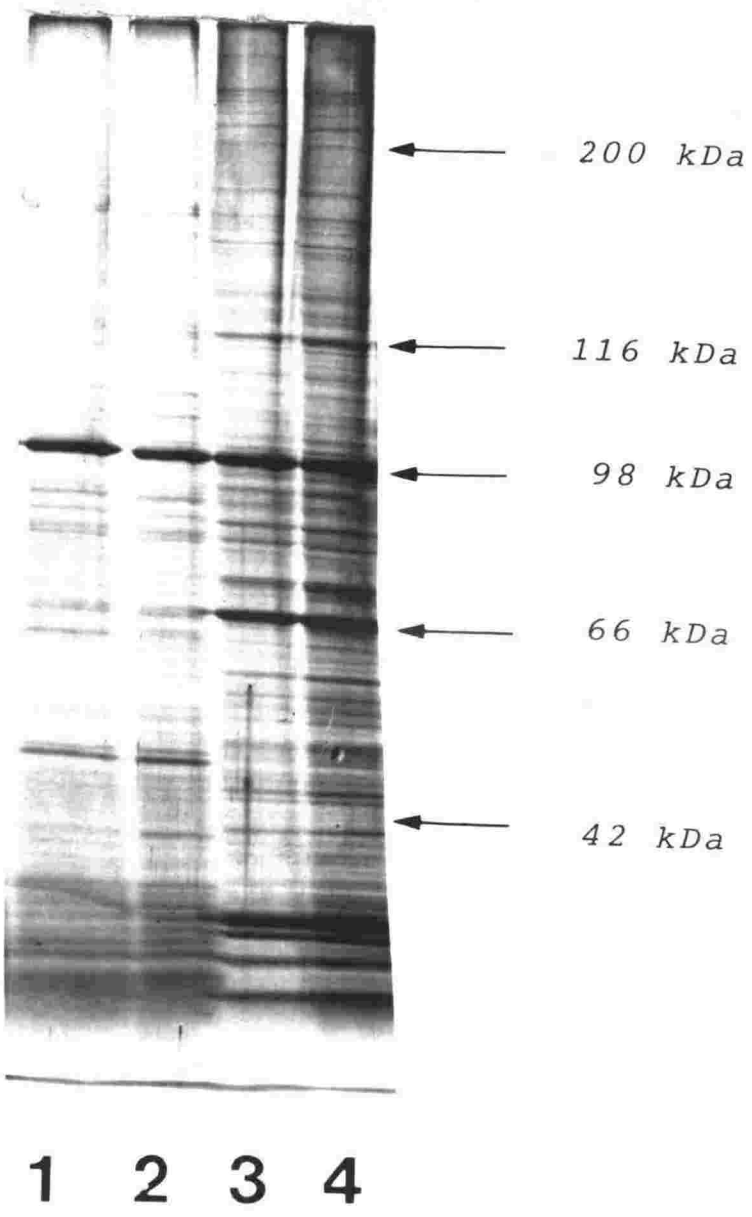
Lane 1 - DOC extract of GMF; Lane 2 - Low molecular weight standards;

Lane 3 - High molecular weight standards; Lane 4 - 8M urea extract of GMF;

Lane 5 - OG extract of GMF.

Figure 6.11.

Electrophoretic profiles of OG and CHAPS extracts obtained from GMF and isolated whole glomeruli.



Samples of OG and CHAPS extracts obtained from GMF and isolated whole glomeruli were electrophoresed on a 5-15% polyacrylamide gel. The gel has been silver-stained.

Lane 1 - OG extract of GMF; Lane 2 - CHAPS extract of GMF; Lane 3 - OG extract of whole glomeruli; Lane 4 - CHAPS extract of whole glomeruli.

6.3.6 Reactivity of anti-GMF monoclonal antibodies with protein extracts of GMF and isolated whole glomeruli.

a. Dot-blots.

Initial screening to determine whether the monoclonal antibodies would react with the GMF extracts was done by dot-blot assay. A summary of anti-GMF activity as assessed by Dot-blot is given in Table 6.4. The reactivity of a series of doubling dilutions of the anti-GMF monoclonal antibodies ASD5 and SC5 with DOC extracts of GMF are shown in Fig. 6.12. The non-kidney binding IgM monoclonal antibody G6 did not react with the DOC extract at any of the dilutions. SC5 was seen to react strongly with the DOC extract to a dilution of 1/200 and weakly at a dilution of 1/400. ASD5 reacted strongly with the extract at a dilution of 1/25 and less strongly at 1/50 and 1/100. It did not react at dilutions greater than 1/100. The reactivity of both SC5 and ASD5 on normal rat kidney, assessed by indirect IF microscopy, was greater than 1/800 (the greatest dilution examined). ASD5 and SC5 were also shown to react with OG and CHAPS extracts of GMF (Fig 6.13). SC5 was seen to react strongly with all three extracts. ASD5 reacted strongly with the DOC extract as already seen and moderately with the CHAPS extract. Only weak reactivity was observed with the OG extract. G6 did not react with any of the extracts examined.

PH7 and BSG11 were also shown to react with all three extracts (Figs. 6.14 and 6.15). Both BSG11 and PH7 reacted strongly with the DOC extract of GMF (Fig. 6.14), with PH7 showing a slightly stronger reaction than BSG11. No reactivity was seen in the control well which received the non-kidney binding monoclonal antibody G6. When examined for reactivity with OG and CHAPS extracts, (Fig. 6.15), PH7 again showed a slightly stronger reaction than BSG11. Both monoclonal antibody preparations reacted with normal rat kidney to a dilution greater than 1/800, as determined by indirect IF microscopy. The control well which did not receive antigen was negative.

ASD5, SC5, PH7 and BSG11 were examined to determine reactivity with a collagenase digest of human GBM and a DOC extract of hGMF. ASD5 reacted with both of these preparations from human kidney (Fig. 6.16). No reactivity with either the collagenase digest or DOC extract of hGMF was observed with SC5, PH7 or BSG11.

Table 6.4.

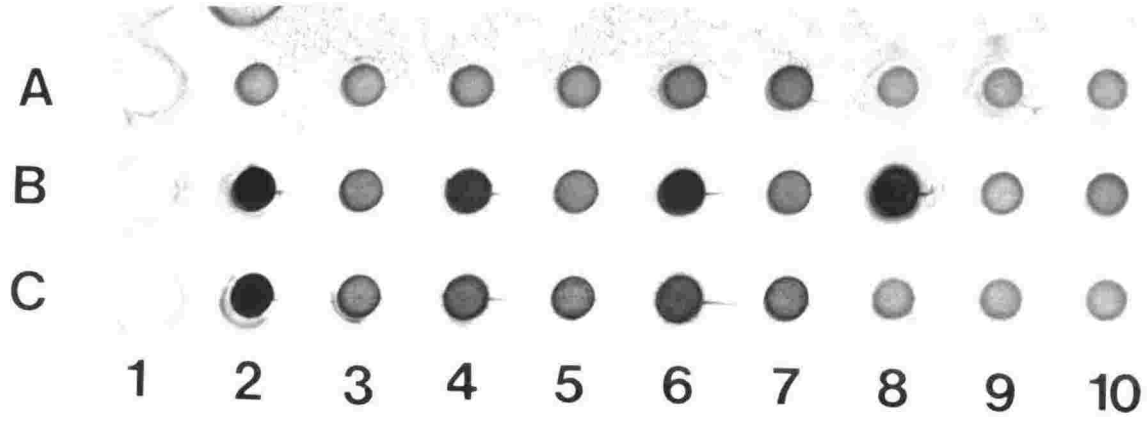
Reactivity of anti-GMF monoclonal antibodies with protein extracts of GMF examined by Dot-blotting.

Monoclonal antibody	Extracts				
	Rat GMF			Human GMF	
	CHAPS	OG	DOC	Collagenase	DOC
G6	-	-	-	-	-
SC5	+ + +	+ + +	+ + +	-	-
ASD5	+ +	+	+ + +	+ + +	+ + +
PH7	+ + +	+ + +	+ + +	-	-
BSG11	+ +	+ +	+ + +	-	-

-	Negative
+	Weak positive
+ +	Moderate positive
+ + +	Strong positive

Figure 6.12.

Dot-blot assay of ASD5 and SC5 monoclonal antibodies
on DOC extract of GMF.

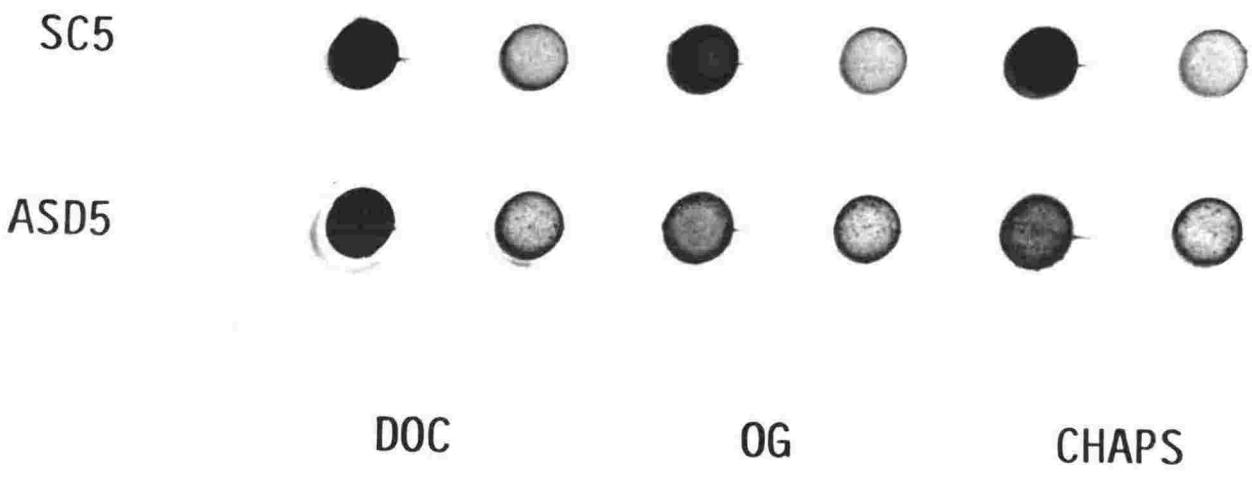


Aliquots (200 μl), of a solution of DOC extract from GMF containing 2 μg, were distributed to the wells in the even numbered vertical rows. The wells in the odd numbered rows received TBS/0.3% BSA only.
Doubling dilutions of SAS fractions of SC5, ASD5 and G6 ascites were made in TBS/0.3% BSA and 200 μl aliquots distributed to the horizontal rows.

Row A - G6; Row B - SC5; Row C - ASD5.

Figure 6.13.

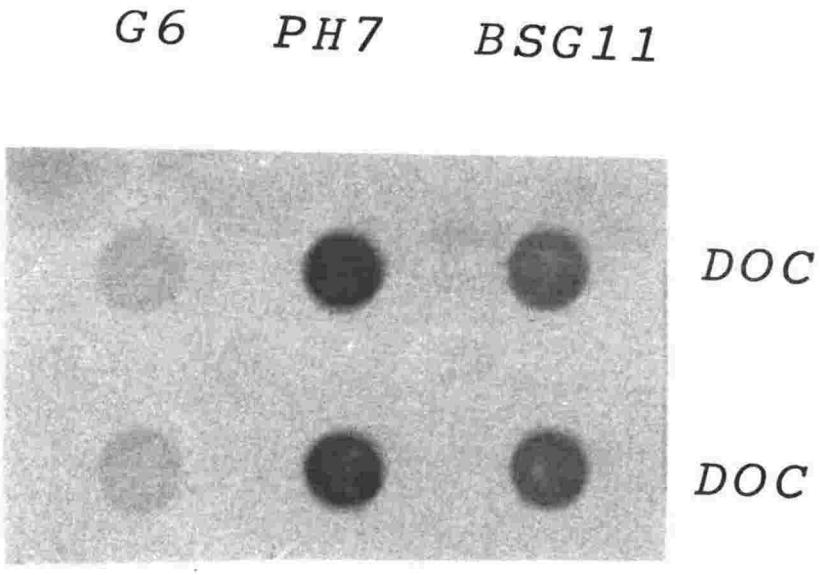
Dot-blot assay of anti-GMF monoclonal antibody reactivity with DOC, OG and CHAPS protein extracts of GMF.



Aliquots of 200 μl containing 2 μg of the GMF extracts were distributed to a group of four wells as indicated. The anti-GMF monoclonal antibodies ASD5 and SC5 were diluted 1/25 in TBS/0.3% BSA and 200 μl aliquots distributed to every second well in the horizontal rows as indicated. The remaining control wells received 200 μl of a 1/25 dilution of the non-kidney binding monoclonal antibody G6.

Figure 6.14.

Dot-blot assay of anti-GMF monoclonal antibodies
PH7 and BSG11, reactivity with DOC extract of GMF.

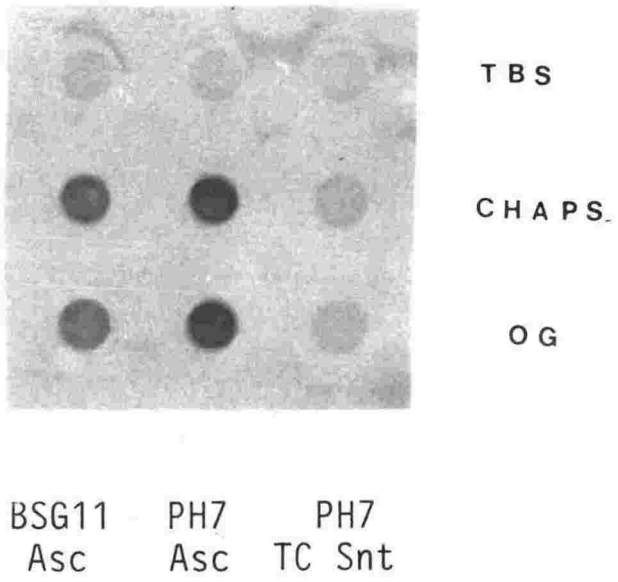


Aliquots of 200 μl containing 2 μg of DOC extract of GMF were distributed to all wells, followed by 200 μl of an SAS precipitate of the monoclonal antibodies diluted 1/25 in TBS/0.3% BSA.

Rows 1 and 2 are duplicates.

Figure 6.15.

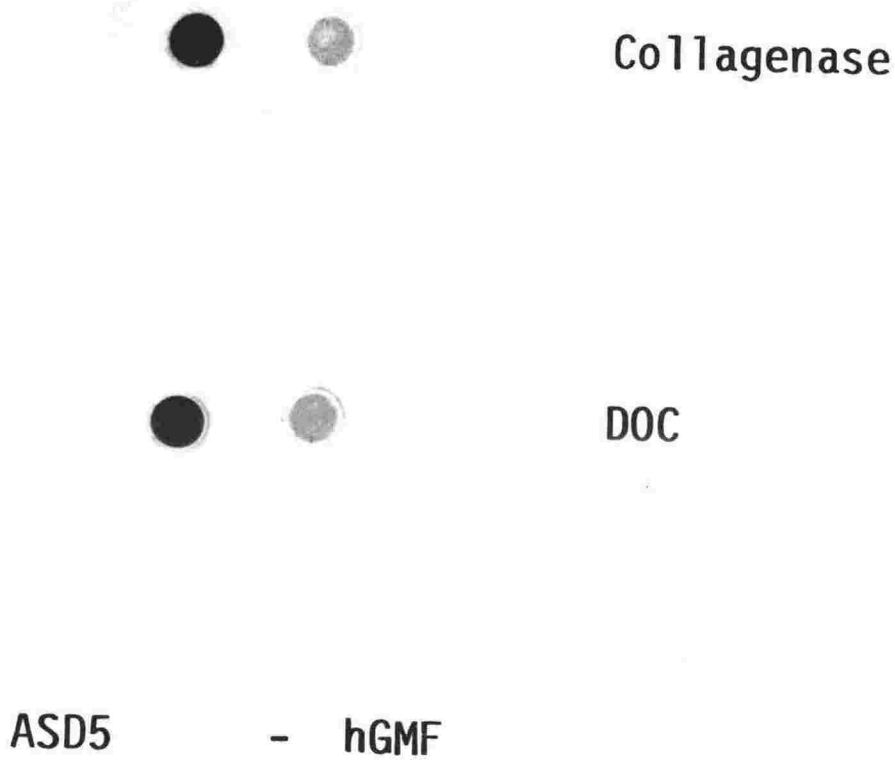
Dot-blot assay of reactivity of anti-GMF monoclonal antibodies PH7 and BSG11 with OG and CHAPS extracts of GMF.



Aliquots of 200 μl containing 2 μg of the GMF extracts were distributed to the horizontal rows as indicated. The top row received 200 μl of TBS/0.3% BSA only, followed by the indicated monoclonal antibody. BSG11 and PH7 ascites were diluted 1/25 in TBS/0.3% BSA; PH7 tissue culture supernatant was used undiluted.

Figure 6.16.

Dot-blot assay of ASD5 against a collagenase digest and a DOC extract prepared from hGMF.



Aliquots of 200μl containing 2 μg of DOC extract or 5 μg of collagenase digest of hGMF were distributed to the test wells. Negative control wells received TBS/0.3% BSA only.

a - Test well.

b - Control well.

b. Immunoblots.

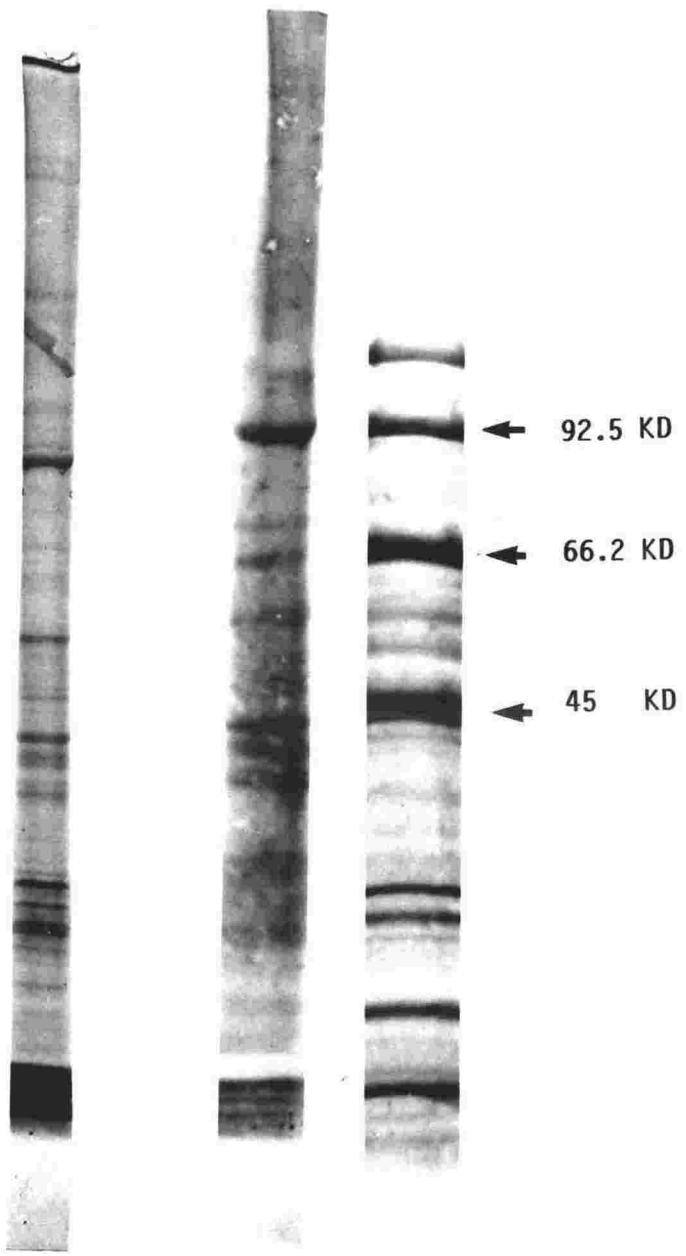
Extracts of GMF were subjected to SDS-PAGE, transferred to nitrocellulose membranes and then probed with the selected anti-GMF monoclonal antibodies. The transfer of proteins from the gel to the nitrocellulose is shown in Fig. 6.17. The polyacrylamide gel was not from the same gel that was transferred to the nitrocellulose. The transferred proteins were clearly visualised by staining with Ponceau S, although the bands were not as compact as observed on the polyacrylamide gel. This may be accounted for by diffusion of the protein bands during transfer. The molecular weight standards were clearly seen to have transferred well, although the 200 kDa standard, myosin, was less intense than observed by either Coomassie blue or silver staining of the polyacrylamide gel, indicating that this protein may not have been transferred as efficiently as the lower molecular weight proteins. A summary of anti-GMF monoclonal antibody reactivity with protein extracts of isolated glomeruli and GMF, defined by immunoblotting experiments, is given in Tables 6.5 and 6.6.

An 8M urea extract, obtained from isolated Lewis rat glomeruli, was electrophoresed, transferred to a nitrocellulose membrane and then probed with SC5 ascites. Reactivity bands were observed at 175, 160, 120 and 53 kDa (Fig. 6.18). Some minor reactivity may have been present with proteins in the 195 - 230 kDa range, though interpretation of this was made difficult by the high background staining. Several attempts were made to reproduce this result with the urea extract on both reduced and non-reduced protein transfers, but the attempts were unsuccessful. Urea extract transfers were also probed with PH7, ASD5 and BSG11. All returned a negative result.

The results obtained with BSG11 on transfers of OG and CHAPS extracts are shown in Fig. 6.19. The samples of extracts were not reduced prior to electrophoresis. Reactivity bands were observed at 100, 65, 62.5, 47, 43, 42, and 15 kDa. A number of immunoblotting experiments were conducted with BSG11 on DOC extracts. Whilst it was possible to reproduce the results obtained with OG and CHAPS extracts with crude ascites and SAS precipitates of BSG11 ascites, results with DOC extracts were consistently negative, even when affinity-purified BSG11 monoclonal antibody and the biotin-streptavidin detection system were used.

Figure 6.17.

Transfer of proteins from polyacrylamide gels to nitrocellulose sheets.



40 μg of a CHAPS extract of GMF was electrophoresed on a 5-15% gradient polyacrylamide gel and then transferred to a nitrocellulose sheet as described in the methods.

a. Polyacrylamide gel of CHAPS extract, silver-stained.

b. Nitrocellulose strip following transfer of the CHAPS extract from the gel.

c. Nitrocellulose strip following transfer of molecular weight standards from the gel.

The nitrocellulose strips were stained with Ponceau S.

Table 6.5.

Immunoblot reactivity of anti-GMF monoclonal antibodies with protein extracts of isolated rat glomeruli.

Monoclonal antibody	Protein extracts			
	Urea	CHAPS	OG	DOC
G6	-	-	-	-
SC5	175 160 120 53	96 32 to 14	96 32 to 14	-
ASD5	-	35	-	-
PH7	-	14 13 11	14	-
BSG11	-	ND	ND	ND

The reactivities of the anti-GMF monoclonal antibodies with protein bands of detergent extracts of isolated glomeruli are shown in kDa.

ND denotes not done.

Table 6.6.

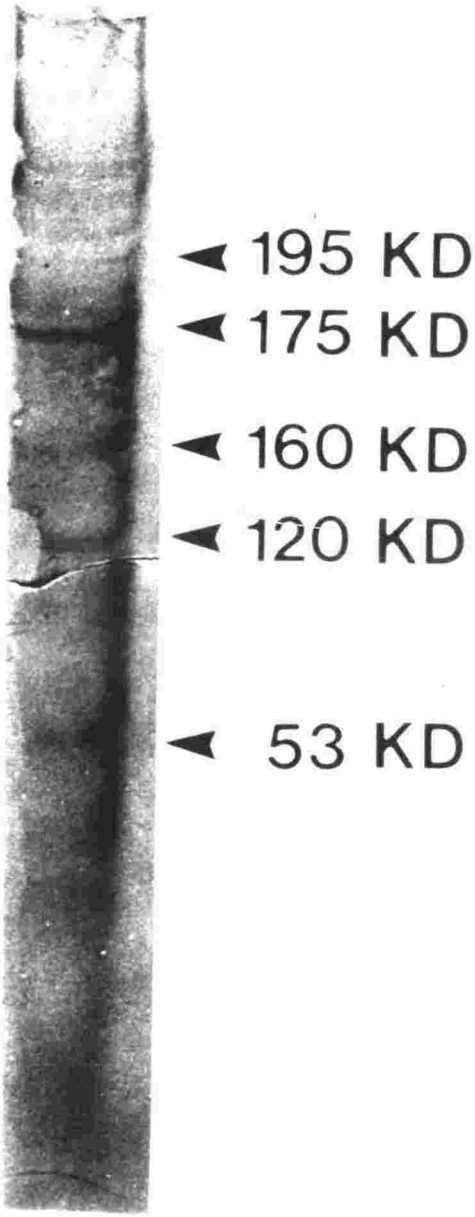
Immunoblot reactivity of anti-GMF monoclonal antibodies with protein extracts of rat GMF.

Monoclonal antibody	Protein extracts			
	Urea	CHAPS	OG	DOC
G6	-	-	-	-
SC5	-	96	78	-
		82		
		78		
		55		
		52		
ASD5	-	44	-	-
		42		
		37		
PH7	-	-	-	-
BSG11	-	100	100	-
		65	65	
		62.5	62.5	
		47	47	
		43	43	
		42	42	
		15	15	

The reactivities of the anti-GMF monoclonal antibodies with protein bands of detergent extracts of rat GMF are shown in kDa.

Figure 6.18.

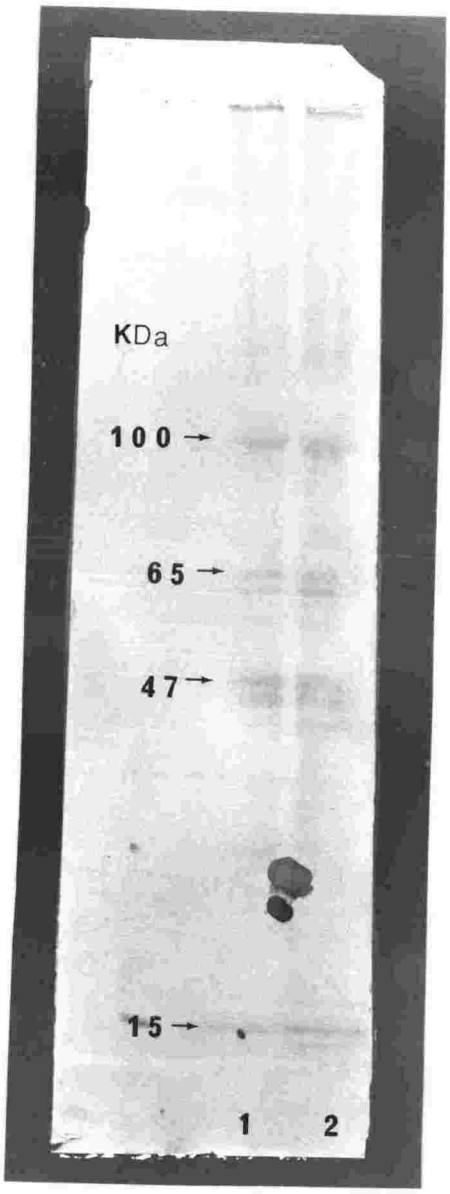
Immunoblot of SC5 ascites against an 8 M urea extract of isolated glomeruli.



An 8 M urea extract of isolated glomeruli was electrophoresed on a 7.5% polyacrylamide gel and then transferred to a nitrocellulose membrane. The transfer was probed with SC5 ascites as described in the methods.

Figure 6.19.

Immunoblot of BSG11 against DOC, and OG extracts of GMF.



Samples containing 50 μg of the protein extracts were electrophoresed on a 5 - 15% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and probed with an SAS precipitate of BSG11 ascites diluted 1/25 in TBS/0.3% BSA as described in the methods.

Lane 1 - OG extract; Lane 2 - CHAPS extract.

Initial immunoblotting experiments with SC5 on extracts of GMF were unsuccessful. The experiments were repeated using extracts obtained from isolated glomeruli. The results show SC5 to be reactive with a large number of protein bands (Fig. 6.20). Reactivity was seen with a protein band at 96 kDa and with a large number of bands between 32 and 14 kDa. The immunoblotting experiments using CHAPS extract from GMF were repeated using the biotin-streptavidin detection system. The results clearly showed that SC5 reacted with protein bands at 96, 82 and 78 kDa; two further reactive bands of lesser intensity were seen at 55 and 52 kDa. A large amount of reaction product is also seen with low molecular weight proteins, this finding, however, was not consistent (Fig. 6.21). Results of immunoblots using OG extracts of GMF were inconclusive. On two occasions a faint band was observed at 78 kDa, but the finding was inconsistent. SC5 did not react with DOC extracts of GMF.

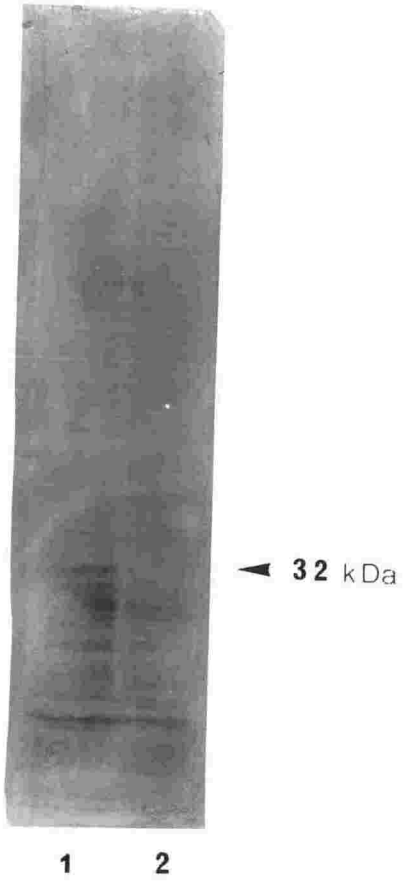
The immunoblot reactivity of PH7 with OG and CHAPS extracts of isolated glomeruli is shown in Fig. 6.22. A reactive band was visible at 14 kDa and two further bands of lesser intensity at 13 and 11 kDa with the CHAPS extract. A very faint band of reactivity may have been present at 14 kDa with the OG extract. No reactivity was observed with the DOC extract. Immunobloting experiments with PH7 against DOC, OG and CHAPS extracts of GMF were consistently negative.

Immunoblotting results with ASD5 were inconclusive. Fig. 6.23 shows ASD5 blotted against a CHAPS extract of GMF. Three faint bands of reactivity were visible at 44, 42 and 37 kDa. The blot was probed with a 1/10 dilution of affinity-purified ASD5, bound monoclonal antibody was detected with HRP-conjugated anti-mouse immunoglobulin antibody. This result was unable to be reproduced using the HRP-conjugated anti-mouse immunoglobulin antibody or with the biotin-streptavidin detection system. In one experiment using CHAPS extract from isolated glomeruli, a faint band was visible at 35 kDa (data not shown). Unfortunately, this result also could not be reproduced. Immunoblotting experiments, undertaken by Mrs M Callus, Scientific Officer, Renal Immunopathology Division, Wellington School of Medicine, revealed that ASD5 did not react with any protein bands present in either the collagenase digest of human GBM or the DOC extract of hGMF.

ASD5, PH7, BSG11 and SC5 were blotted against an extract of rat Fx1A. Repeated attempts, using affinity-purified monoclonal antibody and the streptavidin detection system, failed to detect any reactivity with any of the monoclonal antibodies.

Figure 6.20.

Immunoblot of SC5 against OG and CHAPS extracts of isolated glomeruli.

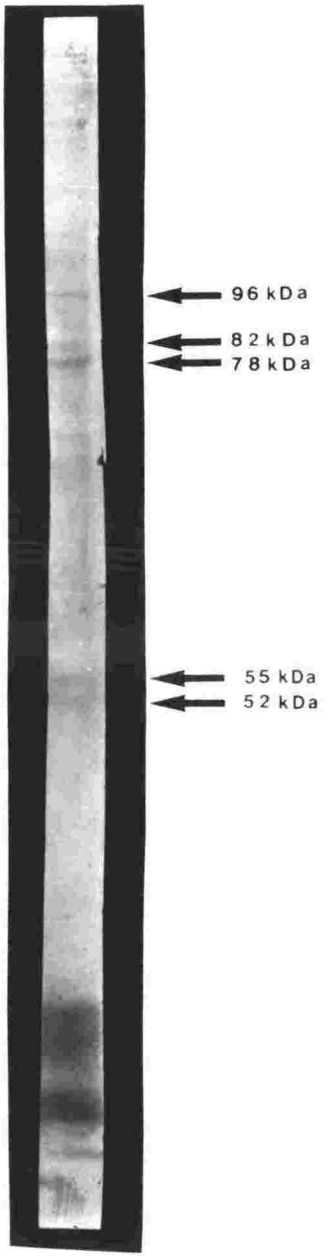


Samples of OG and CHAPS extracts of isolated glomeruli, containing 50 μg of protein, were electrophoresed on a 5-15% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and probed with SC5 ascites diluted 1/25 in TBS/0.3% BSA.

Lane 1 - CHAPS extract; Lane 2 - OG extract.

Figure 6.21.

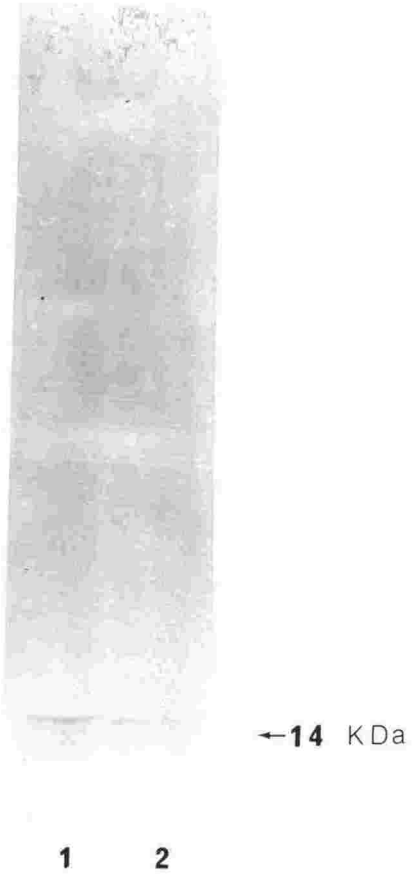
Immunoblot of SC5 against CHAPS extract of GMF



A sample of a CHAPS extract of GMF was electrophoresed on a 5-15% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and probed with an SAS precipitate of SC5 ascites diluted 1/25. Bound mouse monoclonal antibody was detected by a biotin streptavidin system.

Figure 6.22.

Immunoblot of PH7 with CHAPS and OG extracts
of isolated glomeruli.

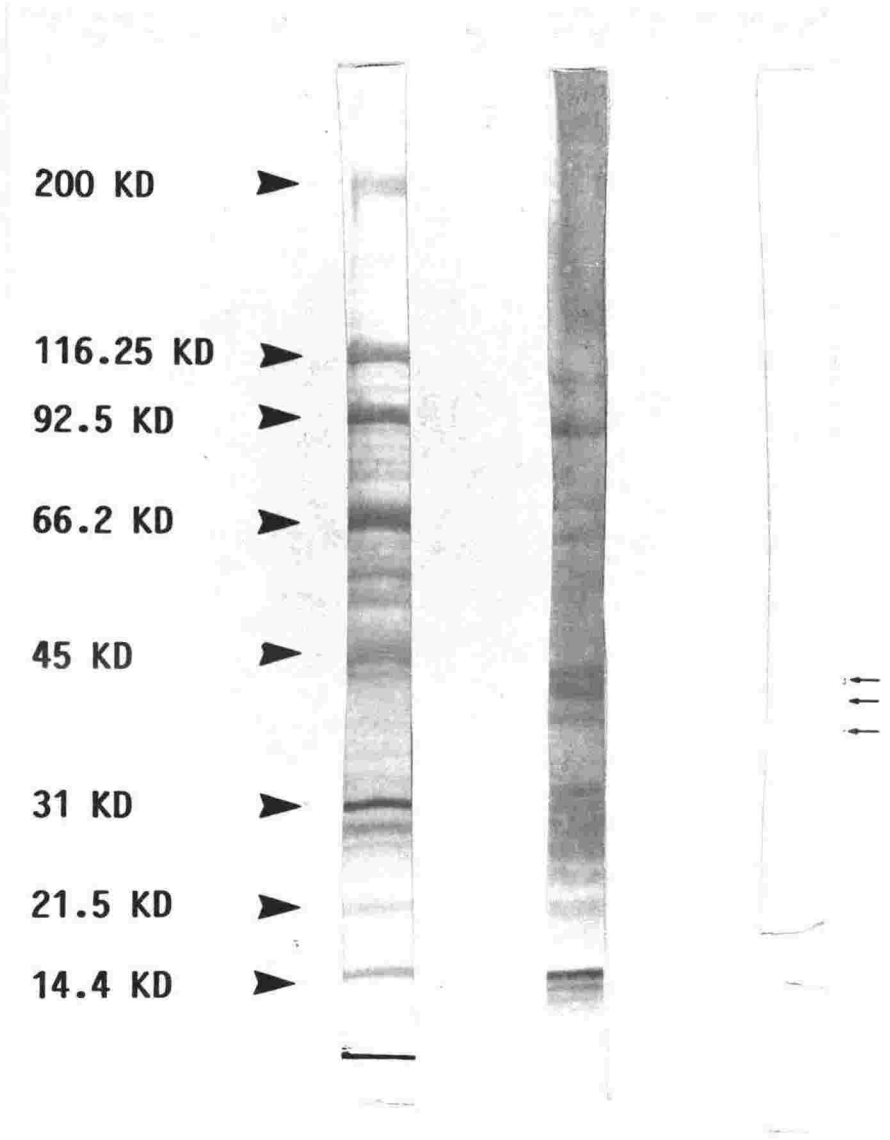


Samples of CHAPS and OG extracts of isolated glomeruli, containing 50 μg of protein, were electrophoresed on a 5-15% gradient polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was probed with a 1/25 dilution of PH7 ascites. Bound monoclonal antibody was detected with a polyclonal HRP-conjugated anti-mouse immmunoglobulin antibody.

Lane 1 - CHAPS extract Lane 2 - OG extract.

Figure 6.23.

Immunoblotting of ASD5 against CHAPS extract of GMF.



A sample of CHAPS extract of GMF containing 45 μg of protein was electrophoresed on a 5-15% polyacrylamide gel. Following transfer to a nitrocellulose membrane, the blot was probed with affinity-purified ASD5 monoclonal antibody diluted 1/10 in TBS/0.3% BSA.

Lane 1 - Nitrocellulose strip, transfer of molecular weight standards stained with Ponceau S.
Lane 2 - Nitrocellulose strip, transfer of protein bands stained with Ponceau S.
Lane 3 - Immunoblot, ASD5.

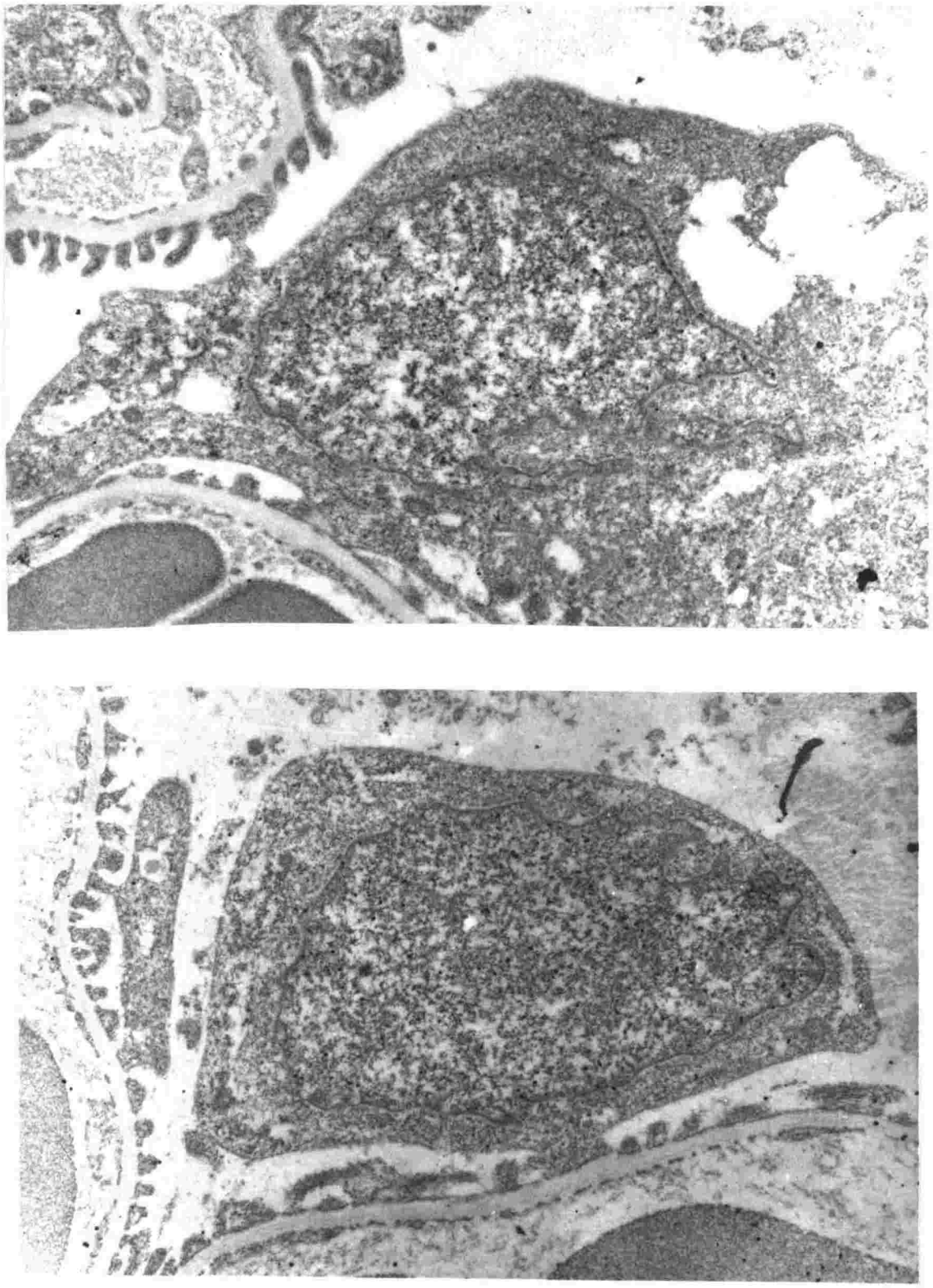
6.3.7 Immunogold electron microscopy (ImAu EM).

Attempts to localise the sites of reactivity of the monoclonal antibodies at the ultrastructural level using both the pre and post-embedding ImAu EM techniques were unsuccessful. The Renal Immunopathology Laboratory at the Welligton School of medicine has extensive experience in the localisation of both polyclonal and monoclonal antibody reactivity at the ultrastructural level by ImAu EM (Fernando et al., 1989; Neale et al., 1988; Boyce et al., 1989). Considerable efforts were made over two years using variations of incubation time and temperature and antibody dilution. Monoclonal antibody-containg ascites, SAS precipitates of these ascites and affinity purified antibody were all used in an attempt to localise binding. In some electron micrographs from post-embedding sections incubated with ASD5 and PH7, localisation of gold particles over the cell nucleus was observed (Fig. 6.24 a and b). All experiments using SC5, BSG11 and the non-kidney binding control monoclonal antibody G6 gave negative results.

6.4 Discussion.

There have been occasional reports in the literature on the production of monoclonal antibodies to renal antigens (Ueda et al., 1981; Falkenberg et al., 1981a, 1981b; Mendrick et al., 1983; Kerjaschki and Farquhar, 1983; Nishi et al., 1984; Ronco et al., 1984a, 1984b; Bander et al., 1985). Although a number of the monoclonal antibodies generated in these studies produced a granular IF pattern of staining, the majority produced linear staining. This could be accounted for by the type of immunogens employed which were generally a complex mixture of kidney cells and membranes. All but one of the monoclonal antibodies generated in the present study produced a granular pattern of immunofluorescence. The only exception was BPC4 which had specificity for an antigen present in the the nuclear membrane. None of the monoclonal antibodies produced a linear IF pattern on the glomerular capillary wall as would be typical of a classical anti-GBM binding pattern. Mendrick et al., (1983) reported that the specificity of 6 out of 11 kidney-fixing monoclonal antibodies resided in the glomerular basement membrane. The monoclonal antibodies in that study were prepared from the splenocytes of a mouse which had been immunised with an homogenate of rat kidney cortex. When examined by IF microscopy for extrarenal binding, the reagents were shown to react with either epithelial or endothelial cells

Figure 6.24.
Post-embedding ImAu EM.



Ultrathin sections of normal Lewis rat kidney were incubated with ASD5 or PH7 affinity-purified monoclonal antibody. Bound mouse monoclonal antibody was detected by a 30 nm gold particle-labelled anti-mouse IgG and IgM. Gold particles are seen over the nuclei in both micrographs. Original magnification of both micrographs was x 25,000.

A. PH7. B. ASD5.

from a variety of tissues, including smooth muscle, cardiac muscle and intestinal mucosa. Nishi et al. (1984) reported the production of a monoclonal antibody A4 which stained the GCW and tubular cell cytoplasm as well as the epithelial cells of the intestinal mucosa. In the present study, PH7 and ASD5 both reacted strongly with intestinal brush border. These two monoclonal antibodies had the widest distribution of reactivity in the tissues examined. Chatelet et al. (1986b) reported on the reactivity of a monoclonal antibody raised against a crude brush border preparation isolated from rat kidney cortex. When administered IV, transient glomerular deposits were observed, being maximal at one to four hours. By immunoblotting, this antibody was shown to react with a 90 kDa protein, and by IF microscopy stained both renal brush borders and glomerular epithelial cells. Non-renal reactivity of this reagent was primarily with intestinal brush border, the biliary pole of hepatocytes and with capillary endothelial cells of heart, lung, liver and spleen.

None of the four kidney-fixing anti-GMF monoclonal antibodies examined in the present study were specific for rat kidney. SC5 showed the greatest restriction by only reacting with rat and human kidney and PH7 the least by reacting with kidney from all three species in addition to rat. These results contrast with the species specificity of the monoclonal antibodies generated against homogenised rat glomeruli by Mendrick et al. (1983), who reported that nine out of fourteen kidney-fixing monoclonal antibodies were considered to be specific for rat kidney.

The monoclonal antibodies SC5, ASD5, PH7, and BSG11 were unable to block the binding of anti-rat Fx1A antibodies or HN eluates to normal rat kidney, neither were the anti-Fx1A serum or the HN eluates able to inhibit the glomerular reactivity of the anti-GMF monoclonal antibodies. These results suggested that the epitopes recognised by these anti-GMF monoclonal antibodies were not significantly involved in the pathogenesis of Heymann nephritis.

The recovery of solubilised proteins, following detergent extraction of GMF, representing 10 to 23% of the starting material, although regarded as an adequate extraction, only provided small amounts of material for use in immunoblotting experiments. The yield was too low in most cases and an alternative source of antigen was needed. Considerably greater amounts of protein were extracted from whole glomeruli; however, a large percentage of this protein would have been unlikely to be present in the immunising GMF preparation. The anti-GMF monoclonal antibodies

would not necessarily therefore react with these non-GMF proteins. The antigens present in the GMF preparation, and to which the monoclonal antibodies were directed, would also be present in the extracts of whole glomeruli. As the protein yield from the extracts of whole glomeruli was much greater than from the GMF extracts, it was felt that this preparation could be used as an alternative target antigen in immunoblotting experiments.

Results from dot-blot experiments indicated that all four of the anti-GMF monoclonal antibodies selected for study reacted with all of the detergent extracts. This indicated that the specific antigens with which the monoclonal antibodies reacted were indeed solubilised by the extraction procedures adopted and that the extracts could therefore be employed as the target antigen in immunoblotting experiments. The result obtained with ASD5 was unexpected. Indirect IF microscopy studies had indicated that this monoclonal antibody did not react with normal human kidney, yet dot-blot experiments clearly showed a strong reactivity with both the collagenase digest of human GBM and the DOC extract of hGMF. To investigate this finding further, Mrs M Callus, Scientific Officer, Renal Immunopathology Division, Wellington School of Medicine, Otago University, undertook to examine ASD5 by microELISA against hGMF and by immunoblotting against the collagenase digest of human GBM and the DOC extract of hGMF, and also repeated the indirect IF microscopy studies on normal human kidney. The results showed that ASD5 did not react with either prepartion in microELISA, nor by immunoblotting, and confirmed the negative result previously obtained by indirect IF microscopy.

The results obtained from the immunoblotting experiments indicated that the monoclonal antibodies, with the exception of ASD5, reacted with more than one protein in detergent extracts. SC5 reacted with a large number of small molecular weight proteins extracted from whole glomeruli, major reactivity was with 96 kDa protein. When examined against an extract obtained from GMF, reactivity was restricted to the 96 kDa band and two other major bands at 82 and 78 kDa. Minor bands of reactivity were seen at 55 and 52 kDa, but no reactivity with lower molecular weight material was seen. The reactivity with the major 96 kDa band occurred in both experiments. The lack of reactivity with the higher molecular weight bands in the whole glomeruli extract may be the result of a decreased concentration of these proteins in extracts of whole glomeruli. None of the reactivities obtained with these anti-GMF monoclonal antibodies in this study corresponded to known reactivities of

other anti-kidney monoclonal antibodies reported in the literature. None of these studies detailing the biochemical nature of the reactive antigens report reactivity with multiple proteins. Bhan et al. (1985) reported the reactivity of two monoclonal antibodies raised against rat Fx1A and a third raised against isolated whole glomeruli. The anti-Fx1A monoclonal 14C1 reacted with glomeruli and brush borders by indirect IF microscopy and also reacted with a single glycoprotein of 330 kDa determined by immunoprecipitation. The second anti-Fx1A monoclonal antibody AG3 stained brush borders but not glomeruli, and all attempts to immunoprecipitate its reactive antigen from isolated whole microvilli were unsuccessful. Ronco et al. have reported the reactivity of monoclonal antibodies with specificity for a 90 kDa antigen (1984a) and a 330 kDa antigen (1984b), both of which were monospecific. Although monoclonal antibodies are specific for a single antigenic epitope, this epitope may be present in a number of antigens, particularly if the epitope is a glycosylated region of the protein. This may be the case with the anti-GMF monoclonal antibodies generated in this study.

Dot-blot assay revealed that ASD5 reacted with both the collagenase and DOC extracts of hGMF. This result conflicted with the indirect IF studies with ASD5 which indicated that this monoclonal antibody did not react with human kidney. Similarly, IF studies revealed that SC5 reacted with human kidney, whereas dot-blot assay, using extracts obtained from hGMF, returned a negative result. The explanation for the first finding is unclear; the possibility exists that the target antigen of ASD5 is an integral membrane protein, and in the human kidney the epitope with which ASD5 reacts is inaccessible before extraction. Extraction procedures release the epitope which is then free to react with the antibody. The most likely explanation for the lack of reactivity of SC5 with hGMF extracts, in spite of showing reactivity by indirect IF microscopy, is that the antigen was denatured or altered during the extraction process.

The failure to localise the binding site of the monoclonal antibodies by ImAu EM was disturbing. The preservation of antigen integrity during tissue processing for histological examination presents a major problem (Stein et al., 1985). Alteration of the antigen during processing could account for the lack of binding encountered with the monoclonal antibodies in this study. The specificity of monoclonal antibodies for a single epitope may also present problems at the electron microscope level. If the epitope density is low, the immunohistochemical signal may be too weak to record (Kerjaschki et al., 1986).

Chapter Seven.

IN VIVO REACTIVITY OF ANTI-GMF MONOCLONAL ANTIBODIES.

7.1 Introduction.

There are few reports in the literature of anti-kidney monoclonal antibody-induced GN. Although there are a number of accounts of the production of monoclonal antibodies to kidney antigens, only a few of these monoclonal antibodies have proved to be nephritogenic to experimental animals in vivo. The studies reported by Thomson et al. (1984), Mendrick et al. (1983), Hirsch et al. (1984), and Mendrick and Rennke (1986, 1988a, 1988b) all detail the induction of proteinuria following the administration of anti-kidney monoclonal antibodies.

Monoclonal antibodies specifically generated against renal antigens may bind to renal structures in vitro, for example by IF microscopy, but when administered IV to experimental animals they may not be detected in the kidney (Mendrick et al., 1983), or form immune deposits identifiable as electron dense deposits by electron microscopy.

The monoclonal antibodies BSG11, ASD5, SC5 and PH7 were selected for study in vivo on the basis of the granular binding patterns observed in vitro by IF microscopy.

7.2 Materials and Methods.

7.2.1 Preparation of monoclonal antibodies.

SAS precipitates of ASD5, SC5, PH7 and BSG11 monoclonal antibody-containing ascitic fluids were prepared as described in Chapter Four. For control experiments an SAS precipitate of ascitic fluid containing the non-kidney binding monoclonal antibody G6 was prepared in an identical fashion. Precipitates were resuspended in

PBS and dialysed extensively (approximately 48 hours) against PBS. The reactivity titres of the anti-GMF monoclonal antibody preparations were determined by indirect IF microscopy of doubling dilutions of the SAS precipitates on normal kidney sections. The immunoglobulin concentrations of the SAS precipitates were determined by absorption spectroscopy at 280 nm in a Beckman UV spectrometer using an extinction coefficient of 14.49 for a 1% solution of mouse gammaglobulin in a 1 cm light path (Hudson and Hay, 1980). The final preparations were stored in plastic vials at 4°C and used within one week of preparation. No preservatives were added to preparations for use in vivo.

7.2.2 Administration of anti-GMF monoclonal antibodies.

All in vivo studies were performed on Lewis rats of either sex between four and six months of age. Body weights of the rats were 180-240 grams. Rats were placed in metabolic cages for 24 hours to determine baseline 24 hour urine protein excretion. Forty-eight to 72 hours later, the rats received monoclonal antibody IV. Immediately prior to administering the monoclonal antibody preparations IV, the solutions were passed through a sterilized 0.22 μm nitrocellulose membrane filter, thereby removing any aggregated material and also rendering the solutions sterile. Vasodilation was induced in the experimental animals prior to IV injection by placing them in a high-sided, circular metal container under an infrared light source (250 v, 250 w, Stella, Holland). IV injection, under light ether anaesthesia, was made over one to two minutes into a tail vein with a 26 G needle. Rats were sacrificed by cervical dislocation under ether anaesthesia at one, 24 or 48 hours post-injection. Those rats which were to be sacrificed 24 hours post-injection were placed in metabolic cages immediately following the administration of monoclonal antibody and 24 hour urine samples collected. Those rats which were to be sacrificed at 48 hours post-injection were returned to regular cages in the animal facility and placed in metabolic cages 24 hours later. At sacrifice, both kidneys were removed, sections of cortical tissue were obtained from each and immediately snap frozen in liquid nitrogen for subsequent study by direct IF microscopy. Cortical tissue was also obtained for light microscopy study from those animals sacrificed at 24 hours or later.

7.2.3 24 Hour urine samples.

For collection of 24 hour urine samples, experimental animals were housed in

metabolic cages. Water was available ad libtum, but food was withheld. Small metal mesh grids placed at the mouth of the urine collecting funnels prevented faecal material entering the collection bottles. All bottles used for the collection of urine samples were sterilised prior to use.

7.2.4 Urine protein estimation.

Urine volume was determined by measurement in graduated glass cylinders. Two ml samples were centrifuged at 1000 x g for 10 minutes. One ml of supernatant was transferred to a 4 ml plastic test tube and an equal volume of 20% trichloracetic acid (TCA) added; the tube was then vortexed to ensure thorough mixing. Tubes were allowed to stand for at least two hours at RT or overnight at 4°C. Proteins precipitated from the urine samples were sedimented by centrifugation at 3000 x g for 20 minutes. The supernatant was discarded and the precipitate redissolved in 500 μl of 0.1 M NaOH. Protein estimation was then determined by one of the following methods.

a. Modified Bradford Assay.

This assay was based on the Sedmak and Grossberg (1977) modification of the dye binding assay described by Bradford (1976). Brilliant blue G 250 dye was prepared as a 0.04% (w/v) solution in 3.5% perchloric acid. Samples (100 μl) of resolubilised TCA-precipitable urine protein were added to test tubes, 1.5 mls of 0.9% NaCl was then added and the tubes vortexed. This was followed immediately with 1.5 mls of dye solution which was added while the tubes were being vortexed. Samples were allowed to stand at RT for 10 to 30 minutes before colourometric readings were made at 620 nm in a spectrophotometer (model 3600, Beckman Instruments, USA).

b. Lowry Protein Assay.

TCA-precipitable urine protein as described above was assayed by the method of Lowry et al. (1951). Samples were read at 750 nm. For both assays, the protein concentration of the experimental samples was determined from a standard curve constructed with known amounts of standardised BSA as described in Chapter Three. Statistical analysis of urine protein excretion prior to and following the administration of anti-GMF monoclonal antibodies was performed using the two-tailed Student's t

test.

7.2.5 Light microscopy.

Immediately following sacrifice, samples of renal cortical tissue, approximately 0.5 x 0.5 x 0.25 cm, were fixed in buffered formalin for 24 to 72 hours. The tissue was then dehydrated in graded alcohols, cleared in chloroform and impregnated with paraffin wax in a Shandon-Elliot SE 400 automated tissue processor (Shandon Scientific Ltd., UK). Following embedding, serial tissue sections, 2-3 μm thick, were cut on a rotary microtome (American Optical Co. Ltd., USA). Eight or ten sections were cut, the block was advanced approximately 20-25 μm and a further eight or ten sections cut. The sections were air dried at 37°C for 24 hours, and then stained with Ehrlich's haematoxylin and eosin by the method of Culling (1974). The sections were viewed in a photomicroscope and glomeruli examined for injury and specifically for the presence of polymorphonuclear leucocytes (PMN). A total of five glomeruli were selected which appeared to have been sectioned at or near their mid-point and the number of PMNs present counted. A further five glomeruli were selected from the second group of sections which had been cut 20-25 μm distant from the first sections and a further PMN count performed. The results were expressed as the mean value obtained from the ten glomeruli counted.

7.2.6 Immunofluorescence (IF) microscopy.

Cortical tissue sections were obtained immediately upon sacrifice and processed as described in Chapter Three. Bound mouse monoclonal antibody was detected with FITC sheep anti-mouse immunoglobulin (Silenus Laboratories, Australia) which had previously been absorbed with normal rat serum.

7.2.7 Electron microscopy.

a. Transmission electron microscopy (TEM).

Cortical tissue was finely diced with a scalpel blade and immediately placed in 2% glutaraldehyde in 0.1M cacodylate buffer and then processed as described in Chapter Three. Ultrathin sections approximately 90 nm thick were cut on an LK13 ultratome (LKB Produkter AB, Sweden), counterstained with lead citrate and uranyl acetate and

viewed in a Philips transmission electron microscope.

b. Immunogold electron microscopy (ImAu EM).

Renal cortical tissue was obtained from monoclonal anti-GMF antibody-injected rats immediately upon sacrifice, finely diced with a scalpel blade and placed in 1% glutaraldehyde for one hour. The samples were then processed and sectioned as described for post-embedding ImAu EM in Chapter Six. The sections were incubated with gold-conjugated anti-mouse IgG-IgM and then counterstained prior to viewing and photographing.

7.2.8 Radiolabelling of monoclonal antibodies.

Quantitation of specific binding of the monoclonal antibodies in vivo was determined by the paired radiolabel technique (Wilson et al., 1971). Monoclonal antibody-containing solutions, prepared from 40% SAS precipitates, were filtered through 0.22 μm membranes. Immunoglobulin protein estimation was determined by absorption spectroscopy at 280 nm as described in 7.2.1. Reactivity titres, before and following radiolabelling, were determined by indirect IF microscopy of doubling dilutions of the monoclonal antibodies on frozen sections of normal Lewis rat kidney. A total of 5-10 mgs of the specific monoclonal antibodies were labelled with ^{125}I and the non-specific G6 monoclonal antibody with ^{131}I by the chloramine T method (McConahey and Dixon, 1966). The protein to chloramine T ratio was 50:1 and reaction time was five minutes. Free iodide was removed by dialysis against large volumes (4-5 litres) of PBS, changed at one hourly intervals at least five times, and then overnight (10-14 hours). Two further changes of PBS were made in the morning, an hour apart. Precipitability of counts was determined by precipitating the protein from a 100 μl aliquot of the radiolabelled proteins with an equal volume of 20% trichloracetic acid (TCA). If $<90\%$ of counts were precipitable, the labelled fractions were dialysed for a further 24 hours with frequent changes of PBS and the precipitability of counts repeated. Only those proteins with $>90\%$ TCA-precipitable counts were used in vivo. The protein concentration of the labelled proteins was determined by the modified Bradford method. The counts contained in a 10 μl aliquot of labeled protein was determined and the counts per μg of protein calculated.

7.2.9 Quantitation of specific monoclonal antibody binding in vivo.

A total of 2 mgs of a specific kidney-binding radiolabelled monoclonal antibody was administered together with 2 mgs of the non-specific G6 monoclonal antibody IV through a tail vein to normal Lewis rats (200-350 g). The rats were housed in cages and allowed food and water ad libitum. Rats were sacrificed at 24 and 48 hrs post-injection. Blood samples were obtained at the time of sacrifice in heparinized tubes. The abdomen was opened through a midline incision and the viscera exposed. The inferior vena cava was identified and transected with a scalpel blade. The thoracic cavity was opened and the organs perfused with PBS via a 21G needle inserted into the left ventricle of the heart. Perfusion was continued until all organs were blanched. Whole kidneys and spleen were removed and specimens of liver, lung and small intestine obtained. The wet weights of all samples were determined. Tissues obtained were homogenised in a Sorval Omnimixer for 60 seconds. The homogenates were then washed in PBS and centrifuged at 3000 x g for 10 minutes. This procedure was repeated until the supernatants were clear. To determine the blood ^{125}I:^{131}I ratio, 100 μl of heparinised whole blood was pipetted into a test tube and counted in an LKB two channel gamma counter programmed to count ^{125}I and ^{131}I. The whole blood was then diluted with 400 μl of PBS and precipitated with 500 μl of 20% TCA, centrifuged at 3000 x g for 10 minutes and the precipitate counted. The post-TCA counts were used to determine the ^{125}I:^{131}I ratio. Following preparation of the tissue homogenates, 5-10 μl samples of radiolabelled specific and non-specific monoclonal antibody were diluted in 200 μl of PBS/1% BSA. Proteins were precipitated by the addition of 200 μl of 20% TCA, centrifuged at 3000 x g for 10 mins and the precipitate counted. All counts were corrected for ^{125}I crossover and specific monoclonal antibody binding determined from the paired label equation of Wilson et al., (1971).

7.3 Results.

7.3.1 Binding of anti-GMF monoclonal antibodies in vivo.

In an initial experiment to determine whether the anti-GMF monoclonal antibodies would bind to kidney in vivo, five groups of Lewis rats, each consisting of four rats, received 5 mgs of one of the respective SAS-precipitated anti-GMF monoclonal antibodies ASD5, SC5, PH7, BSG11, or the negative control G6. Rats were sacrificed

at one hour and the kidneys removed for direct IF microscopy (Table 7.1). A granular deposition of mouse immunoglobulin was detected by direct IF microscopy in the glomeruli of those rats which had received SC5 or PH7. Mouse immunoglobulin deposition in the kidneys of these rats was limited to the glomeruli and was not detected at any other site in the kidney.

Only occasional deposits of mouse immunoglobulin were detected in the glomeruli of those rats receiving ASD5 monoclonal antibody. Immunoglobulin was not detected in all glomeruli, and where evident, occurred only in isolated patches in the glomeruli. No mouse immunoglobulin was detected in the kidney at any site in those rats receiving BSG11.

Negative control experiments were conducted with G6 monoclonal antibody which in vitro did not bind to kidney. No mouse immunoglobulin was detected in the kidneys of rats receiving G6.

The anti-GMF monoclonal antibody SC5 was administered IV to normal Lewis rats. Groups of rats received 10, 20 or 30 mg and were sacrificed at 24 or 48 hours post-injection. Direct IF microscopy of cortical tissue taken at the time of sacrifice of the experimental animals revealed the presence of mouse immunoglobulin in the glomeruli of all rats and at all time points studied (Fig.7.1 a and b). At the 10 mg dose level, a segmental, coarse granular deposition of mouse immunoglobulin was observed in the glomeruli of treated animals (Fig.7.1a). At the 20 mg dose level the same coarse granular pattern of deposition was evident; however, there was a much heavier deposition of mouse immunoglobulin in these rats (Fig. 7.1b). A similar pattern of deposition was found in those rats receiving 30 mg of SC5 IV. Mouse immunoglobulin was detected only in the glomeruli of treated rats and at no other site in the kidney.

Mouse immunoglobulin was also detected in the kidneys of experimental animals following the administration of PH7. At a dose of 5 mg, scant deposition was observed within the glomeruli (Fig.7.2). The segmental, coarse granular pattern of deposition was similar to that observed with SC5 at low doses. No staining of kidney structures other than the glomerulus was evident at this dose. At higher doses there was a heavier deposition of mouse immunoglobulin, and the IF pattern was similar to that observed by indirect IF microscopy studies with this monoclonal antibody in

Table 7.1.

Renal binding of anti-GMF monoclonal antibodies in vivo at one hour.

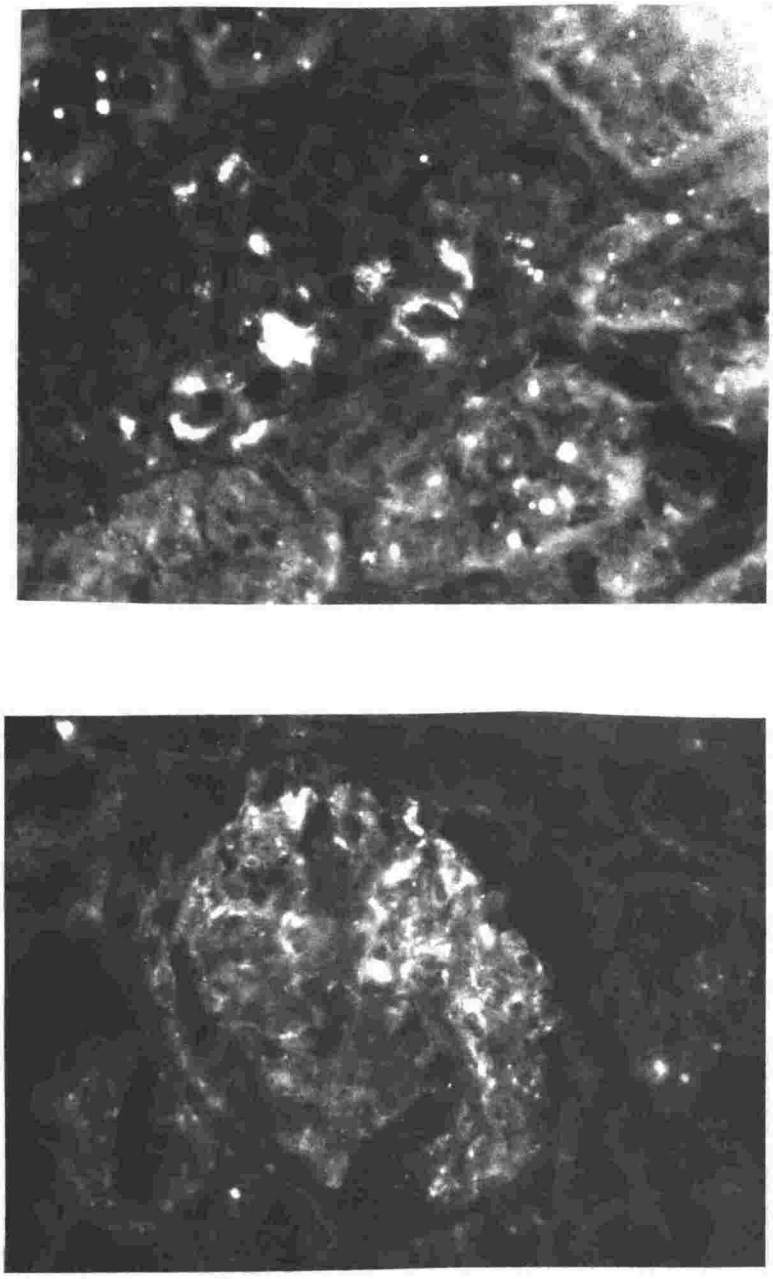
Monoclonal antibody	Renal binding	
	Glomeruli	Tubules
SC5	+	-
PH7	+	-
ASD5	±	-
BSG11	-	-
G6	-	-

Frozen sections of renal cortex, taken from rats one hour post-injection of monoclonal antibody, were examined by direct IF microscopy for the presence of mouse immunoglobulin.

\+ Positive staining
\- Negative staining
± Indeterminate, inconsistent/irregular staining

Figure 7.1.

Deposition of mouse immunoglobulin in rat glomeruli following IV administration of SC5 monoclonal antibody.

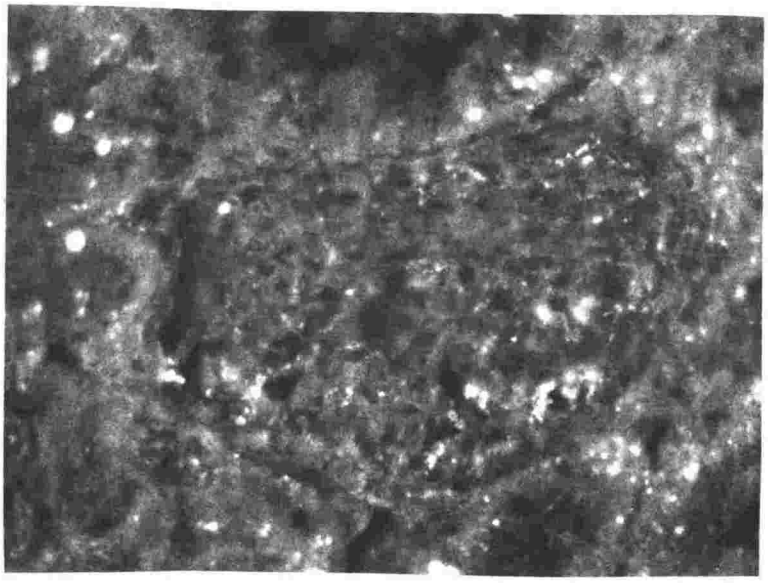


Renal cortical tissue obtained at sacrifice from a rat which had received SC5 monoclonal antibody IV was examined by direct IF microscopy. The sections have been stained with polyclonal anti-mouse immunoglobulin.

a - 24 hours following 10 mg SC5 IV.
b - 48 hours following 20 mg SC5 IV.

Figure 7.2.

Mouse immunoglobulin deposition following
IV administration of 5 mgs of PH7.



Five mgs of an SAS precipitate of mouse ascitic fluid containing the monoclonal antibody PH7 was administered IV to a normal Lewis rat. Renal cortical tissue was obtained at the time of sacrifice 24 hours post-injection. The section has been stained with polyclonal anti-mouse immunoglobulin.

vitro (Fig. 7.3). Staining of the tubular basolateral membrane was observed in those rats which received 20mg or 30 mg of PH7 (Fig. 7.4). The staining was not as intense as that observed in the glomerulus, nor were all the tubules stained. However, some staining of the tubules was observed in all rats which received 20 or 30 mg of PH7.

Mouse immunoglobulin was not detected at any site in the kidney at 24 or 48 hours following the administration of 10 mg of BSG11. A similar negative result was obtained following the administration of both 5 and 10 mg of ASD5.

Control rats received 20 or 30 mg of G6 monoclonal antibody and were sacrificed at 24 or 48 hours post-injection. Mouse immunoglobulin was not detected in the kidneys from any of the animals in this control group.

7.3.2 Urine protein excretion.

Urine protein estimation was performed by the Lowry method (Lowry et al., 1951) on the urines obtained from rats which received 5 mgs of SC5 and those which received 5 mgs of ASD5. All remaining urine protein estimations were made by the modified Bradford method.

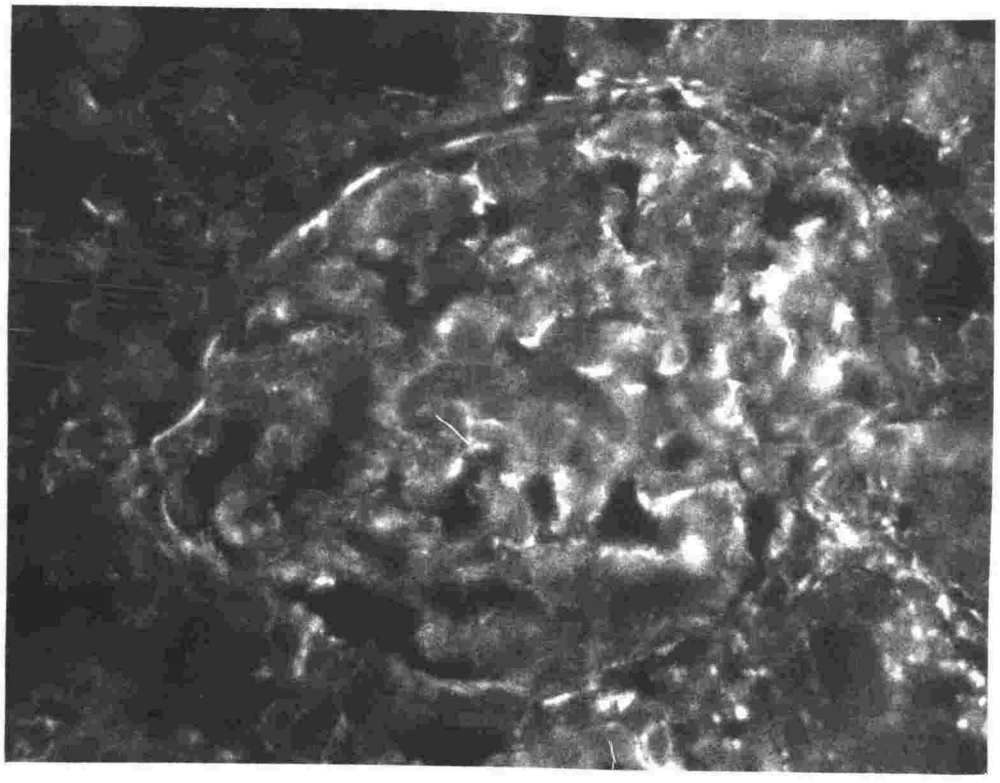
Baseline 24 hour urine protein excretion assessment of 117 normal Lewis rats indicated a mean 24 hour urine protein excretion value of 3.21 (± 2.29) mg/24 hours. All rats excreted less than 10 mg protein/24 hours, with individual values ranging from 0.25 - 9.57 mg/24 hours.

a. SC5.

The results obtained with SC5 are shown in Table 7.2 . Post-treatment 24hr urine protein excretion in those rats receiving 20 mg SC5 and sacrificed at 24 hours was significantly greater than pre-treatment values, $P < 0.005$. The increased urine protein exretion during the 24 to 48 hour period following treatment with 20 mg of SC5 was also significant at the $P < 0.05$ level. At the 30 mg dose level there was no significant difference between pre and post-treatment urine protein excretion during the immediate 24 hour period following treatment ($P = .055$). However, although the pre and post-treatment mean urine protein values were not significantly different statistically, three rats in this group had 24 hour urine protein excretion in excess of

Figure 7.3.

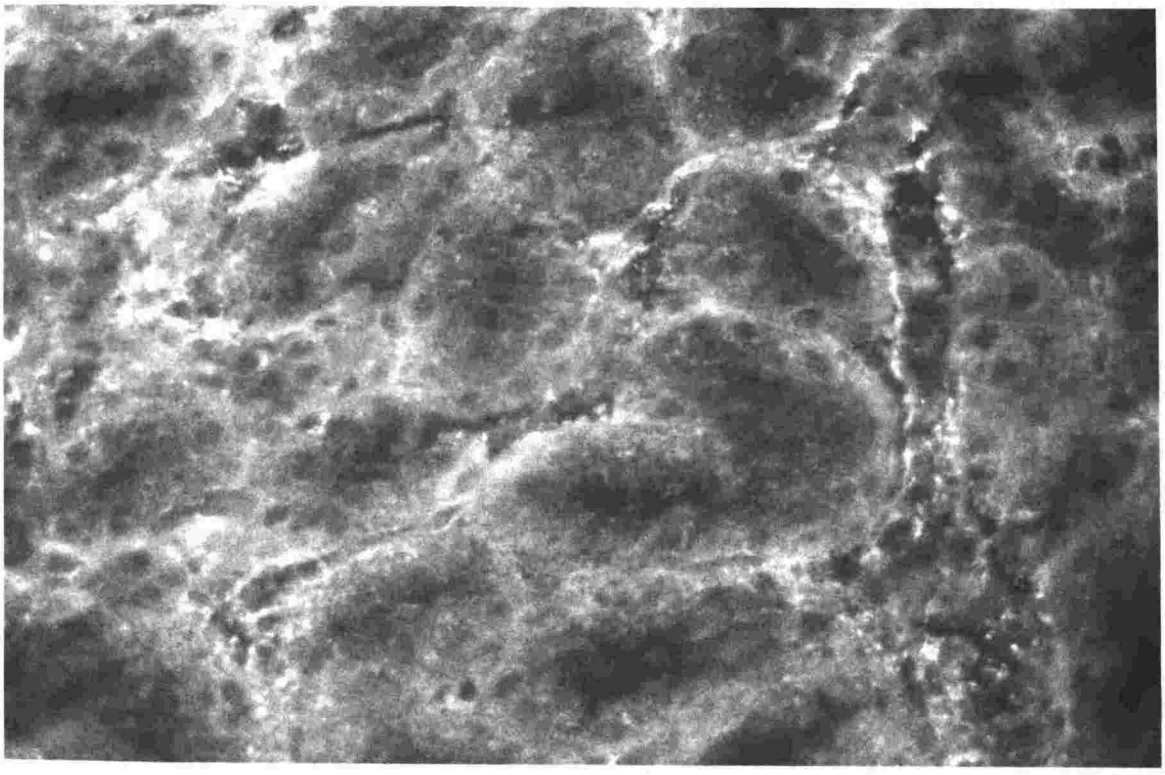
Mouse immunoglobulin deposition following
IV administration of 20 mgs of PH7.



Twenty mgs of an SAS precipitate of mouse ascitic fluid containing the monoclonal antibody PH7 was administered IV to a normal Lewis rat. The rat was sacrificed 48 hours post-injection and renal cortical tissue obtained for study by direct IF microscopy. The section has been stained with polyclonal anti-mouse immunoglobulin.

Figure 7.4.

Immunofluorescence staining of kidney tubules
following IV administration of PH7.



Renal cortical tissue was obtained from a rat which had received 30 mg of an SAS precipitate of mouse ascitic fluid containing the monoclonal antibody PH7. The section has been stained with polyclonal anti-mouse immunoglobulin.

Table 7.2.

24 hour urine protein excretion following IV administration of SC5.

n	Dose (mg)	Time point (hours)	24 hour urine protein excretion (mg/24 hrs) Pre-treatment	SD	Post-treatment	SD	
8	10	24	3.08 (0.29-5.82)	±2.19	4.96 (0.84-11.4)	±3.66	
7	10	48	2.42 (0.36-6.00)	±1.90	4.66 (1.44-7.70)	±2.24	
9	20	24	2.94 (1.20-3.83)	±0.97	14.68 (3.68-34.40)	±10.6	4*
6	20	48	2.89 (1.04-4.80)	±1.33	6.32 (3.36-10.85)	±2.99	*
6	30	24	5.38 (0.25-9.57)	±3.38	15.15 (5.04-35.40)	±10	
4	30	48	2.04 (1.20-2.44)	±0.49	13.27 (9.10-17.55)	±3.03	*

Urine protein excretion values are expressed as the mean.

SD - One standard deviation of the mean.

Figures in parentheses are the range of urine protein excretion values for that group.

* - Signifies a significant difference between pre- and post-treatment urine protein excretion at the $p < 0.05$ level.

15 mg (15.73, 20.06 and 35.40 mg). At the 30 mg dose level, post-treatment urine protein excretion was significantly greater than pre-treatment values during the 24 to 48 hour post-treatment period ($P < 0.01$).

B. PH7.

24 hour urine protein excretion values obtained following IV administration of this anti-GMF monoclonal antibody are shown in Table 7.3. At a dose of 5 mg there was no significant difference between pre-treatment and post-treatment values. At the 10 mg dose regimen, urine protein excretion was increased over control values during the 24 hour post-treatment period. This increase was significant at the $P < 0.05$ level. Although the mean post-treatment value was 8.95 mg/24hrs, three of the seven rats in this group excreted more than 10 mg of protein during the 24 hour post-treatment period. During the 24 - 48 hour period following 10 mg of PH7, there was no significant diffeence between pre and post-treatment values. At the 20 mg dose level, 24 hour time point, post-treatment urine protein excretion was significant at the $P < 0.05$ level. Three of six rats in this group excreted more than 10 mg of protein. There was no significant difference between pre and post-treatment values obtained during the 24 - 48 hour period following administration of 20 mg PH7. The post-treatment urine protein excretion in the group receiving 30 mg of PH7 and sacrificed at 24 hours, ranged from 7.15 - 31.28 mg/24 hours, and was significant at the $P < 0.05$ level. Urine protein excretion in four of the six rats in this group exceeded 10 mg/24 hours. Although the urine protein excretion was also significant at the $P < .05$ level in the group sacrificed at 48 hours following 30 mg of PH7, none of the rats in this group excreted more than 10 mg of protein during the 24 hour period.

C. ASD5.

The mean urine protein excretion values obtained following IV administration of ASD5 are shown in Table 7.4. There were no significant differences between pre and post-treatment urine protein excretion at either dose level examined. No rats in either group excreted more than 10 mg of protein during the post-treatment period.

D. BSG11.

Table 7.5 shows the mean urine protein excretion values obtained in rats receiving this

Table 7.3.

24 hour urine protein excretion following IV administration of PH7.

n	Dose (mg)	Time point (hours)	24 hour urine protein excretion (mg/24 hrs) Pre-treatment	SD	Post-treatment	SD
6	5	24	3.63 (2.60-4.63)	±0.76	3.31 (2.00-4.08)	±0.70
7	10	24	2.87 (0.98-5.43)	±1.35	8.97 (1.80-17.84)	±5.27 *
6	10	48	3.55 (1.89-6.32)	±1.37	4.72 (2.86-7.02)	±1.26
6	20	24	5.64 (1.52-9.00)	±2.57	9.24 (7.32-11.36)	±1.58 *
5	20	48	6.82 (4.54-8.82)	±1.82	7.93 (4.41-11.26)	±2.29
6	30	24	5.11 (2.46-8.16)	±2.18	16.07 (7.15-31.28)	±8.52 *
4	30	48	1.71 (0.92-2.31)	±0.52	3.99 (0.77-7.15)	±2.03 *

Urine protein excretion values are expressed as the mean.
SD - One standard deviation of the mean.
Figures in parentheses are the range of urine protein excretion values for that

Table 7.4.

24 hour urine protein excretion following IV administration of ASD5.

n	Dose (mg)	Time point (hours)	24 hour urine protein excretion (mg/24 hrs) Pre-treatment	SD	Post-treatment	SD
3	5	24	3.58 (1.12-4.86)	±1.74	5.49 (3.12-6.97)	±1.69
3	10	48	2.35 (0.42-4.62)	±1.73	3.28 (0.80-5.25)	±1.85

Urine protein excretion values are expressed as the mean.

SD - One standard deviation of the mean.

Figures in parentheses are the range of urine protein excretion values for that group.

Table 7.5.

24 hour urine protein excretion following IV administration of BSG11.

n	Dose (mg)	Time point (hours)	24 hour urine protein excretion (mg/24 hrs) Pre-treatment	SD	Post-treatment	SD
3	10	24	1.34 (1.04-1.85)	±0.36	1.18 (0.42-1.68)	±0.55
3	10	48	2.21 (1.70-2.59)	±0.37	1.74 (1.45-2.02)	±0.23

Urine protein excretion values are expressed as the mean.

SD - One standard deviation of the mean.

Figures in parentheses are the range of urine protein excretion values for that group.

monoclonal antibody. No significant differences were found between pre- and post-treatment urine protein excretion in either group. As was found with ASD5, no rat excreted greater than 10 mg of protein during the post-treatment period.

E. G6.

Control studies were conducted with the non-kidney fixing monoclonal antibody G6. Following IV administration, urine protein excretion was monitored as for experimental groups. The mean urine protein excretion values obtained following IV administration of G6 are shown in Table 7.6. No significant differences were found between pre and post-treatment values obtained at any time point following administration of either 20 or 30 mg of this monoclonal antibody.

7.3.3 Light microscopy.

The PMN counts obtained from the glomeruli of rats administered SC5 or G6 monoclonal antibody are shown in Table 7.7. and 7.8. The only significant differences in glomerular PMN accumulation found between SC5-treated and G6-treated rats were at the 20 mg dose level at 24 hours, $P < 0.5$, and the 30 mg dose at 48 hours, $P < 0.5$. The histology of all glomeruli examined appeared normal with no hypercellularity apparent.

The glomerular PMN counts obtained following the administration of PH7 monoclonal antibody are shown in Table 7.9. There were no significant differences found between PH7 and G6 treated rats at the $P < 0.05$ level at any dose or time point studied. Glomerular histology also appeared normal in all glomeruli examined.

Glomerular PMN counts were performed on sections from rats treated with either 10 mg of BSG11 and sacrificed at either 24 or 48 hours post-injection, or treated with 10 mg of ASD5 and sacrificed at 48 hours post-injection. No significant PMN differences were observed following injection of either monoclonal antibody when compared with counts obtained from rats given 20 mg of G6 and sacrificed at 24 or 48 hours. Glomerular histology appeared normal in both the ASD5 and BSG11 treated rats.

Table 7.6.

24 hour urine protein excretion following IV administration of G6.

n	Dose (mg)	Time point (hours)	24 hour urine protein excretion (mg/24 hrs) Pre-treatment	SD	Post-treatment	SD
3	10	24	2.26 (1.01-3.85)	±1.18	2.26 (1.80-3.06)	±0.57
4	20	24	5.90 (3.86-8.20)	±1.93	6.14 (4.60-8.20)	±1.35
3	20	48	3.41 (0.65-6.36)	±2.33	2.85 (2.40-3.19)	±0.33
5	30	24	2.15 (0.88-4.22)	±1.11	2.38 (1.55-2.94)	±0.53
6	30	48	1.79 (0.54-2.59)	±0.69	1.60 (1.37-1.92)	±0.25

Urine protein excretion values are expressed as the mean.

SD - One standard deviation of the mean.

Figures in parentheses are the range of urine protein excretion values for that group.

Table 7.7.

Glomerular polymorphonuclear leucocyte cell count following administration of SC5 monoclonal antibody in vivo.

n	Administered Dose (mg)	Time point (hours)	Mean PMN count	SD
3	10	24	1.10	±0.42
4	10	48	0.85	±0.74
9	20	24	1.27	±0.40
3	20	48	1.20	±0.60
6	30	24	1.41	±0.34
4	30	48	1.65	±0.47

SD - Sample standard deviation.

n - Number of experimental animals studied.

Table 7.8.

Glomerular polymorphonuclear leucocyte cell count following administration of G6 monoclonal antibody in vivo.

n	Administered Dose (mg)	Time point (hours)	PMN count	SD
3	10	24	0.86	±0.12
4	20	24	0.70	±0.50
4	20	48	1.05	±0.34
2	30	24	0.90	±0.42
3	30	48	0.73	±0.42

SD - Sample standard deviation.

n - Number of experimental animals studied.

Table 7.9.

Glomerular polymorphonuclear leucocyte cell count following administration of PH7 monoclonal antibody in vivo.

n	Administered Dose (mg)	Time point (hours)	PMN count	SD
4	20	24	0.98	±0.47
3	20	48	1.45	±0.31
6	30	24	0.97	±0.37
6	30	48	0.93	±0.33
3	30	48	0.73	±0.42

SD - Sample standard deviation.

n - Number of experimental animals studied.

7.3.4 Electron microscopy.

a. TEM.

Electron photomicrographs of glomeruli from treated rats were examined for the presence of electron-dense deposits and for morphological integrity. No obvious abnormality was observed in any of the glomeruli of treated rats. Occasional disruption of the endothelium was observed in some electron photomicrographs of SC5 and PH7 treated rats. However, the same picture was also seen in G6 treated control rats and could not therefore be considered a specific result of anti-GMF monoclonal antibody.

b. ImAu EM.

Despite repeated attempts to localise the binding of the monoclonal antibodies in the kidneys of treated rats, neither mouse IgG nor IgM could be detected by ImAu EM in the glomeruli of rats receiving any of the anti-GMF monoclonal antibodies or the control antibody G6. Incubation conditions were varied with regard to temperature, concentration of labelled antiserum, blocking agents and duration of reaction in an attempt to optimise conditions for successful localisation, all without success.

7.3.5 Paired radiolabel studies.

Specific binding of the anti-GMF monoclonal antibodies SC5 and PH7 was determined at 24 and 48 hours and for ASD5 at 24 hours only.

Three rats each received 2 mg of both the ^{125}I-labelled ASD5 anti-GMF monoclonal antibody and the ^{131}I-labelled non-kidney fixing G6 monoclonal antibody. Examination of tissues obtained from these rats at sacrifice, 24 hours following the administration of the labelled reagents, revealed there was no specific binding of ASD5 in any of the tissues studied: kidney, spleen, liver, gut or lung.

Results obtained with SC5 at 24 hours are shown in Table 7.10. Specific binding was greatest in the kidney. There was no significant difference between right and left kidney binding. Specific binding was also found in the spleen and the liver and to a lesser degree in the gut and lung. At 48 hours, specific binding remained greatest in

Table 7.10.

Specific binding of 2 mgs of SC5 at 24 hours as determined by paired radiolabel studies.

Tissue	n	Minimum	Maximum	Mean	SD
R. Kidney	6	0.146	1.295	0.563	±0.490
L. Kidney	6	0.139	1.118	0.571	±0.456
Spleen	6	0.058	0.421	0.285	±0.145
Liver	6	0	0.438	0.134	±0.209
Gut	6	0	0.107	0.018	±0.044
Lung	6	0	0.025	0.007	±0.011

All values are μg ^{125}I-labelled SC5/g tissue.

the kidney but had decreased to <0.5 μg/g tissue (Table 7.11). Specific binding was still present in the spleen and liver but was detected in the gut of only one rat. No specific binding was detected in the lung of any rat at 48 hours.

The results obtained with PH7 at 24 hours are shown in Table 7.12. Specific binding of this anti-GMF monoclonal antibody is also seen in the kidney, spleen and liver. Specific binding in the spleen was found in only one rat at this time point. It was, however, present at the high value of 0.722 μg/g tissue. Specific binding to liver was seen in only two of the six rats studied. In contrast to SC5, specific binding to gut was observed in all rats at 24 hours, kidney being the only tissue in which specific binding was found to be greater. Specific binding of PH7 was also seen in the lungs of all rats, but in all cases was less than that observed in the kidney and gut. As found with SC5, specific binding of the anti-GMF monoclonal antibodies at 48 hours was observed with the same tissues, but in lesser amounts than at 24 hours (Table 7.13). Specific binding remained greatest in the kidney and gut and was found in the spleen in four of six rats. However, splenic binding was <0.1 μg/g tissue in all cases.

7.4 Discussion.

Studies undertaken in vitro, and detailed in Chapter Six had indicated that the anti-GMF monoclonal antibodies PH7, ASD5, SC5 and BSG11 reacted strongly with glomerular structures and also with some extrarenal tissues. Following IV administration of these reagents to normal Lewis rats, only PH7 and SC5 could be demonstrated by direct IF microscopy to have fixed to the glomeruli. This experience is similar to that of Mendrick et al. (1983) who reported fourteen monoclonal antibodies which reacted with rat glomeruli in vitro when examined by indirect IF microscopy but only six of these reagents could be detected in the glomeruli by direct IF microscopy following IV administration.

Both PH7 and SC5 reacted with renal tubular structures in vitro. Following IV administration of SC5, mouse immunoglobulin could be demonstrated in the glomeruli only and not on any other extraglomerular structures. PH7 was detected in the tubules but only following the higher doses; the intensity of reactivity was much less than in the glomeruli. Following IV injection, the anti-GMF monoclonal antibodies would be present in high concentration within the circulation. Under resting

Table 7.11.

Specific binding of 2 mgs of SC5 at 48 hours as determined by paired radiolabel studies.

Tissue	n	Minimum	Maximum	Mean	SD
R. Kidney	5	0.064	0.825	±0.438	0.322
L. Kidney	5	0.085	0.761	±0.436	0.299
Spleen	5	0	0.176	±0.085	0.081
Liver	5	0	0.221	±0.054	0.096
Gut	5	0	0.024	±0.005	0.011
Lung	5	0	0	±0	0

All values are μg ^{125}I-labelled SC5/g tissue.

Table 7.12.

Specific binding of 2 mgs of PH7 at 24 hours as determined by paired radiolabel studies.

Tissue	n	Minimum	Maximum	Mean	SD
R. Kidney	6	0.224	0.363	0.304	±0.056
L. Kidney	6	0.249	0.336	0.294	±0.031
Spleen	6	0	0.722	0.120	±0.295
Liver	6	0	0.532	0.128	±0.210
Gut	6	0.163	0.228	0.201	±0.028
Lung	6	0.001	0.241	0.107	±0.092

All values are μg ^{125}I-labelled PH7/g tissue.

Table 7.13.

Specific binding of 2 mgs of PH7 at 48 hours as determined by paired radiolabel studies.

Tissue	n	Minimum	Maximum	Mean	SD
R. Kidney	6	0.232	0.317	0.282	±0.490
L. Kidney	6	0.230	0.344	0.289	±0.456
Spleen	6	0	0.072	0.031	±0.145
Liver	6	0	0.177	0.061	±0.209
Gut	6	0.108	0.209	0.166	±0.044
Lung	6	0.031	0.097	0.024	±0.011

All values are μg ^{125}I-labelled PH7/g tissue.

conditions the kidneys receive 25% of the cardiac output, thus affording the reagents optimal opportunity to fix to glomerular antigens. If, however, the antigen with which the monoclonal antibody reacted was inaccessible from the vascular space, binding would not occur in vivo, even though the reagent had been shown to fix to glomeruli in vitro.

Urine protein excretion was seen to increase in SC5-injected rats. The upper limit of normal for urine protein excretion was considered to be 10 mg/24 hours. All experimental rats acted as their own controls. In the SC5-treated group, an increase in urine protein excretion was observed which was dose dependent and peaked at 24 hours post-injection. The high dose limit in this group was 30 mg of the SAS-precipitated monoclonal antibody. Urine protein excretion was increased only at the 30 mg dose level in the PH7 treated group and peaked at 24 hours. The post-treatment 24 hour urine protein excretion of the PH7 30 mg group was significantly greater than pre-treatment levels. However, no rats in this group exceeded an excretion of 10 mg of protein, and therefore no abnormal proteinuria was evident. Mendrick and Rennke (1988a) have reported the production of proteinuria in rats with a monoclonal antibody, K9/9, raised against rat renal cortex. Reactivity of K9/9 in vitro was with an antigen present in the GCW, the brush border membrane of the proximal tubule and intestine, as well as cells in the liver and spleen. Urine protein excretion peaked at 24 hours post-injection, reaching 63 mgs/24hours. However, in Mendrick and Rennke's study the rats received a large amount of protein (66 mgs of a 45% SAS precipitate) containing the monoclonal antibody, and dose response studies were not reported.

When glomerular sections were examined post-treatment for the presence of glomerular PMNs, no significant differences were found between controls and any of the groups receiving anti-GMF monoclonal antibodies. This result would indicate that the proteinuria observed in the SC5-treated group and at the 30 mg dose level in the PH7 treated group was unlikely to be PMN dependent. However, PMN depletion studies would have to be performed to confirm this. Examination of post-treatment glomeruli by light microscopy did not reveal any histological changes in any rats receiving anti-GMF monoclonal antibodies.

Electron microscopy studies did not not reveal any morphological abnormalities in the kidneys from any rats treated with anti-GMF monoclonal antibodies, including those

rats which had an elevated urine protein excretion, or with the control G6. No electron-dense deposits were observed at any site in the glomeruli and epithelial cell foot processes appeared normal. Mendrick and Rennke (1988b), however, reported glomerular epithelial cell damage in the kidneys of rats 24 hours following the administration of the monoclonal antibody K9/9 IV.

ImAu EM failed to localise the binding of the anti-GMF monoclonal antibodies following IV administration. Direct IF studies had clearly demonstrated the presence of mouse immunoglobulin in the kidneys of SC5 and PH7 treated animals. As discussed in Chapter Six, it is posssible that the tissue processing necessary for this procedure may have led to an alteration of tertiary structure of the monoclonal antibody and therefore its antigenic properties.

The paired radiolabel studies revealed that demonstrable specific binding of anti-GMF monoclonal antibodies to the kidney in vivo was limited to PH7 and SC5 only. This was consistent with the findings of direct IF microscopy on post-treatment sections which demonstrated the presence of mouse immunoglobulin only in the kidneys of PH7 and SC5 treated rats. ASD5 was shown not to specifically bind to the kidney nor to any of the other tissues studied, though, in vitro, this monoclonal antibody showed strong reactivity with both glomeruli and tubules. In addition to the specific binding to kidney, SC5 was found in high concentration in the spleen of one rat at 24 hours. Indirect IF studies in vitro had shown SC5 reactivity with lung to be inconsistent and to be negative with liver and gut, but the paired radiolabel studies demonstrated low levels of specific binding to lung. Specific binding to the spleen was also demonstrated in PH7 treated rats, as was binding to gut, lung and liver, a result that was consistent with indirect IF studies in vitro. It is likely that the antigen with which PH7 reacts has a wide tissue distribution on epithelial cells throughout the body, but that the SC5 reactive epitope has a more restricted distribution.

Chapter Eight

GENERAL DISCUSSION

8.1 Aim of project.

The aims of the work presented in this thesis were (a) to prepare monoclonal antibodies against rat renal antigens which were discontinuously represented along the glomerular capillary wall; hence a granular pattern of binding of antibody would be expected by IF microscopy, (b) to examine in vivo the monoclonal antibodies produced to determine whether they were nephritogenic and the nature of any monoclonal antibody-induced lesions, (c) to determine the molecular identity and tissue distribution of the antigen(s) with which the monoclonal antibodies reacted. The now well-established animal model of human membranous GN, produced in rats and known as Heymann nephritis, is characterised by a granular immunofluorescence pattern. However, most cases of glomerulonephritis in man, exhibiting a granular deposition of immunoglobulin in the glomeruli are thought to result from the random deposition of circulating immune-complexes. Although presumed to arise from the formation of immune-complexes in situ, the immunopathogenesis of classical human membranous GN has not yet been established. Antigens with which the immunoglobulins react, either in situ or in the circulation, are in most cases unknown.

In a recent review of the subject of in situ immune complex formation, Verroust (1989) identified three models which could be mediated by this mechanism, all of which are characterised by a granular pattern of IF staining. These were (a) Heymann nephritis: in this model anti-Fx1A antibodies react primarily with a 330 kDa glycoprotein present on glomerular epithelial cells to produce a proteinuric glomerular lesion. (b) Chronic serum sickness nephritis: it is postulated that in this model, cationised BSA binds to anionic molecules present in the GCW, subsequently administered anti-BSA antibodies react with the glomerular bound BSA to produce glomerular damage. (c) Lupus nephritis: the mechanism proposed is that circulating DNA binds to the GCW, anti-DNA antibodies which are characteristically present in the serum of afflicted persons then react with the glomerular bound DNA.

Additionally, Jacob et al. (1986, 1987) have reported the cross reactivity of anti-DNA antibodies found in the serum of humans with systemic lupus erythematosus with five polypeptides. These lupus-associated proteins (LAMP) are found on the surface of several cell types including glomerular cells. The monoclonal antibodies which were generated in this study, and reacted in vitro with rat kidney to produce a granular pattern of indirect IF, were examined in vivo to determine whether these monoclonal antibodies could bind to their respective glomerular antigen and therfore form immune complexes in situ.

8.2 Monoclonal Antibodies to Renal Antigens.

Monoclonal antibodies have been applied to a wide range of problems encountered in both experimental and clinical nephrology. These range from studies of renal antigen expression during development (Platt, et al., 1983; Jeraj, et al., 1984; Fukushi, et al., 1986) to the identification of lymphoid infiltrating cells of renal allografts (Hancock, 1984; Bishop, et al., 1986). A number of monoclonal antibodies have been developed which are specific for antigens confined to cells of a single segment of the nephron: for example, Bowman's capsule, glomeruli, proximal and distal convoluted tubules, loop of Henle and collecting tubules (see Bander, 1987 for review). These monoclonal antibodies have been used in immunopathological analysis and diagnostic assays. By using the unique specifications of these reagents and immunocytochemical techniques it has been possible to construct an antigenic map of the human nephron (Bander, 1987).

Mouse monoclonal antibodies specific for human T-lymphocyte subsets have been used to determine the nature of infiltrating lymphocytes in a variety of glomerulonephritides (Hooke et al., 1984, 1987; Patel, et al., 1986; Lin, et al., 1986; D'Agati, et al., 1986; Neale et al., 1988) and have also been used to monitor the expression of Ia antigens (Hinglais et al., 1984). From the literature it is clear a number of fixed glomerular antigens that could potentially serve as the ligand for nephritogenic antibodies are being identified by monoclonal reagents. However, reports of the induction of glomerular lesions in vivo with monoclonal antibodies directed against non-GBM antigens are currently limited to those emanating from the group at Boston, USA, (Mendrick et al., 1983). The monoclonal antibodies generated in this study that reacted in vitro with rat kidney to produce a granular pattern of indirect IF were examined in vivo to determine whether these monoclonal antibodies

had nephritogenic properties.

8.3 Immunogen.

Previous published studies on the production of monoclonal antibodies against renal antigens have used a wide variety of immunogens, including whole or sonicated glomeruli (Mendrick, et al., 1983; Nishi, et al., 1984), plasma membranes prepared from intact renal cortex (Falkenberg, et al., 1981a, 1981b) and classical Fx1A (Ronco, et al. 1984a; Bhan, et al., 1985). A variety of reactivities of the resultant monoclonal antibodies were obtained. In addition to monoclonal antibodies that produced granular IF patterns, monoclonal antibodies were also produced which bound in a linear fashion to the glomerular capillary loops, or reacted with renal tubular structures. When constructing monoclonal antibody-secreting hybridomas, the purity of the primary immunogen has in the past not been considered a critical factor. For this project, however, some partial purification of the immunising antigen, as advocated by Campbell (1984), was considered imperative in order to reduce the chances of generating unwanted monoclonal antibodies against basement membrane and intracellular organelle antigens. The subcellular fraction prepared by differential centrifugation of homogenised glomeruli was shown by TEM to be enriched for cell membranes. Further attempts at purification of this fraction on sucrose density gradients did enhance purity, but in addition significantly decreased the final yield of membrane. For these reasons, sucrose density centrifugation was not included in the procedure for preparation of GMF antigen.

The purity of the antigen which is to be employed for the screening of hybridomas may be important in minimising the amount of time spent screening cell culture supernatants from hybridomas of unrequired specificity. As in most monoclonal antibody production regimens, the primary factor determining success or failure is the efficiency of the screening procedure. The production of hybridoma colonies which initially recorded a positive anti-GMF result by microELISA, but which were subsequently shown to be non-kidney fixing by indirect IF microscopy, requires some explanation. It is likely that microELISA is a more sensitive technique for the detection of anti-GMF activity than indirect IF microscopy. In some cases, therfore, monoclonal antibody concentrations contained in cell culture supernatants may not have been high enough to yield a positive IF result; hence, anti-GMF secreting colonies may have been passed over at the second screening stage using indirect IF microscopy. However, the colonies which were microELISA-positive were grown to

a high density in 2 ml culture wells. The subsequent negative result obtained by indirect IF microscopy could be the result of poor antibody secretion by these colonies, or alternatively, the secreted monoclonal antibody may have had a low affinity for its antigen on cryostat sections of normal rat kidney. Liabeuf, et al. (1981) have reported the masking of antigenic determinants on cell surface molecules as a consequence of their association with adjacent cell surface proteins. The possibility therefore exists that during the preparation of GMF, protein sequences may have been exposed which, in the native state, were masked or otherwise inaccessible to antibody molecules. Monoclonal antibodies reactive with epitopes present on these sequences would yield a positive result when examined by microELISA against GMF, but would register a negative result when examined by indirect IF microscopy on normal rat kidney. Whatever the mechanism for the discrepancy, this finding emphasises that the types of assay and the conditions under which they are performed are critical factors in the screening of monoclonal antibody specificity.

8.4 Fusions.

The failure to generate hybridomas in the first five fusion experiments was almost certainly due to the mycoplasma infection of the myeloma cell lines. Most species of mycoplasma possess an enzyme which cleaves the base-sugar bond of thymidine. It is believed that the resulting thymidine deficiency is the cause of the post-fusion failure of hybridoma growth. A number of reports have appeared in the literature proposing how mycoplasma may be erradicated from infected cell lines (Borup-Chistensen, et al., 1988; Hessling, et al., 1980; Ravaoarinoro and Lecomte, 1988; Roseto, et al., 1984). All the procedures are detailed and time consuming to perform. It has been recomended that elimination of mycoplasma should only be attempted if the infected cell line is particularly valuable (Goding, 1983). In the present study, when mycoplasma infection of the myeloma cell lines was confirmed, fresh stocks of mycoplasma-free cell lines were obtained. As mycoplasma-free myeloma cell lines were readily available, no attempt was made to eliminate mycoplasma from the original myeloma cell lines. Subsequently, routine precautionary screening of all cell lines and hybridomas for mycoplasma infection was undertaken at six to eight month intervals with Hoechst 33258 stain using the fluorescence microscopy method of Chen (1977). As proof that mycoplasma contamination caused the initial failure of fusion experiments, 100% of all subsequent fusion experiments undertaken with mycoplasma-free myeloma cell lines were successful.

Following the first failed fusion, using the method of Gefter, et al., (1977), a number of reported modifications were introduced. All myeloma cell lines were grown in RPMI medium, and all later selective or restrictive media were based on RPMI also. This decision was based on the fact that the majority of regimens reported in the literature detailing the generation of monoclonal antibody-secreting hybridomas utilised this medium. In addition to the use of RPMI, 10% NCTC medium was added post-fusion, as advocated by Claflin and Williams (1978), and insulin was added as described by Feit et al., (1984). The post-fusion growth of hybridomas on peritoneal macrophages has been reported to be beneficial to hybridoma growth (Fazekas de St Groth and Schreidegger, 1980; Goding, 1980; Campbell, 1984), an effect attributed to the phagocytic activity of the macrophages, enabling then to clear the cultures of cell debris. However, a recent report has shown that addition of macrophage-derived growth factor (interleukin 6) to the post-fusion medium directly increases hybridoma production (Bazin and Lemieux, 1989), indicating that macrophages may have a direct effect on hybridoma cultures post-fusion through the secretion of growth-promoting factors. Although a direct assessment of the effects of these modifications was not made, the first fusion undertaken with mycoplasma-free myeloma cells resulted in the successful generation of hybridomas, and the modifications which were introduced were retained in subsequent fusion experiments.

The mean fusion frequency of 19.75 hybridomas per 10^7 splenocytes obtained in fusions 6 to 9, compares favourably to the expected fusion frequencies published by Westerwoudt (1986). Loss of chromosomes from hybridomas during the early post-fusion period has been reported previously (Kohler, 1980; Goding, 1983; Westerwoudt, 1985), and may account for clone mortality during this period. A sudden or gradual loss of antibody secretion of hybridomas has been reported (Yelton, et al., 1978) an observation which may be accounted for by the preferential loss of chromosomes carrying either the heavy or light chain loci. There are few data to be found on hybridoma mortality during the early post-fusion period, although hybridoma instability during this period is well-documented (Clark et al., 1983; Kohler, 1980; Kearney, 1984). Two of the eighteen hybridoma colonies generated in this study, that secreted anti-GMF monoclonal antibody were lost during the post-fusion period. This represents a mortality rate, for specific anti-GMF-secreting hybridomas, of only 11%.

8.5 Monoclonal antibody isotype.

Over 50% of the anti-GMF monoclonal antibodies generated in the present study

were of the IgM subclass, including all of those secreted by hybridomas generated from splenocytes obtained from mice which had been immunised via the intrasplenic route. Spitz et al., (1984) have reported the production of monoclonal antibodies against high molecular weight cross-linked fibrin degradation products and have advocated intrasplenic immunisation as the method of choice for the production of monoclonal antibodies against high molecular weight antigens. As was the case in the current study using glomerular membrane fragments, all of the monoclonal antibodies produced by Spitz et al. using intrasplenic immunisation were of the IgM subclass.

8.6 Monoclonal antibody recovery.

Initial attempts to establish the anti-GMF IgM-secreting hybridomas as peritoneal tumours in Balb/cJ mice were unsuccessful. Campbell (1984) has reported that IgM-producing hybridomas may produce lower titres of antibody in ascitic fluid and that some may be difficult to establish as peritoneal tumours. The sub-lethal irradiation of mice prior to the intraperitoneal administration of hybridoma cells has been reported to enhance the development of peritoneal hybridoma tumours with subsequent production of both human (Kozbor, et al., 1985) and murine (Weissman, et al., 1985) monoclonal antibodies. The mechanism by which hybridoma intraperitoneal growth failure occurs is uncertain; however, the lack of growth without irradiation suggests that an allogeneic rejection phenomenon may be the cause (Samoilovich, et al., 1987). In the current study, the prior exposure of Balb/cJ mice to a sub-lethal total body irradiation dose of 400 Rads resulted in the subsequent growth of all IgM producing hybridomas with recovery of monoclonal antibody-containing ascites.

8.7 Monoclonal antibody purification.

As it was intended that anti-GMF monoclonal antibodies would be administered to rats to examine their effects in vivo, some purification of the monoclonal antibodies was considered desirable. A pure monoclonal antibody preparation would decrease the variabilitiy associated with heterologous protein solutions such as ascitic fluid and reduce the possibility of native mouse antibodies, cross-reactive with rat tissues, producing spurious results when examining in vivo binding of the monoclonal antibodies. A number of techniques which have been used for the purification of immunoglobulins from serum and other complex protein solutions have been applied to the purification of monoclonal antibodies. These include euglobulin precipitation

(Hudson and Hay, 1980), ammonium sulphate precipitation (Goding, 1983), staphylococcal protein A binding (Ey, et al., 1978; Goding, 1978; Kearney, 1983), and diethylaminoethyl (DEAE) chromatography (Goding, 1980).

Different purification procedures favour different antibody subclasses. In this study, the monoclonal antibodies examined were of the IgM subclass. IgM does not have a high affinity for protein A (Ey et al., 1978). IgM may also be denatured by classical euglobulin precipitation (Goding, 1983), although a recent study reported the successful purification of murine IgG3 and IgM by euglobulin precipitation. SDS-PAGE analysis of a fraction precipitated from ascitic fluid by this method indicating a purity of 40% to 90% IgM (Garcia-Gonzalez, et al., 1988). The majority of the methods reported for the purification of IgM exploit the physical characteristics of the molecule, including its high molecular weight which is useful in separation by gel filtration (Angeretti, et al., 1983; Bouvet, et al., 1984), and its high carbohydrate content which aids in its separation by lectin affinity chromatography (Klein, et al., 1979). Belew, et al., (1987) have reported the purification of murine monoclonal antibodies in a single step procedure using salt-promoted chromatography on a thiophilic adsorbant. However, the recovery of IgM using this procedure was considerably less than that of IgG. Similarly, the purification of monoclonal antibodies from ammonium sulphate precipitates of ascitic fluid by caprylic acid (McKinney and Parkinson, 1987; Reik, et al., 1987) has generally been more successful in the purification of IgG than IgM. In addition, monoclonal IgM may not react the same as polyclonal IgM-containing preparations. Recently reported methods for the purification of monoclonal IgM antibodies include anion exchange on Mono Q (Clezardin, et al., 1986), affinity chromatograhy on Cibacron Blue F3GA followed by gel filtration on ACA-22 (Johnson, et al., 1987) and polyethylene glycol precipitation following the removal of lipids by silicon dioxide (Neoh, et al., 1986). Chromatographic separation of monoclonal antibodies on a number of gel and ion-exchange matrices in high performance (HPLC) and fast protein (FPLC) liquid chromatography systems have been reported (Chen, et al., 1988; Danielsson, et al., 1988; Josic, et al., 1988). Particularly interesting was the report of Chen, et al. (1988). These authors reported the recovery of IgM from ascitic fluid and cell culture supernatants with a purity of >99% using a mixed-mode ion exchange matrix, ABx, in an FPLC system. However, most of the procedures reported for the purification of IgM require multiple steps, resulting in a decrease in yield of the purified antibody. In addition, the preparations are frequently contaminated with $\alpha 2$ macroglobulin, a molecule of similar size which exhibits similar elution characteristics in

chromatographic procedures.

The use of hydroxylapatite for the purification of monoclonal antibodies was first reported by Juarez-Salinas, et al, in 1984 and later by others (Stanker et al., 1985; Juarez-Salinas, et al., 1986; Bukovsky and Kennett, 1987). The mechanism by which proteins bind to hydroxylapatite has been determined to be primarily electrostatic interactions between amino groups of basic proteins and the phosphate group of the hydroxylapatite and between carboxyl groups of acidic proteins and calcium sites on hydroxyapaptite. Elution of proteins bound to hydroxylapatite is accomplished either by Debye-Huckel charge screening as occurs using phosphate buffer or by specific displacement by Ca^{2+} and/or Mg^{2+} (Gorbunoff and Timasheff, 1984).

Attempts to purify anti-GMF IgM monoclonal antibodies from ascites or cell culture supernatant by hydroxylapatite chromatography using the method described by Stanker et al. (1985) were not successful in this study. Examination of chromatographic protein peaks by SDS-PAGE indicated the possible elution of free light chains, an observation previously reported by Juarez-Salinas, et al. (1984). The monoclonal antibodies examined by Juarez-Salinas, et al. (1984) were secreted from hybridomas resulting from the fusion of splenocytes with myeloma Sp2/0. This cell line neither synthesises nor secretes either the heavy or the light chains of immunoglobulin. The myeloma cell line NS1 used to generate the anti-GMF monoclonal antibody-secreting hybridomas in the current study, is reported to synthesise, but not secrete, immunoglobulin light chains. However, free light chain secretion from hybrid cells generated with this myeloma cell line has been occasionally reported (Kohler, et al., 1976). None of the elution peaks obtained following chromatography of anti-GMF monoclonal antibody-containing ascites or cell culture supernatants, reacted with rat kidney when examined by indirect IF microscopy; whereas the ascites and cell culture supernatants applied to the column had previously been shown to have a high anti-rat kidney titre. This observation raises the possibility of significant degradation of the monoclonal antibodies during chromatography. If this has occurred, the reason for degradation is not understood.

A number of gel matrices have been used to purify monoclonal antibodies from ascites or cell culture supernatants by exclusion chromatography. Goding (1983) has stressed the importance of selecting a gel which includes IgM, and therefore chromatographs it, rather than just allowing the IgM to pass through the column. The molecular weight of IgM is 900 kDa, and the exclusion limit of BioGel A1.5 M is 1500

kDa. BioGel A1.5 M therefore fulfilled this criterion. Attempts to purify the anti-GMF monoclonal antibodies by exclusion chromatography on BioGel A1.5 M were more successful than using hydroxylapatite chromatography. The appearance of protein in the same molecular weight range as immunoglobulin light chain eluting in all four peaks is unusual. However, since separation of proteins on BioGel A1.5 M is by exclusion the elution profile should reflect the molecular weight of the proteins, with the larger molecular weight components eluting from the column ahead of those of lesser molecular weight. IgG would therefore elute from the column after IgM. As the SDS-PAGE analysis of the protein peaks was performed under reducing conditions, it is possible that those proteins with similar molecular weights to immunoglobulin light chains may in fact be light chains originating from native mouse immunoglobulins which were recovered in the ascitic fluid together with monoclonal antibody. Although anti-rat kidney activity could be demonstrated by indirect IF microscopy in the first two peaks recovered from BioGel A1.5 M chromatography, indicating probable monoclonal IgM presence, SDS-PAGE analysis of these two peaks revealed they were not of a homogeneous protein content although protein bands corresponding to IgM heavy and light chains were present.

In the present study, affinity chromatography on an anti-mouse IgM-agarose conjugate column yielded the highest purity of monoclonal IgM which also exhibited a high titre of anti-rat kidney activity when examined by indirect IF microscopy. SDS-PAGE analysis of the protein peak recovered from anti-GMF-containing ascites following elution of the affinity column revealed the presence of a 52 kDa protein as well as immunoglobulin heavy and light chains. This 52 kDa band was not found in SDS-PAGE profiles of mouse IgM isolated by affinity chromatograhy from cell culture supernatants. The origin of this protein is uncertain. The anti-mouse polyclonal antibody that was covalently attached to the agarose support is reported by the manufacturers to be μ chain specific. This, however, does not rule out the possibility that the polyclonal anti-mouse antibody may have some cross-reactivity with mouse proteins present in the ascitic fluid, which had not been absorbed from the antibody by the manufacturers. If this were the case, the 'unknown' protein would dissociate from the column under acid elution conditions and appear as a contaminant of the IgM preparation. The presence of a double peak in the elution profile of IgM isolated from ascitic fluid by affinity chromatography may have resulted from non-specific, native mouse IgM binding to the column. This native IgM may subsequently have eluted from the column at a fractionally higher pH than the monoclonal IgM. However, the possibility remains that the first peak may represent the elution of the

52 kDa protein. The weak anti-kidney reactivity which was found in this first peak by indirect IF microscopy may be accounted for by the presence of by early eluting monoclonal anti-GMF antibody in the peak. Although the purity of IgM recovered from the affinity column was satisfactory, only small samples could be processed at any one time.

SAS precipitation of anti-GMF monoclonal antibodies from ascitic fluid resulted in the recovery of an IgM-enriched protein fraction with a high anti-rat kidney titre when examined by indirect IF microscopy. Since purification of anti-GMF monoclonal IgM antibody by chromatography on hydroxylapatite was unsatisfactory and since the fraction recovered from chromatography on BioGel A1.5 M was not considered to possess significant advantages over the IgM-enriched fraction prepared by 40% SAS precipitation, the simpler SAS precipitation method was chosen as the standard purification step. Affinity chromatography was not considered because of the costs and time involved in scaling up of the procedure in order to process larger samples. However, the higher purity of the affinity-purified IgM antibody was mandatory for later ImAu EM studies undertaken with the anti-GMF monoclonal antibodies (Kerjaschki, et al., 1986).

8.8 Nephritogenic anti-kidney monoclonal antibodies.

The first reports of the generation of monoclonal antibodies directed against renal antigens appeared in 1981. Ueda, et al. (1981) described the production of a panel of monoclonal antibody secreting hybridomas from the splenocytes of mice which had been immunised with whole cells from established cell culture lines derived from renal carcinomas. Three antigens, designated gp 160, S25 and gp 120r, were identified which were kidney specific and were expressed on both normal and neoplastic kidney cells. Falkenberg, et al. (1981a, 1981b) were the first to use an immunogen derived from normal kidney to generate hybridomas. These authors immunised mice with plasma membranes prepared from whole human kidney cortex. Fusion of the splenocytes obtained from these immune mice with NSI/1 myeloma cells resulted in the production of sixty hybridoma colonies secreting antibody which reacted more or less strongly with a variety of renal antigens. Six of the monoclonal antibodies which were generated were specific for glomeruli. When examined by IF microscopy, one of these monoclonal antibodies produced a linear pattern of fluorescent staining, the remaining five reacted in a discontinuous granular pattern. The monoclonal antibodies produced by Falkenberg, et al. (1981a, 1981b) have subsequently been

employed in microELISA assays for the detection and quantification of kidney-derived urinary antigens in a number of pathological renal conditions (Falkenberg, et al., 1985, 1986). Since these initial studies, a number of investigators have reported the production of monoclonal antibodies directed against antigens present in both human and animal GBM (Michael et al., 1983; Pressey, et al., 1983; Mendrick, et al., 1983; Hancock and Atkins, 1984; Thomson, et al., 1984; Nakamura, 1986; Pusey, et al., 1987). These monoclonal antibodies all produced a linear staining of the GBM when examined by IF microscopy.

The specific aim of the current study was to generate monoclonal antibodies directed against antigens which were discontinuously represented along the glomerular capillary wall, and when examined by indirect IF microscopy, would produce a granular pattern of staining. In order to enhance the chance of success, the primary immunogen was depleted of GBM, thereby decreasing the possibilities of generating monoclonal antibodies which would exhibit linear binding patterns. The non-GBM glomerular antigens which were of interest were likely to be cell surface molecules expressed on either glomerular endothelial, epithelial or mesangial cells (Matsuo, et al., 1987; Andres, et al., 1979). Presumably any membrane fragments contained in the GMF preparation of the present study would have originated from these cell types as the starting material was isolated whole glomeruli and would include membranes derived from intracellular structures such as lysosomes and nuclei. In addition, following homogenisation of the isolated glomeruli, non-disrupted intracellular organelles and subcellular molecules would have been removed in discarded pellets and supernatants respectively during the differential centrifugation procedure employed, thus reducing the chances of generating unwanted monoclonal antibodies against these antigens. However, only one monoclonal antibody, BPC4, reacted with nuclear membranes, idicating membrane derived from intracellular organelles was not a significant antigenic component of GMF. From four successful fusion experiments undertaken in the current study, a total of 18 monoclonal antibodies were produced which reacted with rat kidney by indirect IF microscopy. Seventeen of these monoclonal antibodies produced a granular IF staining pattern. The one exception, BPC4, reacted with an antigen present on nuclear membranes. The fact that none of the reagents appeared to react with linearly disposed GBM structures, as judged by indirect IF, justified the choice of GMF as the immunogen for the production of anti-rat kidney monoclonal antibodies reacting with discontinuously represented glomerular antigens.

Mendrick, et al. (1983) have reported the production of more than fifty monoclonal

antibodies that reacted with normal Lewis rat kidney. Fourteen of these were selected on the basis of glomerular capillary wall binding patterns in vitro, and were examined in more detail. Fifty percent of the selected monoclonal antibodies bound to glomeruli in vivo, of which only two exhibited a non-linear pattern by IF microscopy. Nine of the monoclonal antibodies were specific for rat antigens. In a recent study, the same authors have reported in vitro and in vivo investigations with a monoclonal antibody named K9/9, generated by fusion of splenocytes obtained from a mouse immunised with an homogenate of rat renal cortex (Mendrick and Rennke, 1988a, 1988b). Immunoblotting and immunoprecipitation studies indicated K9/9 reacted with antigens of 118 and 107 kDa. The distribution of these antigens was restricted to the cell surface of all glomerular podocytes; they were not present on any other cell type in the rat, nor on glomerular cells of any other species examined. When administered intravenously, K9/9 induced a dose-dependent proteinuric lesion.

A monoclonal antibody against an 81 kDa protein that is restricted to glomerular mesangial cells has also been reported by Mendrick and Rennke (1986). Following intravenous administration of up to 66 mgs of ascitic fluid containing this monoclonal antibody to normal rats, IF microscopy revealed a pattern of glomerular deposition similar to that observed with the antibody in vitro. Flocculent electron dense deposits were observed in all glomeruli examined. Urine protein excretion was not increased following in vivo administration.

In the current study, anti-GMF monoclonal antibodies SC5, PH7, ASD5 and BSG11 were selected for further study on the basis of their granular IF staining patterns in vitro. None of these monoclonal antibodies, however, reacted with antigens that were restricted to the rat glomerulus, although the binding patterns produced on kidney from other species were similar to those observed in the rat.

Considerable research with monoclonal antibodies produced against renal antigens of both rats and rabbits has been contributed from the laboratories of Ronco and Verroust in France (Ronco, et al. 1984a; 1984b; 1986a; 1986b; Chatelet et al., 1986a; 1986b).

In a study to further investigate immunological injury in Heymann's nephritis Ronco, et al. (1984b) generated twenty-seven hybridomas secreting monoclonal antibody which reacted with rat kidney. The splenocytes participating in the fusion were donated by a mouse which had been immunised with rat Fx1A. The monoclonal antibodies produced reacted with a number of renal structures including proximal tubule brush

border and basolateral membrane, intracytoplasmic antigens, glomerular epithelial cells, and intestinal brush border membrane. Three of the monoclonal antibodies reacted with a 330 kDa protein present on proximal tubule brush borders and glomeruli. Two were specific for a 90 kDa protein present on proximal tubule and intestinal brush border and glomerular endothelial cells. One further monoclonal antibody reacted with an antigen exclusive to tubular and glomerular epithelial cells. The antigen with which this antibody reacted could not be identified by the immunoprecipitation technique employed. Interestingly, this latter antibody was the only GCW-reactive monoclonal antibody that was of the IgM subclass. This monoclonal antibody did not bind to glomeruli in vivo. In contrast, all the monoclonal antibodies which were specific for intracytoplasmic antigens were of the IgM subclass.

The four anti-GMF monoclonal antibodies generated in the current study and examined in vitro and in vivo, were IgM antibodies. Two of these, SC5 and PH7, bound to glomeruli when administered IV to normal rats. This result indicated that in vivo reactivity of anti-kidney monoclonal antibodies is not restricted to antibodies of the IgG subclass. The remaining two monoclonal antibodies, ASD5 and BSG11, did not bind to rat glomeruli in vivo, despite showing strong reactivity with rat glomeruli in vitro. This finding may be accounted for by antigen accessability, the membrane antigens with which ASD5 and BSG11 were reactive may have been available to interact with the antibodies in in vitro preparations, but in vivo epitope accessability may have been restricted by restraints imposed by spatial arrangement. None of these monoclonal antibodies appeared to react with intracellular antigens, unlike the monoclonal antibodies produced by Mendrick et al. (1983) some of which reacted with annumber of intracellular structures.

The renal distribution of the 90 kDa and 330 kDa antigens reactive with the monoclonal antibodies described by Ronco, et al. (1984b) has been investigated using IF and immunoelectron microscopy (Chatelet, et al., 1986a). The 90 kDa antigen was diffusely represented on the cell membrane of glomerular epithelial cells and brush border membranes of proximal tubule cells, vascular endothelial cell membranes and cells of the lamina fenestrata. The antigen was not expressed on the continuous endothelium close to the vascular pole, the afferent and efferent arterioles or interstitial capillaries. The distribution of the 330 kDa antigen was distinctly dissimilar to that of the 90 kDa antigen, being localised to the intermicrovillar membrane and associated with coated pits. The 90 kDa antigen was also shown to be associated with the membranes of intracytoplasmic vesicles. The extrarenal distribution of the 90 kDa

and 330 kDa antigens described by Ronco, et al. (1984b) has also been examined Chatelet, et al., (1986b). The 330 kDa antigen is restricted to epididymal epithelium and the microvillar membrane of type II but not type I pneumocytes. The distribution of the 90 kDa antigen was much more ubiquitous, being found on a variety of cell types including hepatocytes and endothelial cells, but not Kupffer cells, of liver, endothelial cells of spleen, heart and lung, intestinal brush border membranes, and type II but not type I pneumocytes. The conclusion from this study was that the 330 kDa antigen may be involved with receptor mediated endocytosis, the 90 kDa antigen had a more ubiquitous distribution than the 330 kDa antigen between tissues and within cells, and was particularly associated with cells having a high membrane turnover.

8.9 Antigen localisation.

Localisation of the antigens was partially successful in these studies and a general localisation was determined in the present study. Immunofluorescence microscopy revealed that none of the anti-GMF monoclonal antibodies studied were seen to exhibit reactivity that was restricted to the glomerulus. Reactivity with non-renal tissues, however, was limited to cell membranes, indicating that the reactive antigens were cell surface molecules. A wide tissue distribution has also been reported for a 108 kDa glycoprotein antigen (gp 108) isolated from Fx1A. Intravenous administration of anti-gp 108 polyclonal antibody to normal rats induces passive Heymann nephritis with acute and severe proteinuria (Natori, et al., 1986). Non-renal reactivity was reported to be with small intestine, lung, spleen, thymus, liver, epididymis, stomach, pancreas, heart and peripheral blood lymphocytes (Natori, et al., 1987).

In the present study, binding of the anti-GMF monoclonal antibodies in vivo was limited to SC5 and PH7. Direct IF microscopy revealed that the patterns of glomerular binding of the two monoclonal antibodies was similar to that observed in vitro. However, reactivity with tubules was not observed in vivo. Glomerular binding of both SC5 and PH7, as assessed by direct IF microscopy, although present at one hour, was greatest at 24 hours post-injection. This contrasts with the in vivo studies with the IgG2a monoclonal antibody MoAb8 reported by Ronco, et al., (1984a). This monoclonal antibody was reported to exhibit a granular IF binding pattern in vitro and in vivo which was characteristic of anti-Fx1A serum (Ronco, et al. 1984b). The in vivo binding of MoAb8 was greatest in the glomerulus at one to four hours post-

injection, although antibody could still be detected on proximal tubular brush borders at fifteen days post-injection. MoAb8 was not kidney specific; indirect IF microscopy demonstrating reactivity with spleen, lung, heart and liver. Immunoprecipitation and immunoblotting experiments with MoAb8 indicated that the reactivity was confined to a single protein band of 90 kDa, present on both glomerular and proximal tubular brush border membranes. Immunoperoxidase electron microscopy localised the binding of MoAb8 to proximal tubular membrane invaginations and endocytotic vesicles and to glomerular epithelial and endothelial cells. Some reactivity of MoAb8 with the lamina densa of the GBM was also noted. Furthermore, the antigen could be detected in the circulation. Perfusion of isolated kidneys and in vitro binding studies confirmed that MoAb8 did indeed form immune complexes with renal antigens in situ. The results overall indicated that MoAb8 was specific for an antigen which was biochemically and anatomically independent of the gp 330 antigen described by Kerjaschki and Farquhar (1982).

Results obtained in dot-blot experiments in the current study indicated that PH7, ASD5, SC5 and BSG11 reacted with all of the protein extracts prepared from rat GMF. ASD5 was the only monoclonal antibody that reacted with hGMF protein extracts in the dot-blot assay. Indirect IF microscopy studies, undertaken to determine interspecies reactivity of the anti-GMF monoclonal antibodies, had indicated that SC5 and PH7, both of which bound in vivo to rat kidney, also reacted with human kidney. However when the reactivities of these reagents with protein extracts of either human GBM or hGMF were examined by dot-blot, both SC5 and PH7 returned a negative result. The dot-blot result obtained with ASD5 against extracts from human kidney was therfore unexpected. ASD5 did not react with hGMF in microELISA studies nor in immunoblotting experiments, and repeat indirect IF microscopy studies confirmed that this monoclonal antibody did not react with normal human kidney in vitro. Collagenase digests of GBM have been widely used in immunological studies to determine the reactivities of anti-GBM antibodies since the original description of the technique by Marquardt, et al. (1973b), and it is possible that theGBM preparation could contain some human glomerular membrane antigens. The anti-GMF antibodies were generated against rat GMF, a preparation which had been depleted of GBM. In addition indirect IF microscopy had shown that none of the anti-GMF monoclonal antibodies reacted with rat GBM in vitro. It is therefore uncertain as to how ASD5 could exhibit reactivity with the collagenase digest of human GBM. The removal of the reactivity of monoclonal antibodies with proteins which have been exposed to DOC has been reported in the literature (Herrman, et al., 1979, 1982) and may

account for the lack of reactivity observed with SC5 and PH7 against the DOC extracts of hGMF. The negative result obtained with these two monoclonal antibodies against the collagenase digest of human GBM was anticipated since neither reagent demonstrated GBM binding in indirect IF studies. The findings obtained with ASD5 in the dot-blot experiments suggest that exposure of protein antigens to DOC, prior to immunological studies, may also return false positive results. Conceivably, exposure of protein antigens in the GBM and hGMF preparations to DOC may introduce alterations in the tertiary structure of the proteins, creating what may be termed 'pseudoepitopes', that were cross-reactive with ASD5. Alternatively, a non-immunological reaction may have occurred between ASD5 and DOC which may have remained bound to the extract protein. Data obtained with DOC protein extracts must therefore be interpreted with caution.

Attempts to localise the binding of the anti-GMF monoclonal antibodies by ImAu EM were unsuccessful. Kerjaschki and Farquhar (1983) have reported the destruction of protein antigen reactivity following fixation with glutaraldehyde. In the present study free aldehydes were quenched, prior to probing with the monoclonal antibodies, with 1% ammonium chloride as advocated by Brown and Revel (1976). The use of hydrophilic acrylic resins, such as Lowicryl, are preferred to the hydrophobic resins, for example the widely used Epon 812, as the embedding process employed with hydrophobic resins results in the partial or total denaturation of protein antigens (Kerjaschki, et al., 1986). However, Kerjaschki, et al. point out that acrylic resins may not be suitable for the localisation of membrane antigens which are present in low density, and that the use of ultrathin frozen sections may be the method of choice in these cases.

8.10 Identification of antigens reactive with anti-GMF monoclonal antibodies.

An initial immunoblotting experiment conducted with SC5 on an 8M urea extract of isolated whole glomeruli had indicated that this monoclonal antibody reacted with protein bands of 175, 160, 120 and 53 kDa. This result was unable to be repeated. In addition, all immunoblotting experiments carried out with PH7, ASD5 and BSG11 returned negative results against urea extracts. Urea is a denaturing agent and at the concentration used breaks non-covalent bonds to render a mixture of denaturated, dissociated polypeptide chains (Van Renswoude and Kempf, 1984). The 24 hour exposure of proteins to the urea could be considered harsh and may have in severely modified the epitope tertiary structure which could have destroyed antigen specificity.

Mendrick and Rennke (1986) have reported the solubilisation of an 81 kDa protein reactive with a monoclonal antibody which binds to rat mesangial cells in vitro. This protein was only solubilised by 0.2% OG and not by other detergents including CHAPS, NP40 and Triton X-100. The protein profiles obtained following SDS-PAGE of the OG and CHAPS extracts of GMF were similar. Subsequent transfer of the proteins to nitrocellulose sheets and probing with the anti-GMF monoclonal antibodies revealed bands of reactivity only with SC5 and BSG11. BSG11 reacted with seven protein bands which were present in both the CHAPS and OG extracts. SC5 reacted with a single 78 kDa protein band present in the OG extract and with five protein bands in the CHAPS extract, including the 78 kDa protein, but this finding was not consistent. When examined against detergent extracts of isolated whole glomeruli, the reactivity of SC5 with a 96 kDa protein was again observed. However, the four bands of reactivity corresponding to proteins with molecular sizes of 82 - 52 kDa, as seen with the CHAPS extract of GMF, were absent from the immunoblots obtained with extracts of isolated glomeruli. The amounts of protein which were obtained from extracts of isolated whole glomeruli were greater than those obtained from GMF extracts; additionally, the number of protein bands visualised following SDS-PAGE was also greater. A large percentage of the protein extracted from whole glomeruli was likely to be intracellular protein which would not have been present in the GMF preparation. It would, therefore, be unlikely that any of the anti-GMF monoclonal antibodies would react with these non-GMF proteins unless they were preformed cytosolic membrane proteins or they shared GMF protein epitopes. The antigens present in the GMF extracts would also be present in the extracts of whole glomeruli. It could be anticipated, therefore, that the anti-GMF monoclonal antibodies which reacted with protein bands in the GMF extracts would react with the same protein bands in the extracts of whole glomeruli. However, since the GMF preparation was an enriched plasma membrane fraction, proteins which were exclusive to the plasma membrane were likely to be present in higher concentrations in the GMF extracts than in the extracts of whole glomeruli. If the 82, 78, 55 and 52 kDa proteins, which were reactive with SC5 in the GMF extracts, were exclusively membrane proteins, then the concentrations of these proteins in the extracts obtained from whole glomeruli may not have been great enough to be detected by the immunoblotting technique. The possibility that antigen density is an important determinant in immunoblotting studies is suggested by the finding that reactivity of SC5 with protein bands in the GMF extracts was only revealed when the more sensitive biotin-streptavidin system was applied to detect bound monoclonal antibody.

The immunoblotting results indicated that only BSG11 reacted with a protein of similar molecular weight to previously reported reactivities of anti-kidney monoclonal antibodies. BSG11 reacted with a protein band of 43 kDa; the monoclonal antibody K2.7, reported by Kinouchi, et al. (1987), also reacted with a protein of 43 kDa. All of the anti-GMF monoclonal antibodies reacted with more than one protein band in the immunoblotting studies. Although ASD5 was shown to react with a single protein band when blotted against a CHAPS extract of isolated glomeruli, it was also seen to react with three protein bands in a CHAPS extract of GMF. However, neither of these results were reproducible. Monoclonal antibodies are specific for a single antigen epitope, but this highly specific reactivity does not necessarily imply that a given monoclonal antibody is monospecific. The epitope with which it reacts may be present on one or more proteins which may or may not be present in the immunising antigen. The monoclonal antibody could therefore exhibit more than a single band of reactivity when examined by immunoblotting. The dominant reactivity of SC5 was with a 96 kDa protein present in OG and CHAPS extracts of isolated glomeruli and the CHAPS extract of GMF. This protein has a similar molecular weight to the 90 kDa antigen described by Ronco, et al. (1984a, 1984b) which was identified by a monoclonal antibody generated from the fusion of splenocytes obtained from a mouse immunised with rat Fx1A. However, attempts to block the reactivity of SC5 with rat kidney by anti-Fx1A serum in IF microscopy studies were unsuccessful, indicating that the 96 kDa antigen recognised by SC5 is unlikely to be the same as the 90 kDa antigen component of Fx1A described by Ronco, et al. (1984a, 1984b) and others (Bagchus, et al., 1986d; Kawai, et al., 1986). Bakker et al. (1979, 1981) have demonstrated anti-T cell activity in anti-Fx1A serum and identified the anti-T cell reactivity to be directed against a 90 kDa protein. Glomerular reactivity of the anti-Fx1A serum was removed following immunoabsorption of the antiserum with thymocytes as was reactivity with the 90 kDa protein (Bagchus, et al., 1986d). A study of the distribution of gp 330, (the 'Heymann antigen'), and gp 90 in the rat and the mouse was conducted by Assman, et al. (1986). These workers found that gp 90 was diffusely represented along the glomerular capillary walls of both the rat and the mouse. However, gp 330 which was a major constituent of proximal renal tubule brush border membranes of both the rat and the mouse, was absent from the glomerular capillary wall of the mouse. These authors concluded that gp 330 was the target antigen for membranous glomerulonephritis in the rat, whereas in the mouse, where the lesion can also be produced, gp 90 is the target antigen.

The most widely studied experimental animal model of a glomerulonephritis

characterised by granular glomerular deposits of immunoglobulin is Heymann nephritis. The application of the monoclonal antibody technique to the study of Heymann nephritis was first reported by Kerjaschki and Farquhar (1983). These authors generated a monoclonal antibody to a glycoprotein gp 330 which had been purified from a deoxycholate extract of rat kidney proximal tubule microvilli by gel column fractionation and lentil lectin affinity chromatography. The monoclonal antibody specifically stained the brush borders of proximal tubule cells, and reacted with the glomerulus in a faint but distinctive granular pattern. Specificity was confirmed by the immunoprecipitation of a 330 kDa glycoprotein. Ultrastructural localisation of gp 330 by immunoperoxidase electron microscopy with this monoclonal antibody was complementary to results obtained with a polyclonal anti-gp 330 antibody. However, glomerular fixation of the anti-gp 330 monoclonal antibody could not be demonstrated by IF microscopy following intravenous injection. Ronco, et al. (1986a) have reported the formation of immune deposits in rat glomeruli following IV administration of a monoclonal antibody against a 330 kDa glycoprotein. Specific glomerular binding was confirmed by paired radiolabel studies. Urine protein excretion following administration of the monoclonal antibody was not the subject of this work but immunisation with a gp 330 preparation, which had been affinity isolated by three epitope-specific anti-330 kDa monoclonal antibodies, resulted in the development of classical membranous glomerulonephritis in the treated rats.

Bhan et al. (1985), have reported the production of a monoclonal antibody derived from the fusion of splenocytes obtained from a mouse immunised with Fx1A. This monoclonal antibody, named 14C1 was demonstrated to immunprecipitate a glycoprotein from an extract of renal tubular microvilli which had an electrophoretic mobility corresponding to 440 kDa in SDS-PAGE under non-reducing conditions. Immunofluorescence and immunoelectron microscopy studies indicated that 14C1 reacted with an antigen present on podocyte cell membranes in the vicinity of coated pits, and also with material present in the glomerular deposits of rats with Heymann's nephritis. A second monoclonal antibody examined in the study by Bhan, et al., 4H6, was generated from the fusion of splenocytes obtained from a mouse immunised with glomeruli isolated from Heymann nephritic rats. Indirect IF microscopy on normal rat kidney revealed that 4H6 produced a heavy granular staining of glomeruli, whereas 14C1 produced a faint, finely granular punctate staining of the glomeruli. Both monoclonal antibodies reacted with renal proximal tubules. The anti-GMF monoclonal antibodies reported in the current study produced a moderately heavy granular staining of the glomeruli which bore a similarity to that observed with 4H6.

No urine protein excretion studies were reported by Bhan et al. with the 14C1 and 4H6 monoclonal antibodies.

8.11 Nephritogenicity of anti-GMF monoclonal antibodies.

In this study, the four anti-GMF monoclonal antibodies PH7, ASD5, SC5 and BSG11 were examined in vivo to determine nephritogenicity. Initial experiments conducted with ASD5 and BSG11 revealed that these two monoclonal antibodies did not bind to rat glomeruli in vivo, and no increase in urine protein excretion was seen at either a 5 or 10 mg dose. Further study of these anti-GMF monoclocal antibodies was discontinued at this stage, except that paired radiolabel studies were undertaken with ASD5 since an inconsistent, patchy deposition of mouse immunoglobulin had been detected at one hour post-injection. Both PH7 and SC5 bound to rat glomeruli in vivo. Maximum binding of these monoclonal antibodies, as determined by direct IF microscopy, occurred at 24 hours. Indirect studies in vitro had shown that PH7 and SC5 reacted with glomeruli and tubules of normal rat kidney. However, in vivo, monoclonal antibody was only found bound to glomeruli, except in the group of rats which received 30 mg of PH7. This finding again raises the question of antigen accessability. IgM is a large pentameric protein of approximately 900 kDa. If the antigen with which the IgM antibody reacts has a restricted access, the size of the IgM molecule may prevent it from gaining close proximity to the antigen to form an immune-complex.

Urine protein excretion following IV administration of PH7 was greater at 24 hours than at 48 hours at all dose levels examined. All PH7 post-treatment values at 24 hours were significantly different from pre-treatment values at the $P < 0.05$ level. This finding correlates with indirect IF microscopy and paired radiolabel studies which showed that glomerular binding of anti-GMF monoclonal antibody peaked at 24 hours post-injection. The increased urine protein excretion was dose dependent, being maximal at the 30 mg dose level, which was the highest dose administered, and was not associated with an increase in glomerular PMN recruitment. Similarly, IV administration of SC5 monoclonal antibody resulted in a dose-dependent increase in urine protein excretion. Direct IF microscopy and paired radiolabel studies indicated that glomerular binding of monoclonal antibody peaked at 24 hours post-injection. This result correlated with 24 hour urine protein excretion measurements which showed that the increased protein excretion was greater at the 24 hour time point than at the 48 hour time point at all dose levels examined. As found in studies

conducted with PH7 in vivo, examination of glomeruli by light microscopy did not suggest that increased PMN recruiment was a factor in the development of the proteinuria. However, depletion experiments were not undertaken to further investigate this point.

From the literature it is clear that a number of fixed glomerular antigens which could potentially serve as the ligand for nephritogenic antibodies are being identified by monoclonal reagents. However, reports of the induction of glomerular lesions in vivo with monoclonal antibodies directed against non-GBM antigens are currently limited to this study and those emanating from the laboratory of Mendrick and Rennke in Boston, USA, (Mendrick et al., 1983; Mendrick and Rennke, 1986; 1988a; 1988b) and reports by Bagchus, et al. (1986b, 1986c) on an anti-T lymphocyte monoclonal antibody-induced proteinuric renal lesion.

Relating to this latter study, Thy-1 has been reported to be present on a number of tissues including mesangial cells of rat kidney (Paul, et al., 1984). The monoclonal IgG2a anti-Thy-1 antibody, designated ER4, was shown to bind to rat mesangial cells and GBM (Bagchus, et al., 1986a). A single intravenous injection of ER4 antibody into normal rats induced an immediate and severe proteinuria which peaked at forty-eight hours post-injection and returned to normal levels after three weeks. Granular IF staining for mouse immunoglobulin was observed on the GBM and mesangium at one to two hours post injection. Staining intensity decreased thereafter and was not detected at six days. Yamamoto and Wilson (1987) have also reported the induction of complement-dependent glomerular mesangial cell damage with a polyclonal antiserum raised against thymocytes and reactive with a Thy-1-like antigens present on glomerular mesangial cells.

Two IgG1 monoclonal antibodies, K23/1 which reacted with glomerular mesangial cells, and K23/7 which reacted with laminin, were studied in vivo by Mendrick and Rennke (1986). Electron dense deposits were observed in the mesangial matrix of the glomeruli of rats recieving K23/1, but were not in observed at any site in the kidney of those recieving K23/7 or the control monoclonal antibody. Urine protein excretion was not increased following the administration of either reagent, and light microscopy revealed normal glomerular histology.

8.12 Mechanism of proteinuria.

The mechanism involved in the development of proteinuria following the administration of anti-GMF monoclonal antibodies, in the absence of obvious histological damage to the kidney, as seen with PH7 and SC5, was not known in the current study. The direct binding of antibody to GBM antigens has been reported to induce proteinuria independently of complement or inflammatory cell infiltration (Baxter and Small, 1963; Simpson, et al., 1975; Couser and Salant, 1982). Reports have also been published on the induction of proteinuria in isolated perfused kidneys following the perfusion of heterologous anti-GBM antibodies (Boyce and Holdsworth, 1985; Couser, et al., 1985). Couser, et al. attributed the increased glomerular permeability to the direct binding of antibody to the GBM; however, Mendrick and Rennke (1988a) have questioned this hypothesis and, in turn, suggested that the increased glomerular permeability may have resulted from the binding of antibody to glomerular visceral epithelial cells. The antibody used in the study by Couser, et al. was raised against whole rat glomeruli, not purified GBM, and was not shown to be GBM specific. In experimental animal models of GN, damage to the glomeruli, and subsequent proteinuria following the deposition of immunoglobulin, have been reported to be mediated either by the complement system (Cochrane, et al., 1970; Salant, et al., 1980; Cybulsky, et al., 1986; Gabbai, et al., 1988; Quigg, et al., 1988), by the coagulation cascade system (Cochrane and Griffin, 1982), by polymorphonucleocytes (Cochrane, et al., 1965; Naish, et al., 1975;) or by macrophages Holdsworth, et al., 1981; Holdsworth and Neale, 1984). In a recent study Perico, et al. (1988) have demonstrated that platelet activating factor (PAF) can increase the permeability of the glomerulus to protein in the absence of platelets or inflammatory cells. The PAF-induced increase in glomerular permeability was inhibited by a L-652,731, a specific PAF receptor antagonist. The authors concluded that the effect of PAF on the protein permeability of the glomerulus was a direct effect of its biological activity.

Mendrick and Rennke (1988a, 1988b) have described an IgG1 monoclonal antibody, K9/9, generated from splenocytes obtained from a mouse immunised with an homogenate of rat renal cortex and shown to react with two glomerular epithelial cell antigens of 115 and 107 kDa respectively, which induces complement and leukocyte-independent glomerular epithelial cell damage and proteinuria. Intravenous administration of 10 mg of K9/9 induced a proteinuric lesion in normal rats which was maximal at 24 hours post-injection. At a dose of 66 mg of K9/9, the degree of proteinuria increased and was maximal at 48 hours post-injecion. However, the increased urine protein excretion only occurred if the rats received a concomitant

subcutaneous immunisation of a 1:1 (by volume) emulsion of complete Freund's adjuvant and an ascites-derived fraction of non-kidney binding immunoglobulin. Proteinuria did not develop if complete was substituted with incomplete Freund's adjuvant (ie. not containing myobacterium tuberculosis). Light microscopy of kidneys from treated rats revealed normal glomerular cellularity. Diffuse epithelial cell damage was seen in electron micrographs, but electron dense deposits were not observed. The omission of myobacterium tuberculosis did not affect kidney binding of K9/9 as assessed by trace radiolabel studies. Total monoclonal antibody bound to the kidneys at 24 hours following the administration of 10 mg of labelled K9/9 was 40 μg. This is considerably greater than that found with either of the anti-GMF monoclonal antibodies, PH7 or SC5, examined in the current study. Electron microscopy studies in the current study failed to demonstrate electron dense deposits in the glomeruli of proteinuric PH7 or SC5-treated rats. Similarly, Mendrick and Rennke (1988a, 1988b) failed to detect electron-dense deposits in the glomeruli of proteinuric rats following treatment with K9/9 monoclonal antibody. Studies undertaken with polyclonal and monoclonal anti-gp330 antibodies demonstrated that electron dense deposits were only found in the glomeruli of rats treated with polyclonal anti-gp330 (Allegri, et al., 1986). The conclusion from this study was that highly cross-linked immune complexes formed by polyvalent antibodies on the glomerular epithelial cells were necessary for the formation of electron dense deposits. This makes it difficult to explain the lack of deposits in the present study since the anti-GMF monoclonal antibodies were of the IgM subclass, consisting of pentameric molecules and with a theoretical antigen valency of 10. However, since it is unlikely that cell surface antigens would be anatomically arranged in an orientation that would facilitate saturation of the antigen binding sites of IgM, limited antigen accessibility to the large IgM molecule may have contributed to the limitation on formation of large immune complexes.

8.13 Summary.

A total of 17 hybridomas, generated from the fusion of splenocytes obtained from mice immunised with a plasma membrane fraction prepared from glomeruli isolated from Lewis rat kidney, produced monoclonal antibodies which reacted with discontinuously represented antigens in the glomerulus and renal tubules. One further hybridoma secreted a monoclonal antibody which reacted with an antigen present on glomerular and tubular nuclear membranes. No hybridomas were produced which secreted a monoclonal antibody which reacted wth glomerular basement membranes.

Two of these monoclonal antibodies, PH7 and SC5, of the IgM subclass induced a mild proteinuric lesion when administered intravenously to normal Lewis rats. The proteinuria was not associated with histopathological changes at the light or electron microscope level. Attempts to localise the binding of the monoclonal antibodies by immunogold electron microscopy were unsuccessful.

Immunoblotting experiments revealed that SC5 reacted predominantly with a protein band of 96 kDa present in detergent extracts of isolated glomeruli and glomerular plasma membranes. PH7 was shown to react with three low molecular weight proteins of 14, 13 and 11 kDa.

The nephritogenic anti-GMF monoclonal antibodies SC5 and PH7 may prove to be useful tools in the study of animal models of immunologically mediated glomerulonephritis.

Appendix I.

CHEMICALS AND BUFFERS.

All chemicals used in this project for the composition of buffers and other reagents were of commercial reagent or analytical grade. Those chemicals used for the preparation of buffers and other reagents for use in electrophoresis were electrophoresis grade.

Buffers.

1. Carbonate-bicarbonate buffer (0.05 M, pH 9.6).

Stock solutions.	Sodium carbonate	1 M
	Sodium bicarbonate	1 M
	Sodium chloride	0.14 M

The carbonate and bicarbonate solutions were mixed in the proportions 1:9. The pH was adjusted to 9.6 by titration with one of the stock solutions. The final solution was diluted to 0.05 M with sodium chloride (1/19).

The sodium bicarbonate solution was made immediately prior to preparation of the buffer.

2. Citrate buffer (0.1 M, pH 5.0).

Stock solutions.	Citric acid	0.1 M
	Di-sodium hydrogen phosphate	0.1 M

The solutions were mixed immediately prior to use approximately 50:50 and the pH adjusted to 5.0 with one of the solutions.

3. Glycine-HCl (0.1 M, pH 2.8).

Stock solutions.	Glycine	0.2 M
	Hydrochloric acid	0.2 M

250 mls of glycine solution at RT was titrated to pH 2.8 with HCl, the solution was then adjusted to a final volume of 500 mls with distilled deionised water.

The stock solutions were stored at 4°C until used. The glycine solution was used within four weeks of preparation.

4. Homogenisation buffer.

Sucrose	0.25 M
Ethylene diamine tetra-acetic acid (EDTA)	1 mM
Tris (hydroxymethyl) aminomethane (TRIS)	4 mM
Iodoacetamide	1 mM
Phenylmethylsulfonyl fluoride (PMSF)	1 mM
Aprotinin	200 kIU/ml

The components were dissolved in approximately 800 mls of distilled deionised water at RT. The PMSF was dissolved in 1.5-2.0 mls of ethyl alcohol and added to the solution, the final volume was adjusted to 1 litre.

5. Phosphate-buffered saline (0.15 M, pH 7.2).

Sodium chloride	8.00 g/L
Potassium chloride	0.20 g/L
Di-sodium hydrogen phosphate	1.15 g/L
Potassium di-hydrogen phosphate	0.20 g/L

The components were dissolved in approximately 900 mls of distilled deionised water at RT, the pH was checked and the solution made up to a final volume of 1 litre. The buffer was stored at 4° C until used.

6. Phosphate buffer (0.01-0.3 M, pH 6.8).

Stock solutions	Sodium di-hydrogen phosphate	0.5 M
	Di-sodium hydrogen phosphate	0.5 M

The pH of the sodium di-hydrogen phosphate solution was titrated to 6.8 with di-sodium hydrogen phosphate. The solution was then diluted to the required molarity with distilled deionised water.

7. Tris-buffered saline (0.2 M Tris, 0.5 M sodium chloride, pH 7.5).

Tris (hydroxymethyl) aminomethane	2.42 g
Sodium chloride	29.22 g

The components were dissolved in approximately 800 mls of distilled deionised water, the pH was adjusted with 1 M HCl and the final volume made up to 1 litre with distilled deionised water.

8. Tris-glycine transfer buffer (pH 8.3).

Tris (hydroxymethyl) aminomethane	12.12 g
Glycine	57.60 g
Methanol	800 mls

The Tris and glycine were dissolved in 2.5-3.0 litres of distilled deionised water at RT, 800 mls of analytical grade methanol was then added and the final volume adjusted to 4 litres with distilled deionised water. The pH of the solution was checked. The buffer was stored at 4°C until used.

Electrophoreis buffers and reagents.

1. Acrylamide-bisacrylamide stock solution.

Acrylamide	30.0 g
Bisacrylamide	0.8 g

Distilled deionised water was added to a final volume of 100 mls. Approximately 2 to 3 g of activated charcoal was added and the solution stirred for five minutes prior to filtering. The solution was stored at 4°C in a dark bottle for a maximum of four weeks.

2. Electrophoresis running buffer, 0.025 M Tris-0.192 M glycine, pH 8.3.

Tris (hydroxymethyl) aminomethane	9.0 g
Glycine	43.2 g
Sodium dodecyl sulphate	3.0 g

The components were dissolved in approximately 2.5 litres of distilled deionised water, the pH was adjusted with either 0.1 or 1.0 M HCl and the final volume made up to 3 litres. The buffer was stored at 4°C until used.

3. Separating gel buffer (1.5 M, pH 8.8).

Tris (hydroxymethyl) aminomethane (54.45 g) was disolved in approximately 150-200 mls of distilled deionised water. The pH of the solution was adjusted to 8.8 with 1 M HCl and the final volume made up to 300 mls with distilled deionised water. The solution was stored at 4°C.

4. Stacking gel buffer (0.5 M, pH 6.8).

Tris (hydroxymethyl) aminomethane (6 g) was disolved in approximately 60 mls of distilled deionised water. The pH was adjusted to 6.8 with 1 M HCl and the final volume made up to 100 mls with distilled deionised water. The solution was stored at 4°C.

5. Sample buffer.

Distilled water	4.0 mls
Stacking gel buffer	1.0 "
Glycerol	0.8 "
Sodium dodecyl sulphate 10% (w/v)	1.6 "
Dithiothreitol 0.25 M	0.4 "
Bromophenol blue 0.05% (w/v)	0.2 "

The dithiothreitol solution was made up on the day of use.

6. Stacking gel, 4%.

Distilled water	6.1 mls
Stacking gel buffer	2.5 "
Sodium dodecyl sulphate 10% (w/v)	0.1 "
Ammonium persulphate 10% (w/v)	50 μl
N,N,N',N'-tetramethylethylenediamine (TEMED)	10 μl

The solution was de-aired under vacuum immediately prior to addition of the ammonium persulphate and TEMED.

7. Running gel.

Running gel buffer	25.0 mls
Sodium dodecyl sulphate 10% (w/v)	1.0 "
Ammonium persulphate 10% (w/v)	0.5 "
TEMED	50 μl

Acrylamide/bisacrylamide stock solution was added to attain the desired concentration and the final volume brought to 100 mls with distilled deionised water. The solution was de-aired under vacuum immediately prior to addition of the ammonium persulphate and TEMED.

Fixatives.

Karnovsky's fixative (half strength).

Glutaraldehyde	2.5%
Paraformaldehyde	2.0%
Sodium cacodylate	0.1 M
Potassium chloride	5 mM
Sucrose	0.15 M

The pH of the solution was adjusted to 7.4 with 1 M NaOH.

Appendix II.

CELL CULTURE REAGENTS.

Rosewall Park Memorial Institute 1640 (RPMI), Dulbecco's modified Eagle's (DMEM) and NCTC 135 medium were obtained in powder form from Gibco New Zealand Ltd., Auckland. All additives to cell culture medium were of analytical or cell culture grade.

Foetal calf serum was obtained from Commonwealth Serum Laboratories, Australia. This serum had been screened by the manufacturers for its ability to support the growth of Sp2/o and NS1 myeloma cells in culture and also reported to be mycoplasma-free.

Cell culture media additives.

All additives to cell culture media were made up as concentrated solutions. With the exception of aminopterin, the solutions were filter-sterilised and stored in sterile containers at -20°C until used. Aminopterin was obtained in sterile vials from Sigma Chemical Co. (Missouri, USA), the aminopterin was made up to a 50 x concentrated solution by the addition of sterile double glass-distilled water immediately prior to use. The final concentrations of the reagents following addition to the media are detailed below.

L-glutamine	2×10^{-3} M
Sodium pyruvate	1×10^{-3} M
Hypoxanthine	1×10^{-4} M
Thymidine	1.6×10^{-5} M
Aminopterin	4×10^{-7} M
Penicillin	100 μg/ml
Streptomycin	50 μg/ml
2-mercaptoethanol	5×10^{-5} M

2-mercaptoethanol was made up in RPMI medium, the remainder were made up in sterile double-glass distilled water.

Cell culture media.

1. RPMI.

The content of a single satchet, sufficient to make one litre, was dissolved in approximately 800 mls of double-glass distilled water in a glass beaker with continuous stirring. To this medium 3.9045 g of sodium Hepes (15 mM) and 2.0 g of sodium bicarbonate were added. The pH of the medium was adjusted to 7.4 with 1M HCl and made up to a final volume of one litre. The medium was filter-sterilised through a sterile 0.22 μm membrane under positive pressure and stored in 200 or 500 ml aliquots in sterile bottles following the addition of a 100 x concentrated solution of penicillin and streptomycin. The medium was stored at 4°C until used.

2. DMEM.

This medium was prepared from powdered DMEM in an identical manner to that described above for RPMI.

3. NCTC 135.

Cell culture media containing 10% NCTC were made up in 100 ml amounts. A total of 96 mgs of NCTC was weighed into a glass beaker , double-glass distilled water was added to a total volume of 10 mls. This medium was then filter-sterilised as for RPMI and added directly to the respective medium.

4. Cloning medium.

Soluble insulin (100 units/ml)	1 ml
Foetal calf serum	20 mls
NCTC medium (96 mgs)	10 mls
L-glutamine (100 x)	1 ml
Sodium pyruvate (100 x)	1 ml
Hypoxanthine/thymidine (100 x)	1 ml
2-mercaptoethanol (100 x)	1 ml
RPMI	65 mls

5. Freezing medium.

Soluble insulin (100 units/ml)	1 ml
Foetal calf serum	20 mls
L-glutamine (100 x)	1 ml
Sodium pyruvate (100 x)	1 ml
Dimethyl sulphoxide	10 mls
RPMI	66 mls

6. Hypoxanthine/thymidine/aminopterin medium (HAT).

Soluble insulin (100 units/ml)	1 ml
Foetal calf serum	20 mls
NCTC 135	10 mls
L-glutamine (100 x)	1 ml
Sodium pyruvate (100 x)	1 ml
Hypoxanthine/ thymidine (100 x)	1 ml
Aminopterin (50 x)	2 mls
RPMI	64 mls

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