URINARY C5b-9 AND CYTOKINES IN IDIOPATHIC HUMAN MEMBRANOUS NEPHROPATHY

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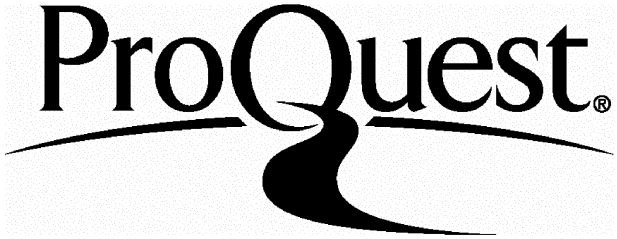

Dedicated to my parents for their continual support

and encouragement.

ABSTRACT

The immunopathogenic mechanisms in idiopathic membranous nephropathy (IMN) are unknown. Disease mechanisms may be similar in IMN and in passive Heymann nephritis (PHN) where proteinuria is mediated by C5b-9. Urinary C5b-9 (uC5b-9) is specific to PHN when compared to other experimental models and accompanies ongoing glomerular deposition of immune reactants. The primary aim of this study was to determine whether uC5b-9 can be used as a marker of active immunological injury in IMN.

An ELISA was developed for the detection of uC5b-9. Discrepancies in the measurement of uC5b-9 reported in proteinuric patients when using different capture antibodies were addressed.

Urinary C5b-9 was measured serially in seventy-six IMN patients who did not receive immunosuppression : fifty-seven patients with stable renal function (SRF) and nineteen patients with declining renal function (DRF). Patients with DRF excreted significantly more C5b-9 than those with SRF ($p < 0.001$). Patients with DRF were less likely to decline further if C5b-9 excretion stopped ($p < 0.05$). Three commonly used immunosuppressive regimens were employed and given to eighteen patients who developed renal impairment. Patients with uC5b-9 had a better clinical outcome if treatment abolished excretion of C5b-9 ($p < 0.005$). All three regimens abolished the excretion of C5b-9. Thus this study suggests that uC5b-9 is a marker of ongoing immunological insult in IMN and can be used in the selection of patients

for treatment and monitoring efficacy.

Membranes in the urine from six IMN patients known to excrete C5b-9 were isolated and characterised: S-protein and CD59 were detected. The cytokines IL-1, IL-6, TNF-α and TGF-$ß_1$ were assayed by ELISA in plasma and urine samples of twenty IMN patients. Of the cytokines studied, only plasma IL-1ß and urinary IL-6 were significantly elevated in the IMN group compared to healthy controls ($p<0.05$), but it did not correlate with uC5b-9. C5b-9 does not appear to release inflammatory cytokines in the glomerular injury of IMN.

Unless otherwise stated, all the work described herein is my own.

I declare that this thesis has not been submitted to this or any other university or institution for a degree or comparable award.

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ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt
AICN	Autologous immune complex nephritis
BSA	Bovine serum albumin
C8bp	C8 binding protein (same as HRF and MIP)
CALLA	Common acute lymphoblastic leukaemia antigen
CD10	see CALLA
CD26	see DPP IV
CD35	see CR1
CD46	see MCP
CD51	see VNR
CD55	see DAF
CD59	see MIRL
cDNA	Complementary DNA
CFD	Complement fixation test diluent
CR1	Complement receptor 1, C3b receptor
DAB	3,3'-diaminobenzidine tetrachloride dihydrate
DAF	Decay accelerating factor
DMSO	Dimethyl sulphoxide
DPP IV	Dipeptidyl peptidase IV, gp90
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESRF	End-stage renal failure
FPLC	Fast protein liquid chromatography
GBM	Glomerular basement membrane
GEC	Glomerular epithelial cell
GFR	Glomerular filtration rate
GPI	Glycosyl phosphatidylinositol
gp90	see DPP IV
HLA	Human leucocyte antigen
HMN	Human membranous nephropathy
HN	Heymann nephritis
HRF	Homologous restriction factor (same as C8bp and MIP)
IC	Immune complexes
IL	Interleukin
IMN	Idiopathic membranous nephropathy
kD	Kilodalton
LDL	Low-density lipoprotein
M_r	Relative molecular weight
mAb	Monoclonal antibody
MAC	Membrane attack complex

MCP	Membrane cofactor protein
MHC	Major histocompatibility complex
MIP	MAC inhibitor protein (same as HRF and C8bp)
MIRL	MAC inhibitor of reactive lysis
MN	Membranous nephropathy
mRNA	Messenger RNA
mw	Molecular weight
NHS	Normal human serum
OD	Optical density
OPD	1,2-phenylenediamine dihydrochloride
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PHN	Passive Heymann nephritis
PMSF	Phenylmethylsulphonyl flouride (dissolved in isopropanol)
poly-C9	Polymerised C9
PVDF	Polyvinylidene diflouride
rIL	Recombinant interkeukin
rTGF	Recombinant TGF
rTNF	Recombinant TNF
RGD	Arginine - glycine - aspartate
ROM	Reactive oxygen metabolites
SC5b-9	Serum C5b-9
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylene diamine
TGF	Transforming growth factor
TNF	Tumour necrosis factor
Tris	Tris (hydroxymethyl) methylamine
Tween 20	Polyoxyethylene-sorbitan monolaurate
uC5b-9	Urinary C5b-9
UV	Ultraviolet
VNR	Vitronectin receptor, $\alpha_v ß_3$
Zwitt	Zwittergent 3-12
α_2-MRAP	α_2-macroglobulin receptor associated protein
>30%	greater than 30%

CHAPTER 1

INTRODUCTION

INTRODUCTION

Membranous nephropathy (MN) is recognised as the most common glomerulopathy causing the nephrotic syndrome among adults in the developed world. It is characterised ultrastructurally by discrete electron dense deposits along the subepithelial surface of the glomerular basement membrane (GBM), which correlate using immunofluorescence techniques with diffuse granular deposits of IgG and C3 (Figures 1 and 2). On light microscopy there is uniform thickening of the capillary basement membrane without significant hypercellularity of the glomerular tuft. The extensions of the GBM between the immune deposits are seen as 'spikes' in the periodic-acid silver methanamine technique. This supports the hypothesis of an immune pathogenesis (Arnaout 1982). Furthermore the idiopathic form of the disorder in humans is strongly associated with HLA DR3 (Klouda 1979). In animal models, it is the histopathological result of a variety of pathogenic mechanisms. In humans, however, it is a histological diagnosis and not a discrete disease entity. No precipitating cause can be identified in 75% of patients in the UK (Medical Research Council Registry), and although it is commonly a benign disorder, up to 25% will reach end-stage renal failure (ESRF) or die due to other causes within ten years of diagnosis (Honkanen 1986). It is estimated that 0.5 - 1.3 cases of MN per million population annually enter renal replacement programmes throughout Europe (EDTA Registry 1990).

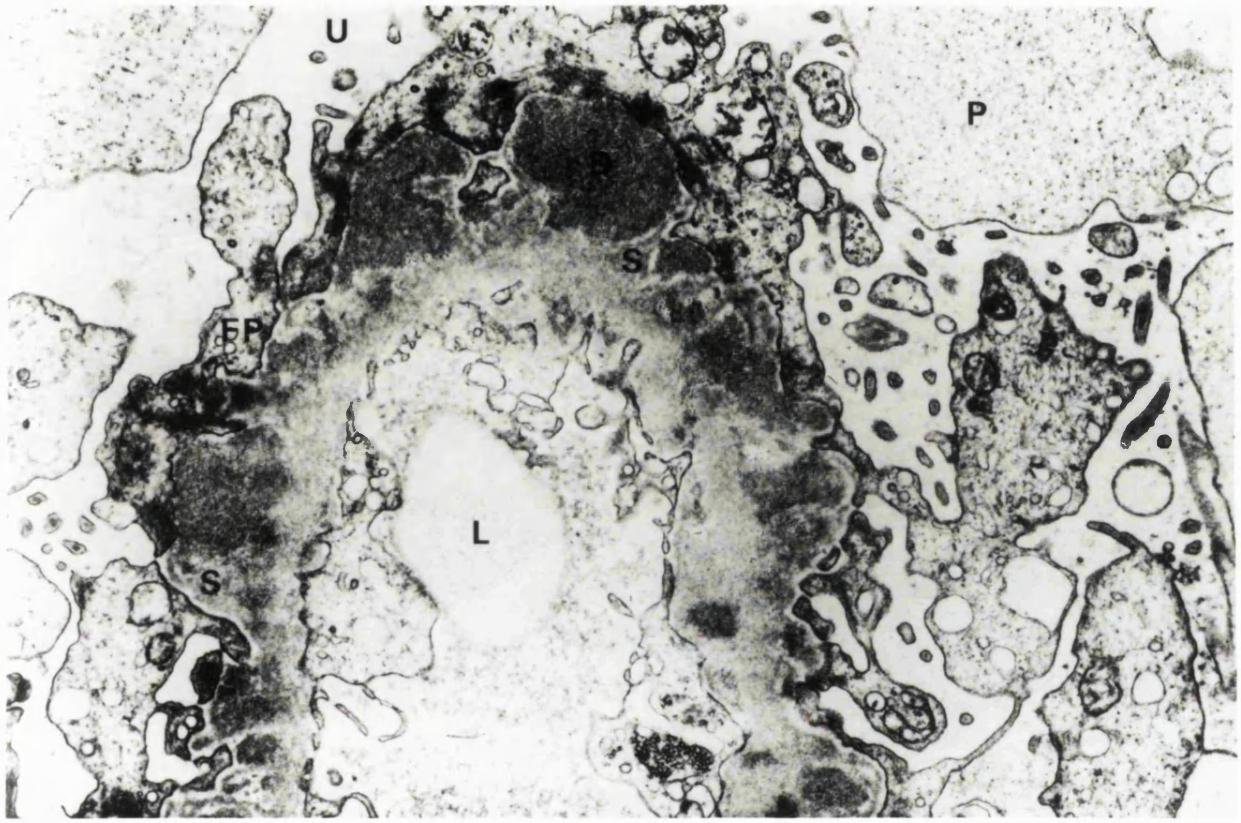

Fig. 1. Electron microscopy. Membranous glomerulonephritis. Large subepithelial deposits **(D)** and spikes **(S)** along the basement membrane. The foot processes **(Fp)** are effaced. **(U)** urinary space; **(P)** podocyte; **(L)** capillary lumen.

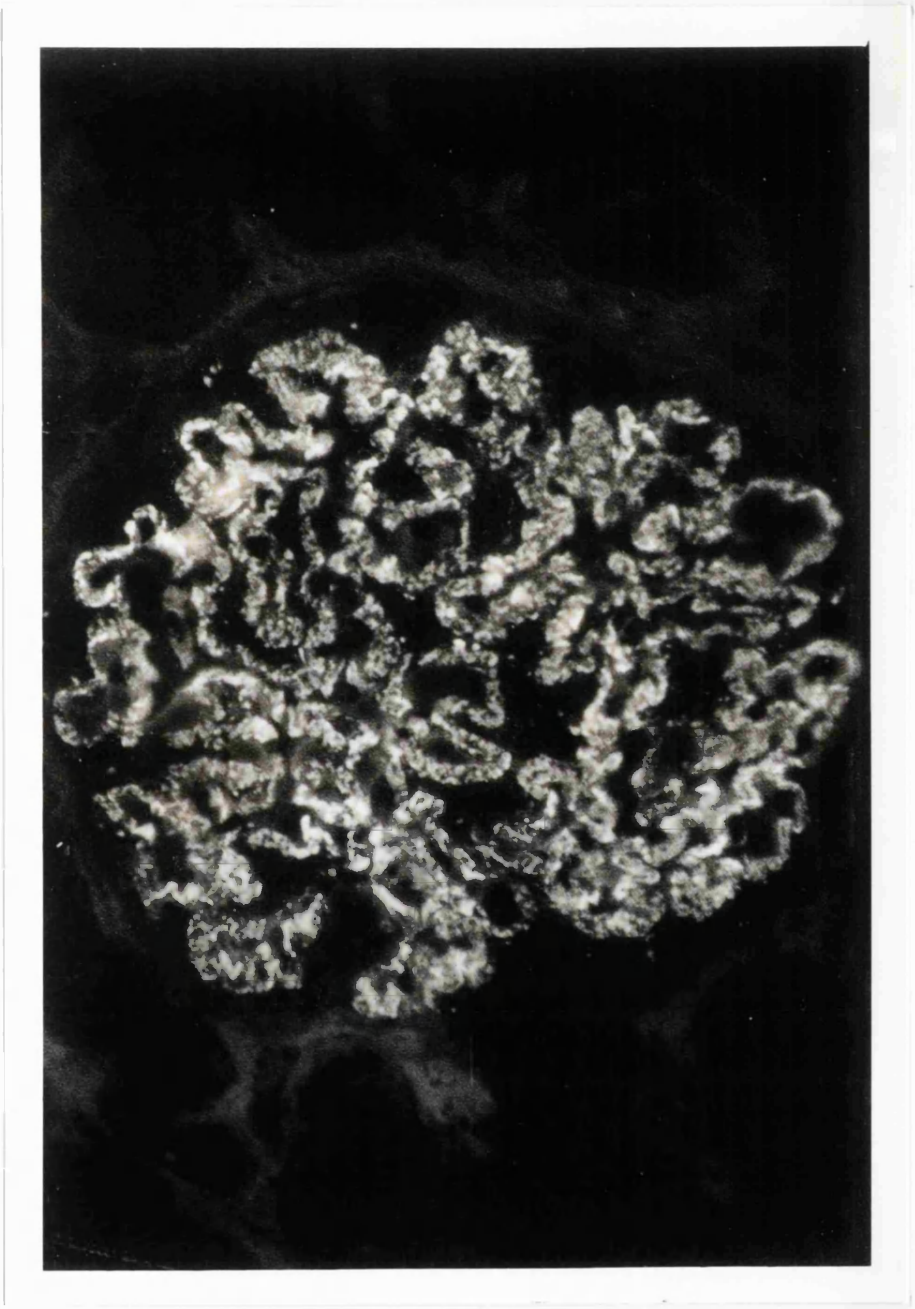

Fig. 2. Immunofluorescence. Diffuse granular deposits of IgG along the capillary walls (x260).

The immune mechanisms which mediate MN in man are unknown. Therapy is largely empiric and guided by measurements of inferred immune injury, such as proteinuria and changes in renal function, rather than by assessment of activity of the underlying immunopathogenic mechanism. Proteinuria may continue for months or years and it is uncertain whether this reflects active disease or is the residual effect of previous injury. Patients may exhibit fluctuating levels of proteinuria in the course of the disease and the uncertain prognosis, both with respect to the persistence of proteinuria and deterioration of renal function, poses a management dilemma in idiopathic MN (IMN). No data establishing any direct relationship between the immune disease mechanism and its consequences such as proteinuria and loss of renal function have been provided. Besides persistent heavy proteinuria which has been shown to be helpful in predicting chronic renal insufficiency (Pei 1992[1]), there is no other measure to identify those patients who show putative evidence of an active immunological insult. If immunosuppressive drugs do have a role in the therapy of MN, it would seem a priori that they would be used to maximum effect where there is evidence of an ongoing immunological damage and thus such patients should be identified as early as possible. An understanding of the immunopathogenic mechanisms in IMN will help the clinician in the management and in the choice of treatment of MN patients with proteinuria without either treating patients who may enter spontaneous remission or waiting for evidence of a decline in glomerular filtration rate (GFR). Experimental models have been helpful in the understanding of possible immunopathogenic mechanisms operative in human membranous nephropathy (HMN), but in the human situation such mechanisms remain enigmatic.

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[1]see page 172 for reference

A. EXPERIMENTAL IMMUNE COMPLEX DISEASE

(i) Mechanisms of immune complex formation

Early this century, an immune basis for glomerulonephritis was suggested following the observation that nephritis may follow scarlet fever or tonsillitis. Schick (1907) observed that this post-infectious nephritis occurred after a similar time interval as that seen in hypersensitivity reactions. Subsequently, the immunofluorescence studies of human tissue from glomerulopathic kidneys supported this hypothesis. Immune deposits were seen in glomeruli of poststreptococcal glomerulonephritis and other diseases (Mellors 1956).

Deposition of preformed immune complexes

Experimental verification of the immune basis of nephritis following exposure to foreign antigen was provided by studies in acute and chronic bovine serum albumin (BSA) serum sickness in rabbits (Germuth 1955, Germuth 1957, Dixon 1961). The deposits were formed only in the presence of circulating immune complexes (IC) and conventional immunofluorescence techniques at that time could not detect antigen and antibody alone in glomeruli. Therefore, it was postulated that preformed IC in the circulation were passively trapped in the GBM. There have been several reports of circulating antigen-antibody complexes in Heymann nephritis (Naruse 1978, Abrass 1980). This pathogenic mechanism remains controversial as subepithelial IC deposits of low avidity antibodies (Germuth 1979, Germuth 1982) or non-covalently linked IC made with cationic antigens (Gallo 1981) could have dissociated intravascularly with subsequent reformation in-situ.

In-situ immune complex formation

Van Damme (1978) and Couser (1978) have proposed an alternative, in-situ, mechanism for the formation of subepithelial immune deposits. This hypothesis was based on experiments where nephritogenic antibodies formed typical subepithelial glomerular immune deposits when perfused through isolated rat kidneys in the passive Heymann nephritis (PHN) model. This method avoided the formation of circulating IC. The antigen responsible for this lesion is now believed to be a glycoprotein, gp330, a relative molecular weight (M_r) of 330,000 (Kerjaschki 1982 and 1983). This theory of an antibody reacting with an intrinsic glomerular antigen has been suggested as a model of HMN. Spontaneous nephropathy has also been demonstrated in rabbits with epithelial cell foot process antigen (Neale 1984). A second mechanism of in-situ subepithelial IC formation has been reported and is due to planted extrinsic antigen as shown by exogenous antigens used in the chronic BSA serum sickness model (Border 1982, Oite 1982) and ferritin (Vogt 1982).

Other animal models also show membranous changes on renal biopsy. They include autoimmunity induced by administration of toxins like mercuric chloride (Druet 1982), D-penicillamine (Tournade 1990) and gold (Tournade 1991), graft versus host reaction (Bruijn 1988), or trypanosomal infection (Bruijn 1987). In these models, it appears that different antibodies are pathogenetic, directed variously against gp330, gp90, laminin, collagen type IV and probably other yet undetected glomerular antigens or planted extrinsic antigens. The B cells in these models appear to be triggered in a non-specific manner.

An outline of the mechanisms of glomerular subepithelial immune deposit formation is shown in Table 1.

Table 1. Mechanisms of subepithelial immune complex formation.

1. Circulating immune complex trapping
   (a) directly
   (b) after dissociation

2. In-situ immune complex formation
   (a) against intrinsic (Heymann-like) antigen
   (b) against planted extrinsic antigen
      - endogenous
      - exogenous

(ii) Heymann nephritis (HN)

No animal model parallels human MN as closely as that of HN in rats. This is because of its morphological characteristics on light and electron microscopy and its association with the nephrotic syndrome (Hoedemaeker 1988). Active HN was induced by immunising susceptible rats with autologous (Heymann 1959) or heterologous (Edgington 1967) homogenates of renal cortex. The lesion induced was characterised by granular deposits in the glomerular capillary wall of endogenous rat IgG and subepithelial electron dense deposits after 3 to 4 weeks. Proteinuria developed in 30% to 80% of rats within 8 to 10 weeks of immunisation. This actively induced HN is also called autologous immune complex nephritis (AICN).

Similar glomerular lesions were induced by a single intravenous dose of heterologous anti-proximal tubular brush border antiserum, sheep anti-Fx1A, into normal rats

(Barabas 1974). Heterologous IgG deposits accumulate in glomeruli over hours and days (Kerjaschki 1987a) and proteinuria ensues in almost all animals within 5 days. This variant is referred to as PHN as the immune response was passively transferred to the animal. This 'heterologous phase' is followed by an 'autologous phase' during which rat IgG antibodies with specificity for the heterologous glomerular-bound IgG are deposited, and induce a further increase in proteinuria (Edgington 1969).

That active and PHN are caused by the same antigen is known because IgG eluted from glomeruli in rats with active HN induced PHN when injected intravenously into normal rats (Neale 1982, Madaio 1983). However PHN is the more commonly studied model of early events in the formation of immune deposits.

In PHN the in-situ subepithelial immune deposits is due to IgG antibody reacting with antigens on the glomerular epithelial cell (GEC) membrane. The best characterised antigen is gp330 which is located in the clathrin-coated pits of GEC (Kerjaschki 1982). The newly formed IC detaches from the cell membrane and this shed IC is stabilised in the GBM by covalent bonds. The immune deposit grows in size by repeated cycles of in-situ IC formation and shedding into the lamina rara externa, until it encroaches on the area of the slit diaphragm (Kerjaschki 1987a). This is illustrated in Figure 3. The antigen-antibody complexes result in discontinuous subepithelial immune deposits as they remain tightly bound to the GBM. Proteinuria follows and the lesion is non-inflammatory and indistinguishable from HMN.

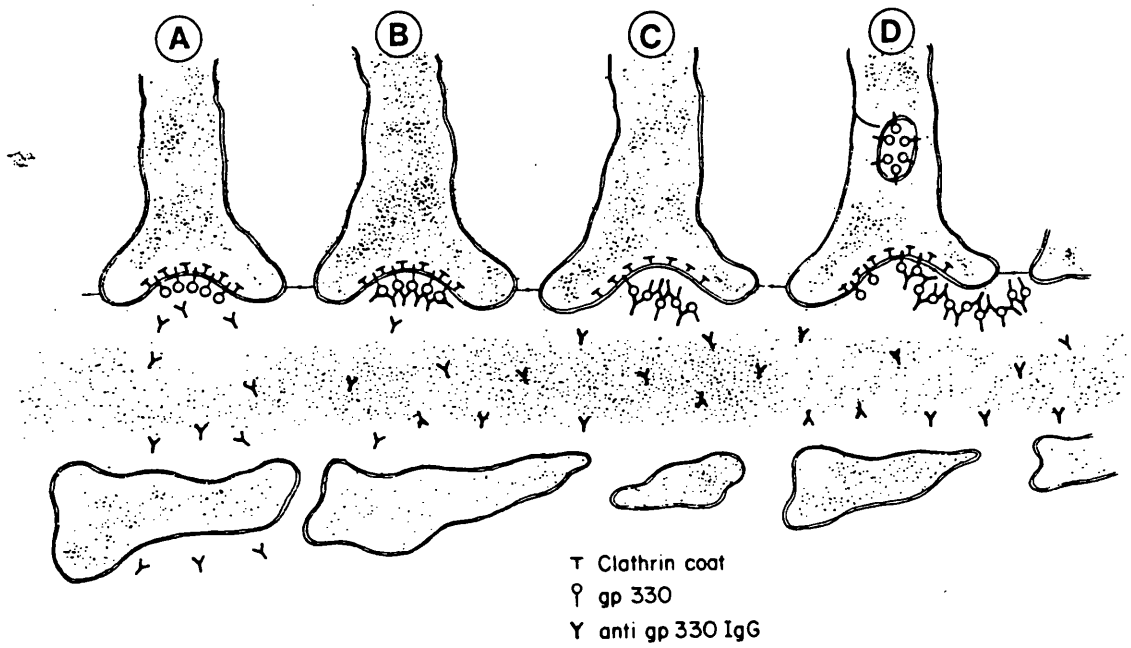

Fig. 3. Summary of early events of immune deposit formation in PHN. **A.** Circulating anti-gp330 penetrate GBM to approach gp330 on epithelial foot process. **B.** Antigen-antibody bind via 'pathogenic epitopes' in the coated pits. **C.** The initial IC detaches and is shed into the lamina rara externa. **D.** The immune deposit grows in size by repeated cycles of in-situ IC formation until it encroaches on the slit diaphragm area. New molecules of gp330, synthesized de novo by the GEC, are delivered via vesicles which fuse with the membrane at the base of the foot process.

(From Kerjaschki D, 1987a)

Much effort was made to identify further antigens in PHN, the paradigm for in-situ formation of IC in the glomerular capillary wall. It was suggested that other antibodies present in anti-Fx1A antiserum could synergise with anti-gp330 to induce glomerular injury (Abrass 1987). Besides gp330, which is present in both rat tubular brush border and rat podocyte, additional antigens have been identified. From the multitude of other antigens present in renal tubular epithelium, only a few were eligible candidates as most of them are not present in rat glomeruli. The enzyme dipeptidyl peptidase (DPP IV, CD26, gp90) is one of them (Natori 1987). A synergistic effect of antibodies against gp330 and DPP IV has been shown in PHN (van Leer 1992). The production of monoclonal antibodies (mAb) to rat podocytes has enabled the identification of a further antigen present in both rat tubular brush border and podocyte, i.e. neutral endopeptidase which is identical with the common acute lymphoblastic leukaemic antigen (CALLA, CD10, Jongeneel 1989). However, these attempts to identify glomerular antigens in PHN may or may not be relevant to the human disorder.

(iii) Roles of C5b-9

A number of factors are involved in the pathway from formation of glomerular immune deposits to the phenomenon of pathophysiological tissue injury. The pivotal role of complement became established in the 1960s (Cochrane 1965). Activation of complement by either the classical or alternative pathway leads to the formation of the membrane attack complex (MAC). Classical pathway activation is initiated by C1 which binds to immunoglobulins. Alternative pathway can be activated immunologically by a variety of activator substances. This pathway requires C3b.

MAC is assembled from five precursor molecules present in the serum in the inactive form. Proteolytic cleavage of C5 by the C5 convertase of either the classical or alternative pathway generates C5b. C5b combines with C6 and C7 to form the C5b67 complex, an amphophilic molecule which has binding sites for the lipid bilayer of cell membranes. With binding of C8 and multiple C9 molecules, the C5b-9 complex inserts into the cell membrane to form a transmembrane channel (Hansch 1988a). This is illustrated in Figure 4.

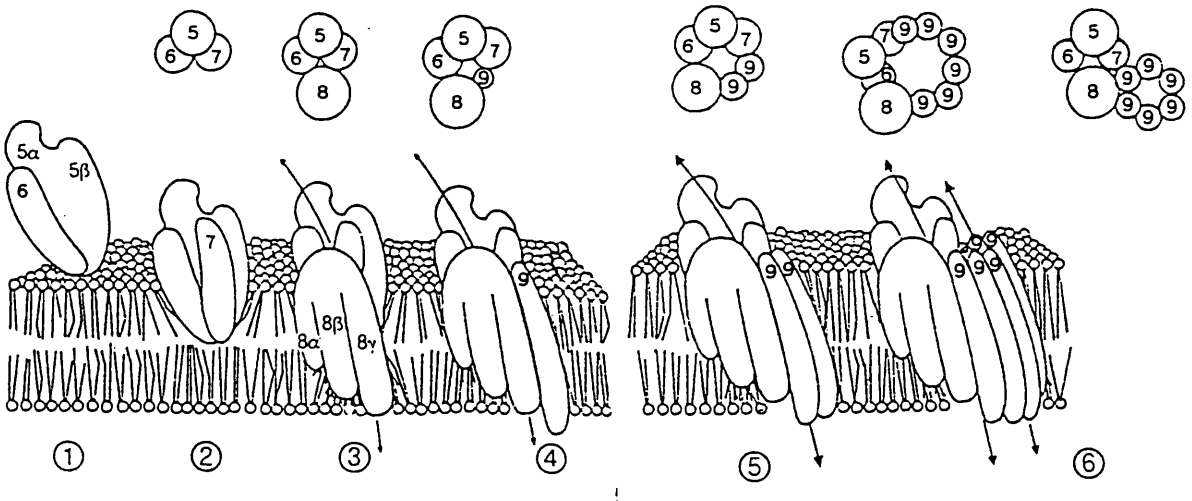

Fig. 4. Formation and insertion of C5b-9 complex. **1.** The C5b6 complex interacts reversibly with the cell membrane. **2-4.** Upon subsequent binding of C7,C8 and C9, newly expressed hydrophobic chains allow insertion of the complex. **3.** Pore formation occurs already at the C5b-8 stage, the upper panels show the top view of the complex. **5.** The pore enlarges with more C9 molecules. **6.** In presence of large amounts of C9, poly-C9 channels are formed.

(From Rother 1991)

(a) C5b-9 as a mediator of proteinuria

Following IC formation, complement is activated. The evidence that C5b-9 induced glomerular injury came from studies in PHN. Complement depletion using cobra venom factor (Salant 1980) or inhibition with specific antibody to rat C6 (Baker 1989) abrogated proteinuria by preventing C5b-9 assembly. However IgG and C3 deposition was unaltered. These studies were in experimental animals and the phenomenon was subsequently also demonstrated in the isolated perfused kidney (Cybulsky 1986a). Proteinuria did not develop when anti-Fx1A antibody deposition occurred in the presence of serum deficient of C6 or C8, but proteinuria appeared immediately following addition of normal serum.

In addition to the PHN model, C5b-9 was also shown to be the principal mediator of proteinuria in other experimental models of subepithelial IC deposits. In the autologous phase of PHN of both the intact animal (Couser 1991) and in the isolated perfused kidney (Cybulsky 1986b), glomerular injury was C5b-9 mediated. In the cationic BSA serum sickness model proteinuria was delayed in C6-deficient rabbits (Groggel 1985).

Glomerular deposits of C5b-9 showed a similar distribution to IgG in PHN (Adler 1984) and chronic serum sickness (Koffler 1983). In sequential studies, the movement of C5b-9 could be followed (Kerjaschki 1989). It was initially detected in the clathrin-coated pits where the putative antigen is expressed. In contrast to IC deposits, C5b-9 was endocytosed by the cell and transported intracellularly in large multivesicular bodies before being exocytosed into the urinary space. Freeze-fracture

studies of the GEC in early PHN reveal clusters of large 20-25nm intramembrane particles which have the size and appearance of membrane inserted C5b-9 complexes.

(b) C5b-9 as an index of disease activity

The immuno-ultrastructural data of the intracellular transport and exocytosis of C5b-9 into the urinary space described above led to quantification of urinary C5b-9 (uC5b-9). Schulze (1989) showed that uC5b-9 was raised in PHN before the onset of proteinuria and its excretion was abolished by complement depletion with cobra venom factor. Urinary C5b-9 was not seen in other proteinuric models induced by exogenous antigens (despite equivalent glomerular C5b-9 deposits), or in models of nephrotoxic nephritis, subendothelial IC deposits, anti-mesangial cell membrane antibody-induced nephritis or in two non-immune nephropathies.

The same group of workers went on to show that uC5b-9 was a marker of ongoing immunologic disease activity in PHN (Pruchno 1989). In the heterologous phase, uC5b-9 fell with the cessation of immune deposit formation despite continuing proteinuria. In the autologous phase, uC5b-9 correlated with the amount of antibody in the serum. uC5b-9 excretion resolved when the antibody disappeared despite proteinuria and the persistence of glomerular deposits of antigen, antibody and C5b-9. Glomerular C3 deposits paralleled C5b-9 excretion. Reinitiation of active immune deposit formation by a second injection of anti-Fx1A produced new deposits of C3 and a marked rise in uC5b-9. Complete abrogation of deposit formation by transplanting PHN kidneys into normal rats halted C5b-9 excretion.

Urinary C5b-9 was also elevated in AICN which is a more indolent model of HN, thus more analogous to HMN (Pruchno 1991). Recurrent deposits of C3 were demonstrated when a normal kidney was transplanted into AICN rats with elevated uC5b-9, whereas kidneys placed in AICN rats with comparable proteinuria but no detectable uC5b-9 did not develop subepithelial deposits. Persistent proteinuria in PHN for weeks or months following the cessation of immune disease activity has been well documented (Makker 1989).

The animal models suggest that uC5b-9 reflects the presence of complement-fixing antibodies to GEC and represents a sensitive, dynamic marker of active immune disease and ongoing subepithelial immune deposit formation. In contrast, proteinuria and positive tissue fluorescence for antigen, antibody and C5b-9 persist long after C3 deposit formation ceases and are therefore not suitable for assessing disease activity.

(c) C5b-9 as a non-lethal mediator

Insertion of C5b-9 (termed the MAC) into cell membranes results in rapid lysis of non-nucleated cells such as erythrocytes. Lysis of nucleated cells is more difficult to achieve. In contrast to erythrocytes, one single channel is not sufficient for killing of nucleated cells (Koski 1983). Nucleated cells eliminate MAC by internalising membrane-bound C5b-7, C5b-8 or sub-lethal doses of C5b-9 (Carney 1985). Another possible mechanism was shown to be exocytosis of MAC following vesiculation in rat GEC in vitro (Camussi 1987). A third possibility was the transcytotic route of MAC elimination in the GEC in vivo as described by

Kerjaschki 1989 (detailed above).

Mechanisms have evolved for protecting self against complement-mediated injury. The plasma inhibitor, S-protein or vitronectin, binds to the nascent MAC and prevents polymerisation of C9. This renders the complex inactive and it remains water-soluble (Podack 1978). These complexes are termed serum C5b-9 (SC5b-9). S-protein has been demonstrated in subepithelial immune deposits in HMN (Bariety 1989). Probably most glomerular C5b-9 deposits are inactivated to SC5b-9 deposits which remain extracellularly with the IC. However, a small fraction of the total glomerular C5b-9 deposits succeed in inserting into cell membranes and cause disease manifestations.

In addition to fluid phase inhibitors, membrane regulators inhibit the functional activity of complement at the C3 cleavage step. Inasmuch as C3b is a common constituent of the C5 convertases in both the classical and alternative pathways, these regulators also inhibit C5 activation. Other membrane proteins have the capacity to regulate MAC assembly, at least in part, by inhibiting the binding and polymerisation of C9. A summary of the membrane-associated regulatory proteins which inhibit MAC formation are shown in Table 2. These proteins have been identified in a variety of nucleated cells and have been shown to be functionally active in the GEC (Rooney 1991). These findings suggest that most MACs formed by homologous complement in nucleated cells are inactivated by inhibitory proteins prior to their removal from the cell surface. Decay accelerating factor (DAF), complement receptor type 1 (CR1) and membrane cofactor protein (MCP) show

significant sequence homology and genetic polymorphism. They belong to the same gene superfamily known as regulators of complement activation, their loci being located in the long arm of chromosome 1 (Rey-Campos 1987, Lublin 1988).

Table 2. Membrane-bound MAC inhibitory proteins.

CD	Name	Abbreviation	Authors
-	Homologous restriction factor, C8 binding protein or MAC inhibitory protein	HRF C8bp MIP	Zalman 1986 Schonermark 1986
CD35	Complement receptor type 1	CR1	Fearon 1980
CD46	Membrane cofactor protein	MCP	Seya 1986
CD55	Decay accelerating factor	DAF	Nicholson-Weller 1981
CD59	Membrane inhibitor of reactive lysis	MIRL	Davies 1989

(iv) Cellular effects of C5b-9

As nucleated cells are highly resistant to lysis by homologous C5b-9, a great deal of interest has arisen regarding the non-lethal effects of MAC. There are three possible mechanisms by which MAC attack on the GEC could cause altered glomerular barrier function resulting in proteinuria:-

(a) release of inflammatory mediators by the GEC,

(b) alteration in synthesis or degradation of extracellular matrix (ECM) by the GEC,

(c) change in GEC shape and detachment of the GEC.

(a) Inflammatory mediators

Since MAC has been localised in immune deposits and has been demonstrated in the urine of PHN rats, its potential role as a proinflammatory mediator has been studied. The GEC, the primary target in PHN, suffers nonlytic injury, appearing as effacement of foot processes and vesiculation of the plasma membranes. MAC-induced proteinuria could involve the activation of the GEC with release of potentially toxic inflammatory mediators by the cell itself without cell death (Cybulsky 1986b).

Eicosanoids

At sublytic concentrations of C5b-9, both rat and human mesangial cells in culture have been shown to produce eicosanoids (Lovett 1987). This same effect has been documented in rat GEC (Hansch 1988b). However, the increase in eicosanoid synthesis by GEC may not account for the altered permeability as administration of thromboxane synthesis inhibitor had no effect on C5b-9 induced proteinuria in PHN (Stahl 1987).

Reactive oxygen metabolites (ROM)

A second group of potential mediators of glomerular injury induced by C5b-9 are ROM. Rat and human mesangial cells in culture released ROM following complete MAC formation (Adler 1986). Administration of a hydroxyl radical scavenger, dimethyl sulphoxide (DMSO), decreased proteinuria in PHN (Lotan 1984).

However, no direct effect of C5b-9 on GEC production of ROM has yet been demonstrated.

Cytokines

A third group of potential mediators induced by C5b-9 are the cytokine family. Cytokines could act on cells which secrete them (autocrine) or on nearby cells (paracrine). Sublytic doses of C5b-9 on rat and human mesangial cells induce production of interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) (Lovett 1987), although it is not known whether sublytic doses of C5b-9 can induce GEC production of cytokines.

GEC display receptors for IL-1 (Hansch 1990). Both IL-1 and TNF-α stimulate mesangial cell proliferation (Lovett 1983, Silver 1988). The co-culture of mesangial cells with IL-1 and TNF-α, either alone or in combination, directly stimulates de novo synthesis of several molecules relevant to the inflammatory response in glomerulonephritis. These include a type IV collagenase (Martin 1986), collagen (Foellmer 1986), and prostaglandin endoperoxide synthetase (Topley 1989). Stimulation of GEC with IL-1 has been shown to increase collagen synthesis (Torbohm 1989). Moreover, both IL-1 and TNF-α induce production of ROM in mesangial cells (Radeke 1990). These biologically functional molecules may play a vital role in the induction of proteinuria.

Interleukin-6 (IL-6) production is induced by a variety of cytokines including IL-1 (Shalaby 1989) and TNF-α (Van Damme 1987). Non-lethal levels of MAC have

been shown to release IL-6 from human rheumatoid synovial cells (Daniels 1990). The effects formerly ascribed to IL-1 are now known to be the result of combined IL-1 and IL-6 action (Shalaby 1989). IL-6 has also been found to act as an autocrine growth factor for rat mesangial cells (Kishimoto 1989).

Glomerular production of transforming growth factor-ß (TGF-ß) in a rabbit model of anti-GBM disease has been associated with the fibrotic stage of the disease, and urinary levels of TGF-ß (Coimbra 1990) were raised. TGF-ß has been shown to promote the accumulation of ECM in sclerosing tissue in vitro (Roberts 1986) and in vivo (Ignotz 1986). Furthermore the production of ECM and progression to sclerosis was prevented in an animal model of mesangial proliferative glomerulonephritis by the administration of an antibody to TGF-ß (Border 1990). GEC are known to express receptors for TGF-ß (MacKay 1989). Attention is now focused on the relevance of TGF-ß to glomerulosclerosis in humans, sclerosis being a poor prognostic feature in membranous biopsies (Ehrenrich 1986).

Thus, activated glomerular cells produce cytokines which may act in an autocrine or paracrine manner. Most of the cell-cell interactions are upregulatory and result in cell proliferation, secretion of inflammatory products and cell detachment (detailed below). This suggests a role for cytokines as major inflammatory mediators in glomerular injury.

Proteinases

The fourth possible group of mediators of glomerular injury would be GBM-degrading proteinases. Enzymes which can digest type IV collagen have been shown to be derived from the GEC (Watanabe 1990) or from glomeruli (Baricos 1990). However, like the ROM and cytokines, proteinases have yet to be shown to be induced by sublytic C5b-9 attack on the GEC.

(b) Change in ECM

By releasing mediators, C5b-9 could produce inflammation even in the absence of infiltrating leucocytes: it is notable that cellular infiltration is not a feature of MN. It is conceivable that after the initial triggering event, inflammation becomes a self-perpetuating process. One consequence of tissue destruction is tissue repair. ECM production could lead to the thickened GBM characteristic of MN. The expansion appears to result primarily from increased deposition of ECM along the outer surface of the GBM, in the form of spike-like projections of the GBM between the GEC. Some studies suggest that collagen type IV is a principal component of the expanded GBM (Kim 1991). Human GEC are known to produce collagen type IV under normal conditions (Killen 1979). C5b-9 has been shown to alter collagen synthesis in cultured GEC (Torbohm 1990). TGF-ß is able to stimulate the expression of protease inhibitors so that enzymes which degrade ECM are decreased (Edwards 1987). GEC respond to immunologic injury by altered synthesis of ECM components. The thickened GBM then exhibits persistent abnormal permeability to proteins and may lose ultrafiltration properties as well (Shemesh 1986, Schneeberger 1979).

(c) Change in GEC shape

In MN there are changes in GEC shape with loss of interdigitating foot processes (Heptinstall 1983). Evidence suggests that cells maintain close contact with their surrounding ECM and with each other via specialised cell surface receptors called integrins (Hynes 1987). Specific members of this family of integrins ,$\alpha_3\beta_1$ and $\alpha_v\beta_3$, have been demonstrated on human GEC (van Goor 1991). Integrins such as vitronectin receptors ($\alpha_v\beta_3$) control cell shape, motility and adhesion to other cells and to the substratum (Burridge 1988). TGF-ß has been shown to upregulate expression of β_1 (Heino 1989a) and $\alpha_v\beta_3$ integrin (Ignotz 1989). In certain cell types, TGF-ß alters the characteristic integrin expression, resulting in a measurable loss of adhesion to matrix (Heino 1989b). With detachment of the GEC from the GBM, proteinuria may ensue. Currently no data is available to show that sublytic C5b-9 attack alters integrin expression on the GEC.

In summary, a dual role of C5b-9 has been proposed. C5b-9 may stimulate the release of inflammatory mediators in the initiation of glomerular injury. Secondly, by releasing the same mediators and by inducing collagen synthesis, C5b-9 may contribute to the progression of the acute glomerular injury leading to chronicity and eventually to sclerosis as well as GEC detachment resulting in proteinuria. The studies reviewed provide evidence that the C5b-9 portion of the complement system remains the central player in the mediation of glomerular disease. The subsequent effector mechanisms for induction of proteinuria following complement activation remain speculative. Although subepithelial C5b-9 deposition is seen in many models of MN, uC5b-9 excretion appears to be unique to PHN (where the initiating antigen

is an integral part of the GEC). Moreover uC5b-9 seems to reflect dynamically the continuing IC deposition at ultrastructural level.

Table 3 summarises the mediators released following glomerular cell activation by sublytic doses of C5b-9.

Table 3. Mediator release from cells and altered protein expression in cells stimulated by sublytic doses of C5b-9.

Mediator	Glomerular mesangial cell	Glomerular epithelial cell
Eicosanoids	+	+
Reactive oxygen metabolites	+	?
Cytokines	+	?
Proteinases	?	?
Collagen type IV	?	+
Integrins	?	?

B. HUMAN MEMBRANOUS NEPHROPATHY (HMN)

(i) Mechanisms of immune complex formation

No human equivalent of the Heymann autoantibody has been identified so far. There is evidence for polyclonal B cell activation in systemic lupus erythematosus, chronic

bacterial or parasitic infections (Levy 1986), drug-induced glomerulopathy (Fillastre 1988) and bone marrow transplant recipients (Barbara 1992). There is no convincing evidence of circulating IC which then get deposited in the glomeruli. One possible mechanism in the human disorder is via antigen(s) planted in the lamina rara externa, their passage being facilitated by electrostatic interaction with fixed glomerular polyanion on the GBM. Such an example could hold true for carcinoma-associated MN in humans (Brenchley 1992a). These planted endogenous antigens then become the focus for an autoimmune response resulting in IgG deposition.

Several attempts have been made to apply the observations in HN to IMN. The nephritogenic antigen of HN is gp330. A human analogue of gp330, gp400, was similarly localised in human proximal tubules but was absent from human GEC (Kerjaschki 1987b). A separate molecule of M_r about 40,000 was shown to be associated with the gp330 in the rat kidney to form the HN antigenic complex. This 40 kD protein showed 73% sequence homology with the low-density lipoprotein receptor-related protein (Raychowdhury 1989) and with the α_2-macroglobulin receptor-associated protein (Herz 1991). Antibodies specific to the nephritogenic epitope in the 40 kD subunit when injected into rats resulted in subepithelial deposits (Kerjaschki 1992). The α_2-macroglobulin receptor-associated protein (α_2-MRAP) has not been identified in the human glomerulus or GEC. The general consensus is that Heymann-like antigens are unlikely to be the candidates for antibody deposition in HMN (Brentjens 1989). Since MN is not a homogenous disease, it is quite possible that there is no universal antigen in HMN comparable to gp330 in rats.

(ii) Clinical course

The clinical course in IMN is variable (Honkanen 1986) with regards to proteinuria and to prognosis. It may result in (a) full recovery, (b) improvement leading to persistent non-nephrotic proteinuria, (c) static disease, (d) deteriorating renal function leading eventually to ESRF or (e) remitting-relapsing disease. The variability of the clinical course suggests that IMN may not be a homogenous entity. In a meta-analysis of 648 patients, after a follow-up period ranging from 4 to 14 years, 10% had renal death, another 5% died from other causes and 17% had renal insufficiency (Honkanen 1992).

Our ability to identify high risk patients at the time of diagnosis is far from satisfactory. The identified clinical risk factors predicting progression in MN are impaired renal function, heavy proteinuria, adult age, male sex, hypertension and the presence of tubulointerstitial damage on biopsy. However, there is still no measurement of immune activity in HMN to identify high risk patients.

(iii) Treatment

Since 20% - 50% of adults with nephrotic syndrome due to IMN go into ESRF within 10 years (Honkanen 1992), treatment would seem to be worthwhile providing side effects from therapy were minimal. It is also relevant to consider that persistent and progressive renal disease have significant non-renal morbidity.

(a) Who should we treat?

There is general agreement that nephrotic IMN should be treated if one could

identify those individuals who have progressive disease. Such patients cannot be identified at presentation other than from the broad clinical categories mentioned above.

(b) When should we treat?

If treatment is to be effective, it should probably be initiated early so that any window of opportunity is not missed (Fuiano 1989). In current practice this may mean more accurate measurement of GFR by e.g. isotopic assessment, a technique which is expensive and labour intensive.

(c) What should we use?

(d) How long should we treat?

(e) What end-points should we use?

These three questions will be discussed together as they are intrinsically linked. Currently used regimens carry risks. If progression to renal failure is to be halted and morbidity minimised, ideally treatment should be tailored to pathogenesis. Moreover more than one injurious process may be present at any one time. The results of some controlled trials of various therapeutic options are analyzed below, in an attempt to answer whether treatment would affect outcome. The therapeutic regimens used are:-

(i) prednisolone alone (Tables 4 and 5) - adapted from Cameron 1992

(ii) prednisolone and chlorambucil (Tables 6 and 7) - adapted from Cameron 1992

(iii) prednisolone and cyclophosphamide (Tables 8 and 9)

Table 4. Controlled trials of steroid treatment in adult patients with idiopathic membranous nephropathy.

Study	Drug	Dosage	Duration of Rx
Collaborative Study, USA (Coggins 1979)	Prednisone	125 mg alt days	8 weeks
Toronto Glomerulo-nephritis Study Group (Cattran 1989)	Prednisone	45 mg/m^2 alt days	6 months
Medical Research Council MRC, UK (Cameron 1990)	Prednisolone	125 mg alt days	8 weeks

Table 5. Results of steroid treated group; post-treatment status of nephrotic patients.

Study	Follow-up (months)	Trial n	Complete remission n	Proteinuria n	ESRF n	p
USA	26 - 52	C 38	5[1]	22	11	
		Rx 34	7[4]	25	2	<0.05
Toronto[a]	48 ± 3	C 54	20[4]	28	6	
		Rx 54	23[8]	24	7	>0.05
MRC	52 ± 6	C 51	7	29	15	
		Rx 52	12[7]	31	11	>0.05

[a]Only patients who had proteinuria >3.5 g/24h are considered.
Patients who died of incidental causes or who were lost to follow-up are considered as at their last observed renal status.
C, control; Rx, treatment group; ESRF, end-stage renal failure (serum creatinine >400 μmol/l); Superscript = number of patients who subsequently relapsed.

Treatment was more prolonged in the Toronto trial but the dose was higher in the USA and UK trials (Table 4). Only the USA trial showed benefit of treatment with steroids, the other 2 trials concluded otherwise (Table 5).

Table 6. Controlled trials of steroid and chlorambucil (chloramb) treatment in adult patients with idiopathic membranous nephropathy.

Study	Drug	Dosage	Duration of Rx
Italian I (Ponticelli 1984 & 1989)	Oral methylpred alternating monthly with	0.4 mg/kg/day	4 weeks x 3
	oral chloramb & monthly injections	0.2 mg/kg/day	4 weeks x 3
	of 3g IV methylpred		6 months
Italian II (Ponticelli 1992)	Italian I v.		
	Oral methylpred & monthly injections	0.4 mg/kg/day	6 months
	of 3g IV methylpred		6 months

Table 7. Results of steroid and chlorambucil (chloramb) treated group; post-treatment status of nephrotic patients.

Study	Follow-up (months)	Trial n	Complete remission n	Proteinuria n	ESRF n	p
Italian I	60	C 39	7[5]	28	4	
		Rx 42	23[4]	18	1	<0.05
Italian II	39 ± 15	C 45	17[6]	?	2	
		Rx 47	20[6]	?	0	>0.05

Abbreviations as in Table 5.

Both these trials had chlorambucil in one arm but the control group in trial I received only placebo, whilst the control group in trial II received steroids alone (Table 6). The steroid and chlorambucil regimen proved beneficial (Table 7). The behaviour of the control group however is noted to be different from other trials. More patients went into ESRF in the control groups of the Collaborative Study, USA and the MRC, UK trials whereas very few did so in the control groups of both the Italian trials.

Table 8. Controlled trials of steroid and cyclophosphamide (cyclo) treatment in adult patients with idiopathic membranous nephropathy.

Study	Drug	Dosage	Duration of Rx
Toronto (West 1987, Jindal 1992)	Prednisone	125 mg alt days	20 ± 4 months
	v.		
	Prednisone	125 mg alt days	7 ± 2 months
	& oral cyclo	2 mg/kg/day	23.3 ± 4.7 months
Glomerular Disease Collaborative Network, USA (Falk 1992)	Prednisone	2 mg/kg alt day	8 weeks
	v.		
	IV methylpred	7 mg/kg/day	3 days
	& Prednisone	1 mg/kg alt day	2 months
	& monthly IV cyclo	0.5 g/m^2/day	6 months

Table 9. Results of steroid and cyclophosphamide (cyclo) treated group; post-treatment status of nephrotic patients.

Study	Follow-up (months)	Trial n	Complete remission n	Proteinuria n	ESRF n	p
Toronto[a]	64 ± 7	C 15	1[1]	6[2]	8	
	83 ± 13	Rx 6	3[2]	2[1]	1	<0.05
USA[b]	29.2 ± 17.1	C 13	?	?	4	
		Rx 13	?	?	4	>0.05

[a]Only patients who had steroid treatment are considered in the control group; only patients who had both steroid and cyclophosphamide are considered in the treatment group. All patients had renal insufficiency before treatment.
[b]It is unclear from the data how many patients had proteinuria >3.5 g/24h, but there were at least 4 in each arm. The other 9 in each arm had renal insufficiency ± proteinuria >3.5 g/24h.
Abbreviations as in Table 5.

All patients in the Toronto trial had significant proteinuria and treatment was more prolonged (Table 8). They had renal insufficiency (135 - 180 μmol/l) before the commencement of oral cyclophosphamide. The patients in the USA trial had a mean serum creatinine of 230 μmol/l before treatment with intravenous cyclophosphamide. However, it is unclear from the data how many of the patients had proteinuria >3.5 g/24h, but there were at least 4 such patients in each arm. The control groups in both trials received steroids. These 2 trials reached different conclusions (Table 9).

In the light of conflicting data, the clinician is no better informed. The length of treatment in different protocols varied. The end-points chosen were proteinuria and

renal function. Such discrepant results may have arisen because there is no marker of ongoing immunological injury and we are obviously treating some patients with inactive disease in addition to those who may have remitted spontaneously. Thus the clinician's dilemma of who to treat, when to treat, what to treat with, how long to treat for and what tool to use to monitor the efficacy of treatment remains unresolved.

(iv) Role of C5b-9

The membranous lesion, characterised by the granular IgG deposits exclusively in the subepithelial site, lacks inflammatory cell infiltrates, yet is associated with marked increase in capillary wall permeability and proteinuria. This is in marked contrast to the anti-GBM antibody deposition where a prominent neutrophil infiltrate is seen in glomeruli within 15 to 30 minutes and the proteinuria which ensues is proportional to the number of neutrophils in glomeruli. Proteinuria can be essentially abolished by neutrophil depletion. Generalised complement depletion abolishes the neutrophil infiltrate and can greatly reduce proteinuria (Cochrane 1965). This is an example of complement-neutrophil mediated glomerular injury.

Besides HMN, complement activation is thought to be an essential part of the immune pathogenesis in IgA nephropathy (Tomino 1981). With deposits in the mesangium, the mesangial matrix expands and there is mesangial proliferation but no neutrophil infiltrate. There is a direct effect of complement, independent of neutrophils, although IgA and HMN differ in their intrarenal site of IC deposition. High levels of C3d fragments in the plasma but not in the urine of patients with IgA

nephropathy has been reported, on the contrary high levels of complement products in the urine but not in the plasma of patients with IMN has been noted (Brenchley 1992b). With mesangial complement deposition, C3dg with a M_r of 43,000 could have immediate access to the capillary lumen whereas C5b-9 with a $M_r > 1x10^6$ is likely to be retained in the circulation and would not appear in the urine in IgA nephropathy. A further study from the same group has shown that patients with IMN who excrete C5b-9 had a worse clinical outcome (Coupes 1993a). Schulze (1991) also reported elevated uC5-9 in a subset of patients with IMN (28%) and lupus MN.

(v) Role of inflammatory mediators

To date there has been no evidence to link the non-lethal effects of C5b-9 on HMN with the production of inflammatory mediators, in particular the cytokines.

C. AIMS OF RESEARCH REPORTED HERE

The key to understanding the immunological mechanism(s) in IMN must be in the study of those patients in whom an immunologically active lesion is present, most likely in the early or relapsing phases of the disease. Sequential uC5b-9 have been analyzed in all IMN patients and these results will be correlated with the evolving clinical course of the patients. The primary aim is to determine the reliability of uC5b-9 as a marker of active immunological insult in HMN.

The cytokines, TNF-α, IL-1ß, IL-6 and TGF-ß, have been measured in paired plasma and urine samples at different stages of the disease. The sequential pattern of this cytokine expression in relationship to uC5b-9 may aid our understanding of

the cellular effects of C5b-9 on the GEC in vivo.

Patients who were given immunosuppression drugs for this disorder were studied. The effect of different drug regimens (prednisolone, cyclophosphamide and chlorambucil) on the marker, uC5b-9, will be correlated with the clinical outcome of these patients. This may also help to determine whether a particular treatment regimen is more efficient in suppressing the immune activity in HMN.

The qualitative nature of the uC5b-9 has been studied in an attempt to show whether the transcytotic mechanism of C5b-9 removal, as shown in PHN, operates in the human GEC in vivo. Characterisation of the uC5b-9 may identify possible pathogenic antigens in HMN which have already been described in the GEC of PHN rats besides gp330 (ie CD10 and CD26). Furthermore, characterisation of uC5b-9 may help our understanding of how the GEC handles C5b-9 in vivo with respect to its membrane-associated MAC inhibitory proteins and to the fluid-phase inhibitors. The differences in expression of these proteins in individuals may explain the variable clinical course in HMN. Upregulation of these proteins may prevent the non-lethal effects of C5b-9 and therefore some patients may follow a benign clinical course. Table 10 summarises the aims of this research on patients with IMN.

Table 10. Aims of this research in human idiopathic membranous nephropathy.

1. To develop an in-house ELISA to measure uC5b-9
2. To investigate uC5b-9 as a marker of active immunological insult
3. To analyze the effect of different immunosuppression regimens on uC5b-9
4. Characterisation of uC5b-9
5. Correlation of cytokine expression with uC5b-9

CHAPTER 2

MATERIALS AND METHODS

A. MATERIALS

Unless stated otherwise, all chemicals were from BDH Chemicals or Sigma Chemical Co. All buffers used in fast protein liquid chromatography (FPLC) and preparation of MAC contained 0.05% sodium azide.

B. PATIENTS

(i) Patient selection

The study period was from 1984 to March 1993. All adult patients who had MN diagnosed by renal biopsy and were under the care of Manchester Royal Infirmary during this period were eligible for consideration.Those included had no clinical, laboratory or histologic evidence of underlying systemic disease or occult malignancy, had not been exposed to proven glomerulotoxic drugs and did not have diabetes mellitus. Laboratory criteria for exclusion were a positive status for antinuclear or anti-DNA antibody or hepatitis B surface antigen, a positive VDRL test, or a low level of C3 or C4. Mid-stream urine specimens were sterile. Patients with signs or symptoms suggestive of renal vein thrombosis were excluded. Renal venography was not, however, a requirement in those in whom renal vein thrombosis was not suspected. Specific enquiry was undertaken to exclude exposure to heavy metals and drugs as potential aetiological agents. Patients treated with immunosuppressive agents other than prednisolone in the twelve months before entry were excluded from the study. The duration of disease before the biopsy was not considered.

(ii) Renal histology

A renal biopsy had been undertaken in all patients and had to contain adequate glomeruli for examination by light and immunofluorescence microscopy to allow a confident diagnosis. The diagnosis of MN was made using the criteria of Churg (1965). The biopsies were not staged. Electron microscopy had only been performed in biopsies where the diagnosis remained unclear after light microscopy and immunofluorescence staining.

(iii) Study design

The history and physical examination, recorded in full at the initial visit, were all reviewed and confirmed by the author in the outpatients clinic during the course of the study wherever possible. All haematological, biochemical and serological investigations were performed using standard techniques. Patients with stable renal function (serum creatinine and proteinuria) were seen at four monthly intervals, others more frequently as indicated by their clinical status. Patients with declining renal function were seen more often. Paired plasma and urine samples were collected at each visit. Measurements of proteinuria, serum albumin and creatinine were made at each visit.

Patients with stable renal function were not treated with immunosuppression unless they were severely nephrotic (proteinuria $>$ 10 g/24 hr and serum albumin $<$ 25 g). These patients who received treatment were not included in the study as the data analysis for response to treatment was based on patients who had declining renal function during the study period.

Patients with declining renal function during the study period were considered for immunosuppression treatment. If no treatable or self-limiting causes, such as renal vein thrombosis (Wagoner 1983), were found and no contraindication to immunosuppression present, the patient's consent was obtained. They were all rebiopsied within twelve months before the start of treatment to exclude diuretic-induced interstitial nephritis (Jennings 1986) and coincident anti-GBM disease (Pettersson 1984). Biopsies were not routinely performed to assess effect of treatment on histopathology. Patients were assigned in a non-random manner to one of the three treatment groups.

(iv) Treatments

Patients in group I received methylprednisolone, 1 g intravenously daily for five days, followed by a tapering dose of oral prednisolone over eight months. The doses were 100 mg, 75 mg, 50 mg, 25 mg, 20 mg, 15 mg, 10 mg and 5 mg on alternate days for consecutive four-week periods (Short 1987).

Most experience in Manchester was gained from patients in group I. However from 1989, patients who relapsed on the above regimen and new patients with declining renal function were assigned to groups II and III. Patients in group II received three cycles of treatment with methylprednisolone 1 g intravenously daily for three days and then 0.4 mg/kg/day of oral prednisolone for 27 days. Each cycle was followed by one month of treatment with oral chlorambucil (0.2 mg/kg/day). The total duration of treatment was six months (Ponticelli 1984).

Patients in group III received oral cyclophosphamide (2 mg/kg/day) for two months followed by a tapering dose of oral prednisolone as in group I (West 1987). Some patients received cyclophosphamide and prednisolone concurrently for the first two months, followed by the same tapering dose of prednisolone.

Anti-hypertensive treatment (including angiotensin-converting enzyme inhibitor), diuretics, lipid lowering drugs and anticoagulants were given as clinically indicated in all patients.

(v) Definitions

Declining renal function was defined by a sustained greater than 30% (>30%) rise in serum creatinine during the previous twelve month period. Stable renal function was defined by a maintenance of serum creatinine at a normal ($\leq$110 μmol/l) or impaired level.

Remission in those with declining renal function but who were not given immunoactive drugs was defined as stabilisation of serum creatinine so that the value did not continue to rise >30% by the end of the study period. Response to treatment was defined by an improvement in renal function or stabilisation of serum creatinine so that the value did not continue to rise >30% by the end of the study period. Relapse was defined by a sustained >30% rise in serum creatinine over twelve months despite treatment. ESRF was defined by a serum creatinine >500 μmol/l or dialysis dependency.

(vi) Data analysis

Standard statistical techniques were used throughout the study period as indicated in the results section.

C. COLLECTION OF PLASMA AND URINE SAMPLES

Blood samples were drawn in EDTA and kept at 4°C. The samples were centrifuged at 2,500 rpm for 10 min and aliquots of plasma were stored at -70°C.

Freshly voided urine was collected into EDTA at 4°C, centrifuged, aliquoted and stored at -70°C. The creatinine concentration of the freshly voided urine samples was also determined.

D. ELISAs FOR C5b-9 USING DIFFERENT αC9 CAPTURE ANTIBODIES

(i) Falk assay

This assay was previously described by Brenchley (1992b). It is an enhanced chemiluminescence immunoassay which employs the antibody to C9 neoantigen (gift from Dr RJ Falk 1983), and is reported to bind both SC5b-9 and C5b-9. The mAb was coated at 10 μg/ml in 50 mM carbonate buffer pH 9.6 onto microFLUOR plates (Dynatech) overnight at 4°C. After blocking, a 1/5 dilution of urine was added to the wells and incubated overnight at 4°C. Biotinylated goat anti-C7 (ICN Biomedicals) at 5 μg/ml was left for four hours, followed by avidin-peroxidase (Sigma) diluted 1/200 for two hours. Enzyme substrate was added (Amerlite reagent, Kodak) and the chemiluminescence was detected using a luminometer (Kodak). A standard containing SC5b-9 was prepared by incubating fresh normal

human serum (NHS) with zymosan. Arbitrary units of C5b-9 were read off the standard curve (range = 3 to 100 units). The concentrations of C5b-9 were expressed as a ratio of units per mg of creatinine in the freshly voided urine sample.

(ii) Manchester assay

This assay was developed as part of the project (as detailed in chap 3). It utilised a commercially available mAb (aE11) from Dako Ltd. The IgG mAb was coated at 1 μg/ml in carbonate buffer pH 9.6 onto Immulon plates (Dynatech) overnight at 4°C. The plates were washed twice in phosphate buffered saline with polyoxyethylene-sorbitan monolaurate (PBS and 0.05% Tween 20) and blocked with 2% BSA in PBS for one hour. After washing twice, 1/20 dilution of urine in 2% BSA was added and incubated overnight at 4°C. The plates were washed four times, goat anti-C7 serum (ICN Biomedicals) diluted 1/500 in 2% BSA was added and left for four hours. Peroxidase-conjugated donkey anti-goat IgG (Jackson Immunoresearch Labs) was incubated with an equal volume of mouse serum for 30 min at room temperature as it showed cross-reactivity to the mouse mAb. After further washing, this treated peroxidase-conjugated antibody 1/2000 in 2% BSA was added to the plates and left for two hours. The plates were washed four times and developed for 15 min with 1 mM ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt) in 0.1 M citrate phosphate buffer pH 5.0 containing 0.1 mM hydrogen peroxide. The absorbance at 420 nm was measured using a Titertek Multiskan plate reader (Flow Labs). Dilutions of the zymosan-activated serum were used to construct a linear standard curve over an optical density (OD) range of 0.2 to 1.0 (background 0.1) from which arbitrary units of

C5b-9 were read.

Possible components associated with urine pellets were screened by a variation of this two-site ELISA method, using the Dako mAb as the first antibody and different polyclonal antibodies as the second antibody (see under immunoblotting). This two-site ELISA was reversed to screen the urine pellets with mAbs, using the goat anti-C7 antibody to capture the sample and the different mAbs as the second antibody. Appropriate positive and negative controls were included.

(iii) Quidel assay

The ELISA kit was prepared by Quidel Labs (Kolb 1988). Urine samples diluted 1/5 in assay buffer were added to wells lined with a mAb to human SC5b-9. After incubating for one hour, the wells were washed five times. Goat polyclonal antibody to complement components C6 and C7 labelled with horseradish peroxidase was added for one hour. The wells were washed again five times and chromogen added. After 30 min the light absorbance at 405 nm was measured. A standard curve was generated using known amounts of SC5b-9 provided with the kit.

(iv) B7 assay

This fourth assay was kindly performed by Dr BP Morgan on identical urine samples which were measured in the above three assays. This enabled a comparison to be made of all the assays. The method was previously described by Morgan (1988). Briefly, the first antibody was a mAb B7 and the second antibody was anti-C9 mAb MC-47. The peroxidase-conjugated goat anti-mouse IgG antibody (ICN Biomedicals)

at 1/1000 dilution was followed by the peroxidase substrate (1,2-phenylene diamine dihydrochloride, OPD). After 15 min, extinction at 492 nm was read. A standard curve was developed using inulin-activated serum.

E. ELISAs FOR CYTOKINES

(i) Tumour necrosis factor - α (TNF-α)

This assay was previously described by Lamb (1992). The mAb TNF-α (clone 5-2) was coated onto microFLUOR plates overnight. After blocking, plasma and urine samples were added neat to the wells and incubated overnight. The rabbit anti-human TNF-α antiserum reacted with the mouse mAb. The cross-reactivity was blocked prior to use by a 30 min incubation with an equal volume of mouse serum. This polyclonal antibody was then diluted 1/1000 in 2% BSA containing 10 mM dithiothreitol (DTT) to break up IgM pentamers of rheumatoid factors (rheumatoid factors give false positive results) and left for two hours. The peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Labs) was also preabsorbed with mouse serum in a similar way. It was diluted 1/2500 and left for one hour. The plates were developed with Amerlite substrate and read on the luminometer. The standards were pooled samples of either plasma or urine from TNF-α negative controls spiked with recombinant TNF-α (rTNF-α, British Biotechnology). The working range was 3.9 pg - 1.0 ng/ml.

(ii) Interleukin - 1ß (IL-1ß)

The monoclonal anti-human IL-1ß (Genzyme) was coated onto microFLUOR plates overnight. After blocking, neat samples of plasma or urine were added and incubated

overnight. The rabbit anti-human IL-1ß antiserum (Genzyme) and the peroxidase-conjugated donkey anti-rabbit antibody were preabsorbed with mouse serum as described above. The anti-IL-1ß antibody was left for two hours. The peroxidase-conjugated anti-rabbit antibody was diluted 1/2500 and left for one hour. Amerlite reagent was added and the plates were read on the luminometer. The standards were pooled plasma or urine samples from IL-1ß negative controls spiked with recombinant IL-1ß (rIL-1ß, British Biotechnology). The working range was 30 pg - 2 ng/ml.

(iii) Interleukin - 6 (IL-6)

This enhanced chemiluminescent assay is similar to the TNF-α assay. The monoclonal anti-human IL-6 (clone B-E4 from Nichols Institute) was coated at 2 μg/ml in carbonate buffer pH 9.6 onto microFLUOR plates overnight. After blocking, neat samples of plasma or urine were added and incubated overnight. The rabbit anti-human IL-6 antiserum and the peroxidase-conjugated donkey anti-rabbit antibody were preabsorbed with mouse serum as described above. The anti-IL-6 antibody was diluted at 1/1000 in 2% BSA containing 10 mM DTT and left for two hours. The peroxidase-conjugated anti-rabbit antibody was diluted 1/2500 and left for one hour. Amerlite reagent was added and the plates were read on the luminometer. The standards were pooled plasma or urine samples from IL-6 negative controls spiked with recombinant IL-6 (rIL-6, British Biotechnology). The working range was 30 pg - 2 ng/ml.

(iv) Transforming growth factor - $ß_1$ (TGF-$ß_1$)

This assay also used the enhanced chemiluminescence detection system as described by Coupes (1993b). MicroFLUOR plates were coated with a mAb to active TGF-$ß_{(1,2,3)}$ (Genzyme) at 2.5 μg/ml in carbonate buffer pH 9.6 overnight, followed by blocking with 2% BSA in PBS for one hour. Plasma and urine samples diluted 1/5 were added and incubated overnight. Chicken anti-human TGF-$ß_1$ (British Biotechnology) at 2 μg/ml was added and left for four hours, followed by peroxidase-conjugated rabbit anti-chicken IgG (Jackson Immunoresearch Labs) at 1/2000 dilution for two hours. The plates were developed with Amerlite substrate. The standards were dilutions of pure recombinant TGF-$ß_1$ (rTGF-$ß_1$) from British Biotechnology. The working range was 0.1 - 50.0 ng/ml.

F. COLUMN CHROMATOGRAPHY

(i) Urine collection and solubilization of membrane proteins pelleted in urine

Twenty-four hour urine specimens were collected in buffer containing a mixture of protease inhibitors - 6 mM benzamidine, 8 mM ϵ-aminocaproic acid, 5 mM EDTA, 5 mM Tris/HCl pH 7.5 and 0.05% sodium azide. Urines were pelleted at 25,000 g for ten hours at 4°C in a DuPont Sorvall RC28S centrifuge. The pellets were washed in PBS and centrifuged again at 25,000 g for 30 min. The washed pellets were solubilized in 1% Zwittergent 3-12 (Zwitt from Calbiochem) in PBS for 30 min at 25°C with frequent mixing and recentrifuged to remove undissolved membranes. The resultant supernatant was concentrated to 2-3 ml by ultrafiltration on an Amicon PM-10 membrane. Aliquots were stored at -70°C for gel filtration. Spun urine was also stored at -70°C.

(ii) Gel filtration

Gel filtration was performed using the FPLC system. Peristaltic pumps, glass columns, gels, accessories and ultraviolet (UV) monitor were from Pharmacia. The apparatus was controlled by Pharmacia's 'FPLC Manager' software programme. The fraction collector used was a Gilson FC203. The samples were filtered with a Dynagard 0.2 μm filter before loading onto a 1 x 30 cm superose 6 column (Pharmacia). Superose is a highly crosslinked agarose based matrix. The column was precalibrated using proteins of known molecular weights (mw). All runs were at a flow rate of 0.5 ml/min in borate buffer (5 mM sodium borate pH 8.8, 0.2% Zwitt, 50 mM NaCl) at 20°C. Fractions were collected in a 96-well plate in 300 μl volumes, and assayed for C5b-9 by ELISA using the Manchester assay.

G. PROTEIN ASSAY

Fractions obtained from the superose 6 column after the void peak were pooled, concentrated to 1 ml (on an Amicon PM-10 membrane) and their protein concentration measured using a commercial Bio-Rad DC Protein Assay. This was based on a method described by Lowry (1951). This colorimetric assay can be used for protein concentration following detergent solubilization. It utilises the shift in absorbance from 405 nm to 750 nm of the reduced Folin reagent by copper-treated protein.

Five μl samples were mixed with 25 μl of alkaline copper tartrate solution in sodium dodecyl sulphate (SDS) followed by 200 μl Folin reagent. Dilutions of BSA in 1% Zwitt ranged from 0.2 - 1.4 mg/ml protein. The OD of the wells in a 96-well

ELISA plate (Dynatech) was determined using a Titertek Multiskan plate reader at absorbance 750 nm.

H. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

PAGE was performed in the presence of SDS (SDS-PAGE) using a discontinuous buffer system and methods described by Laemmli (1970). Gel cassette kits, casting and running apparatus and power pack were from Pharmacia. Slab gels of dimension 110 x 75 x 1.5 mm were used. The acrylamide concentration in the resolving gel and stacking gel were 7.5% and 3.5% respectively. The composition of the resolving gel and stacking gel solutions used were (Hames 1990) :-

Stock solution	Resolving gel[a]	Stacking gel[a]
Acrylamide-bisacrylamide (30:0.8)	7.5	2.5
Stacking gel buffer stock [b]	-	5.0
Resolving gel buffer stock [c]	3.75	-
10% SDS	0.3	0.2
1.5% ammonium persulphate	1.5	1.0
Water	16.95	11.3
TEMED	0.015	0.015

[a]Volumes (ml) of various reagents required to make 30 ml of gel mixture
[b]Stacking gel buffer stock : 0.5 M Tris/HCl, pH 6.8
[c]Resolving gel buffer stock: 3.0 M Tris/HCl, pH 8.8

Samples were dissolved in 2 x concentrated sample buffer (4% SDS, 20% glycerol, 125 mM Tris/HCl pH 6.8, bromophenol blue), boiled for 3 min and subjected to electrophoresis for four hours at 200 V on 7.5% SDS gels. The reservoir buffer used was 25 mM Tris, 190 mM glycine, pH 8.3. Molecular weight calibration was performed with prestained SDS- PAGE standards (Sigma).

I. SILVER STAINING

A modification of the silver staining method was used (Heukeshoven 1986).

Fixing solution:	250 ml methanol 100 ml acetic acid Made up to 1 L with distilled water
Incubation solution:	75 ml methanol 17 g sodium acetate 0.5 g sodium thiosulphate 1.3 ml glutaraldehyde (25% w/v)* Made up to 250ml with distilled water
Silver solution:	0.25 g silver nitrate 50 μl formaldehyde* Made up to 250 ml with distilled water
Developing solution:	6.25 g sodium carbonate 25 μl formaldehyde* Made up to 250 ml with distilled water
Stop solution:	3.65 g EDTA $Na_2.2H_2O$ Made up to 250 ml with distilled water

* These reagents were added immediately before the solution was used.

Staining was performed using the following method:-

1. Gel immersed in fixing solution for 30 min.
2. Gel placed in incubation solution for 30 min.
3. Gel washed with three changes of distilled water for 20 min each.
4. Gel placed in silver solution for 40 min.
5. Gel washed briefly in distilled water and placed in developing solution for 15 to 30 min.
6. The developing reaction was stopped by placing the gel in stop solution for 5 to 10 min.

7. All incubation and washing steps were performed with gentle shaking.

J. IMMUNOBLOTTING

After SDS-PAGE, the stacking gel was removed from the resolving gel. The separated proteins were electrophorectically transferred to polyvinylidene difluoride (PVDF) membrane from Micropore Corporation using the method of Towbin (1979). The PVDF membrane was soaked in 100% methanol and then washed in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol). Electroblotting was performed by the Novablot transfer apparatus (LKB) and a constant current of 1 mA per cm^2 of gel surface area was applied for four hours.

The remaining protein binding sites were blocked with 2% BSA in PBS and 0.2% Tween 20 for one hour. After washing with PBS, the unlabelled polyclonal or monoclonal antibodies were incubated with the membrane for two hours in order to characterise and identify components which were associated with the C5b-9 in urine pellets. The complement components C6, C7, C8 and C9 were identified by goat antisera (ICN Biomedicals), whilst C5 was identified by rabbit antisera (Dako Ltd). Polyclonal antibodies used were rabbit anti-vitronectin (a gift from Dr BP Morgan and another from Calbiochem), rabbit anti-CD59 (MIRL, a gift from Dr BP Morgan), rabbit anti-$ß_3$ integrin (Chemicon) and goat anti-clathrin (Sigma). MAbs used were anti-$ß_1$ integrin (Chemicon), anti-CD10 (CALLA, Dako Ltd), two anti-CD35 (CR1, C3b receptor - from Dako Ltd and from Serotec), anti-CD46 (MCP, Serotec), two anti-CD51 (anti-$\alpha_v ß_3$ integrin, vitronectin receptor, a gift from Dr D Cheresh and another purchased from Chemicon), anti-CD55 (DAF, Serotec), and

anti-CD59 (Davies 1989, a gift from Dr BP Morgan).

Bound antibodies were detected after incubation with the appropriate peroxidase-conjugated second antibody (Jackson Immunoresearch Labs) for a further two hours. This was followed by the peroxidase substrate 3,3'-diaminobenzidine tetrachloride dihydrate (DAB) at 1 mg/ml and 0.1% hydrogen peroxide (30% w/v) in PBS. The staining was stopped by washing the membrane in water. The membrane was washed with PBS between steps.

K. ENHANCED CHEMILUMINESCENCE FOR WESTERN BLOT ANALYSIS

Enhanced chemiluminescence (ECL) was used in the Western blotting system for the detection of the CD59 antigen. The method is similar to the immunoblotting described above except for several variations. The acrylamide concentration in the resolving gel was 15% as the M_r of the CD59 antigen is 20kD only. The anti-CD59 mAb (gift from Dr BP Morgan) at 1/2000 dilution was incubated with the PVDF membrane for two hours. The bound mAb was detected after incubation with the peroxidase-conjugated anti-mouse antibody (Jackson Immunoresearch Labs) for a further two hours. This was followed by immersing the PVDF membrane in Amersham ECL reagent for one minute. The blot was then covered with Hyperfilm-ECL for 15 minutes and developed. The membrane was washed in PBS between steps.

The markers used were low mw biotinylated standards (Bio-Rad). Marker tracks were developed by incubating the blot with peroxidase-conjugated avidin at 1/5000

dilution for one hour. After washing three times in PBS, the blot was photographed (exposure time = 5 sec).

L. PREPARATION OF MAC FROM ERYTHROCYTE MEMBRANES

This was based on the work described by Ware (1981). Sheep blood (150 ml) containing 11 x 10^{11} erythrocytes was washed in complement fixation test diluent (CFD from Oxoid, Unipath) and then centrifuged at 2,500 rpm for 5 min to pack the cells. The erythrocytes were sensitized with the haemolytic serum Amboceptor 6000 (Behring) diluted 1/2 in PBS. After 30 min incubation at 4°C, the cells were washed in CFD and spun at 2,500 rpm. The sensitized sheep erythrocytes were incubated with 1800 ml of a 1/6 dilution of fresh NHS (300 ml NHS in 1500 ml CFD) at 37°C for 60 min with frequent mixing.

The complement lysed erythrocyte membranes were pelleted at 27,000 g for 30 min at 4°C. The membranes were washed three times in membrane washing buffer (5 mM sodium borate pH 8.8, 10 mM EDTA, 1 mM PMSF). The washed membranes were solubilized in 1% Zwitt in membrane washing buffer at 25°C for 30 min with frequent mixing. After sedimentation to remove undissolved membrane (27,000 g, 30 min, 4°C), the supernatant was concentrated to 15 mls (Amicon PM-30 membrane) and filtered (ICN Flowpore 0.22 μm filter) before gel filtration. Gel filtration was performed on the superose 6 column as described above. Fractions were assayed for C5b-9 by the Manchester ELISA and the fractions after the void peak were pooled, concentrated to 15 ml (Amicon PM-10 membrane) and the protein concentration measured using the Bio-Rad DC Protein Assay.

CHAPTER 3

COMPARISON AND DEVELOPMENT OF C5b-9 IMMUNOASSAYS

A. STABILITY OF URINARY C5b-9

The potential usefulness of uC5b-9 measurement in the assessment of IMN depends firstly on the effects of collection and storage conditions on uC5b-9 levels. In order to determine conditions which would validate the measurement of C5b-9, the effects of temperature, storage time and freeze-thawing on uC5b-9 levels were studied.

(i) Effect of temperature on uC5b-9 levels

To test for in vitro production or degradation during the period after collection but before storage, the following experiment was conducted. Urine samples were collected in EDTA and protease inhibitors and left at 4°C, room temperature (20°C) and 37°C. Aliquots of the same urine were taken at various time points. The urine aliquots were stored at -70°C until time of analysis. All the urine aliquots were then measured together in the Falk assay (see methods).

Figure 5 shows the results from serial aliquots taken from urine allowed to stand at 4°C, 20°C and 37°C. No significant difference in uC5b-9 levels between 0 - 7 days was seen at 4°C and 20°C. However at 37°C, uC5b-9 was lower at 24 hours post-incubation.

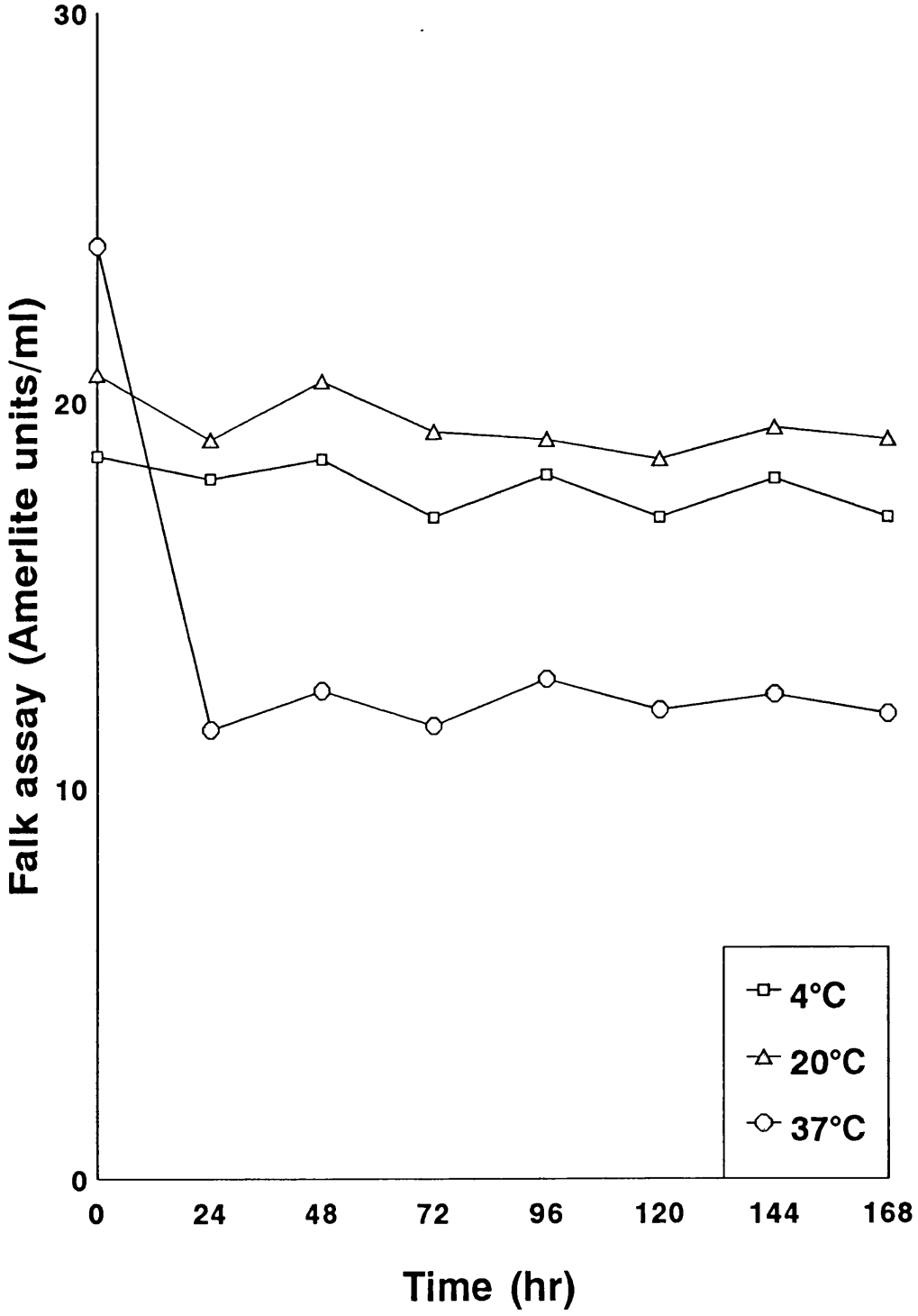

Fig. 5. Effect of temperature on urinary C5b-9 levels when allowed to stand for varying lengths of time.

(ii) Effect of storage time on uC5b-9 levels

To test for in vitro production or degradation during storage, the following experiment was conducted. Aliquots of the same urine collected in EDTA, protease inhibitors and sodium azide were stored at 4°C, -20°C and -70°C for various lengths of time. At the end of each time point, the urine aliquots stored at the 3 different temperatures were measured together using the Falk assay.

Figure 6 shows the results from the urine aliquots stored for various lengths of time. Urinary C5b-9 levels measured in the same urine specimens remained stable over a two year period at the three different storage temperatures.

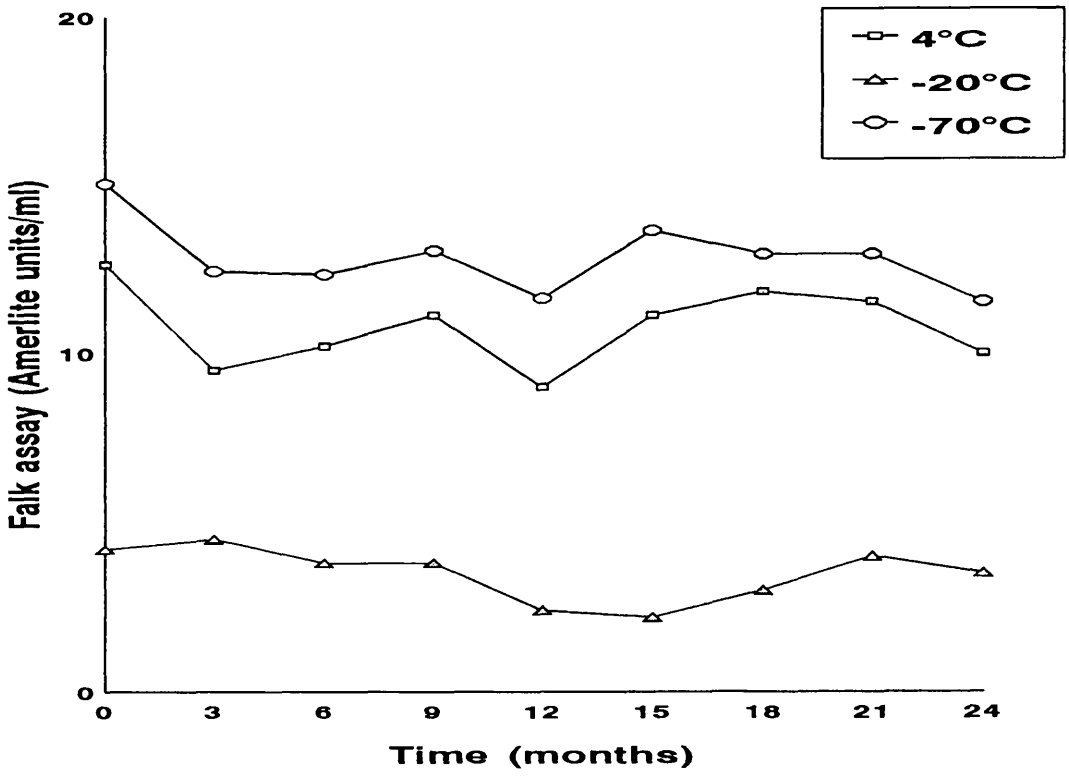

Fig. 6. Effect of time on urinary C5b-9 levels when stored at varying temperatures.

(iii) Effect of freeze - thaw on uC5b-9 levels

The effect of four cycles of freeze-thawing on uC5b-9 levels was studied. At each thaw, an aliquot of the same urine was taken and stored at -70°C until time of analysis. The urine aliquots were measured together using the Falk assay.

Figure 7 shows the results of serial urine aliquots taken after each freeze-thaw cycle. Freeze-thawing did not artefactually raise uC5b-9.

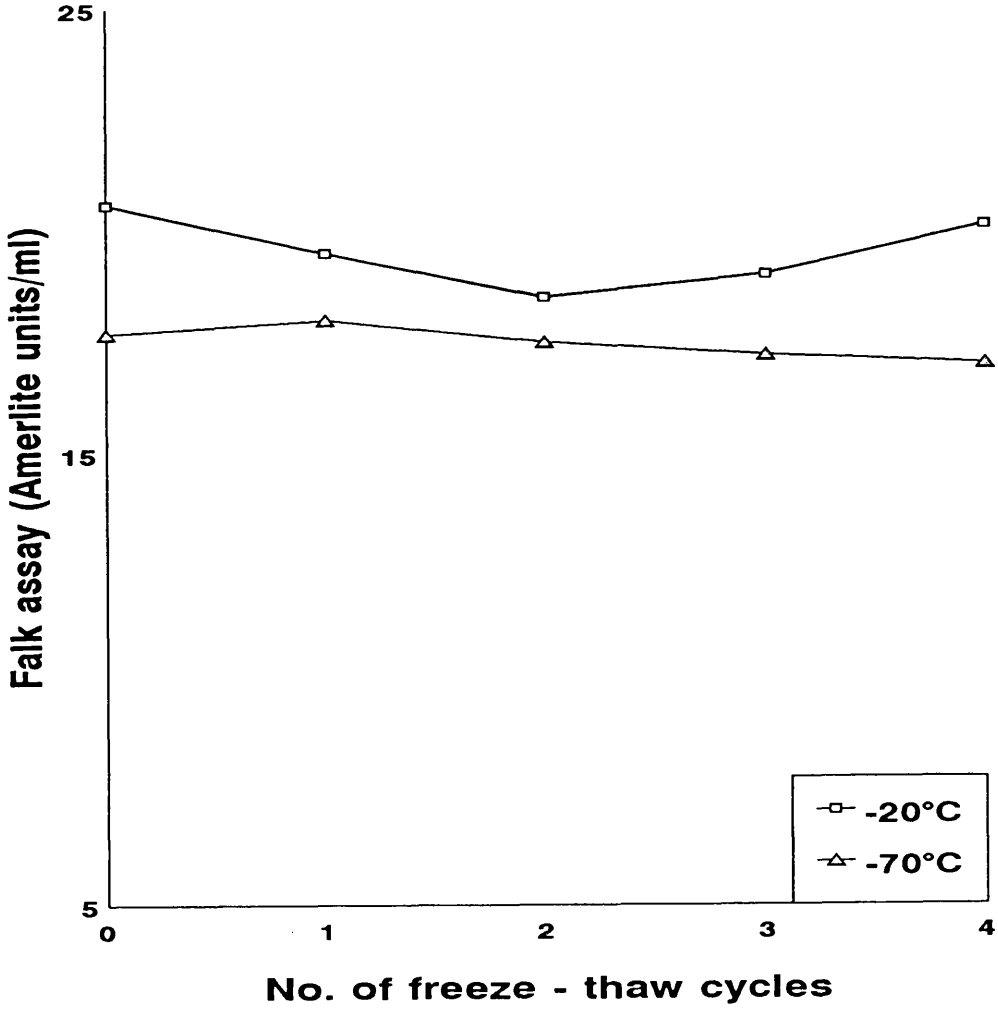

Fig. 7. Effect of freeze - thaw on urinary C5b-9 levels.

(iv) Discussion

These studies show that C5b-9 is a stable molecule. Neither prolonged incubation of urine at room temperature nor repeated freeze-thawing affected the levels of uC5b-9. This is in contrast to that observed by Ogrodowski (1991) where freezing urine at -70°C raised uC5b-9 levels. Stability of uC5b-9 at room temperature documented even at 7 days overcomes the variability in time between specimen collection and storage, an inherent problem in clinical practice. The usual temperature for storage of biological specimens is -70°C. C5b-9 levels remained constant in urine specimens which have been stored over a period of 2 years.

B. <u>DIFFERENT C5b-9 IMMUNOASSAYS</u>

Sensitive ELISAs, based on neoepitope-specific mAbs which are able to detect C5b-9 in plasma, have been described (Falk 1983, Mollnes 1985, Kolb 1988, Morgan 1988, Wurzner 1991). Recently these assays have been shown to represent a valuable tool to assess C5b-9 concentrations in urine. Schulze (1991) reported that uC5b-9 excretion was elevated in a subset of IMN or lupus MN. Brenchley (1992b) has shown that uC5b-9 excretion is associated with disease activity in IMN and is only rarely present in other nephropathies. This study employed the Falk assay. However Ogrodowski (1991) have found uC5b-9 in other non-MN patients and this was related partly to proteinuria. This study employed the Quidel assay. Kusunoki (1991) also reported the presence of uC5b-9 in almost all patients with heavy proteinuria. If highest uC5b-9 levels are seen in patients with heavy proteinuria, this would then invalidate the clinical usefulness of uC5b-9 in determining immunological activity of IMN in humans. To investigate this apparent discrepancy, three parallel

immunoassays were run. The role of the different reagents employed in these assays were then investigated in an attempt to provide an answer to the discrepancies noted.

(i) Comparison of 3 immunoassays

38 urine samples were measured. There were:-

(i) 11 samples with high uC5b-9 levels measured by assay A (11 MN patients),

(ii) 11 samples with no uC5b-9 measured by assay A (obtained from 6 MN patients),

(iii) 11 samples as proteinuric controls (obtained from 8 non MN patients),

(iv) 5 normal samples (5 healthy controls).

The same urine samples were run simultaneously in 3 different immunoassays by a single person. The assays were:-

(A) Falk assay - detailed under methods,

(B) Quidel assay - ELISA kit prepared by Quidel Labs, detailed under methods,

(C) Dako assay - assay as in assay (A) but employing a mAb to C5b-9 (Dako Ltd) at a coating concentration of 1 μg/ml, the IgG fraction having been isolated from a 1 ml protein G column (Pharmacia).

There was good correlation between assays A and C (Kendall's rank $p<0.001$), but not between assays A and B across all proteinuric groups (Table 11). There was no correlation of proteinuria with uC5b-9 levels in any of the 3 assays (Kendall's rank $p>0.05$, $n = 33$). Figure 8 shows the comparison of uC5b-9 levels between assays A and B for all the patient groups (Kendall's rank $p>0.05$, $n = 38$) whilst Figure

9 shows the comparison between assays A and C (Kendall's rank $p < 0.001$, $n = 38$).

These results confirmed the discrepancy in the measurement of uC5b-9 between the Quidel assay (employed by Ogrodowski 1991) and the Falk assay (employed by Brenchley 1992b). However, there was good correlation between the Falk assay and the Dako assay. Proteinuria did not correlate with uC5b-9 levels in all three assays in this study. This is in contrast to that reported by Schulze (1991) and Ogrodowski (1991). Since heavy proteinuria does not affect the level of uC5b-9, uC5b-9 could be a useful marker of immunological activity in IMN because proteinuria has not been shown to reflect disease activity. In an attempt to explain the cause of the discrepancy in the measurement of uC5b-9, the different reagents employed in these assays were then investigated further.

Table 11. Proteinuria in membranous and non-membranous patients, and statistical analysis of uC5b-9 levels in the four patient groups between the 3 immunoassays.

	Group I n = 11	Group II n = 11	Group III n = 11	Group IV n = 5	All Groups n = 38
P'uria (g/24hr)	11.2	9.8	11.8	0	9.5
Assay A v Assay B	n.s	n.s.	n.s.	n.d.	n.s.
Assay A v Assay C	$p<0.001$	$p<0.001$	$p<0.05$	n.d.	$p<0.001$

Group I - MN patients with high uC5b-9 measured by assay A
Group II - MN patients with no uC5b-9 measured by assay A
Group III - non MN patients as proteinuric controls
Group IV - healthy controls

Assay A - Falk assay
Assay B - Quidel assay
Assay C - Dako assay

n.s., $p>0.05$ (Kendall's rank); **n.d.**, not done as all values in assay A were zero.

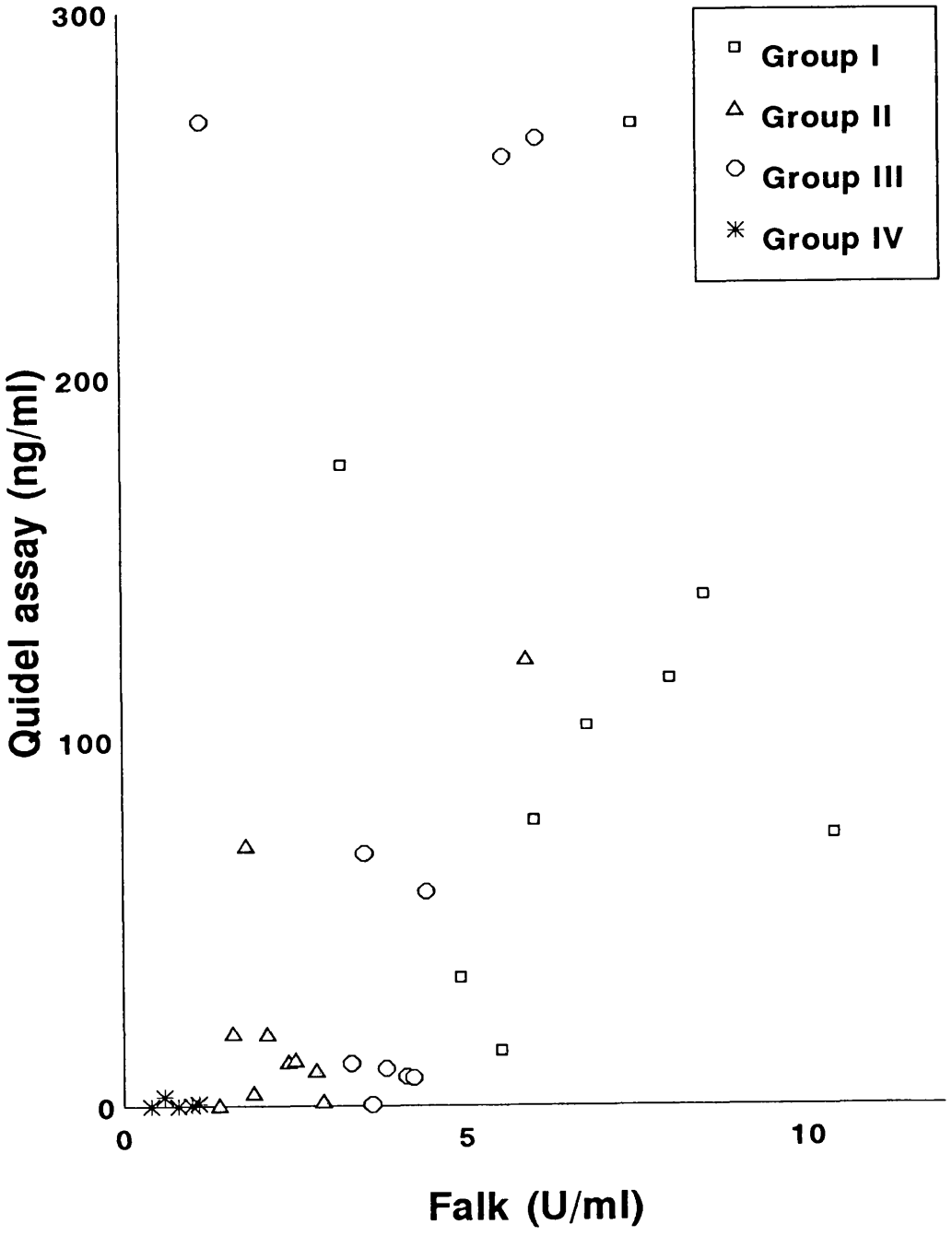

Fig. 8. Comparison of uC5b-9 levels in Falk versus Quidel assay for all patient groups. Kendall rank correlation coefficient tau=0.43, $p>0.05$, n.s.(n = 38).
Group I - MN patients with high uC5b-9 measured by assay A
Group II - MN patients with no uC5b-9 measured by assay A
Group III - non MN patients as proteinuric controls
Group IV - healthy controls

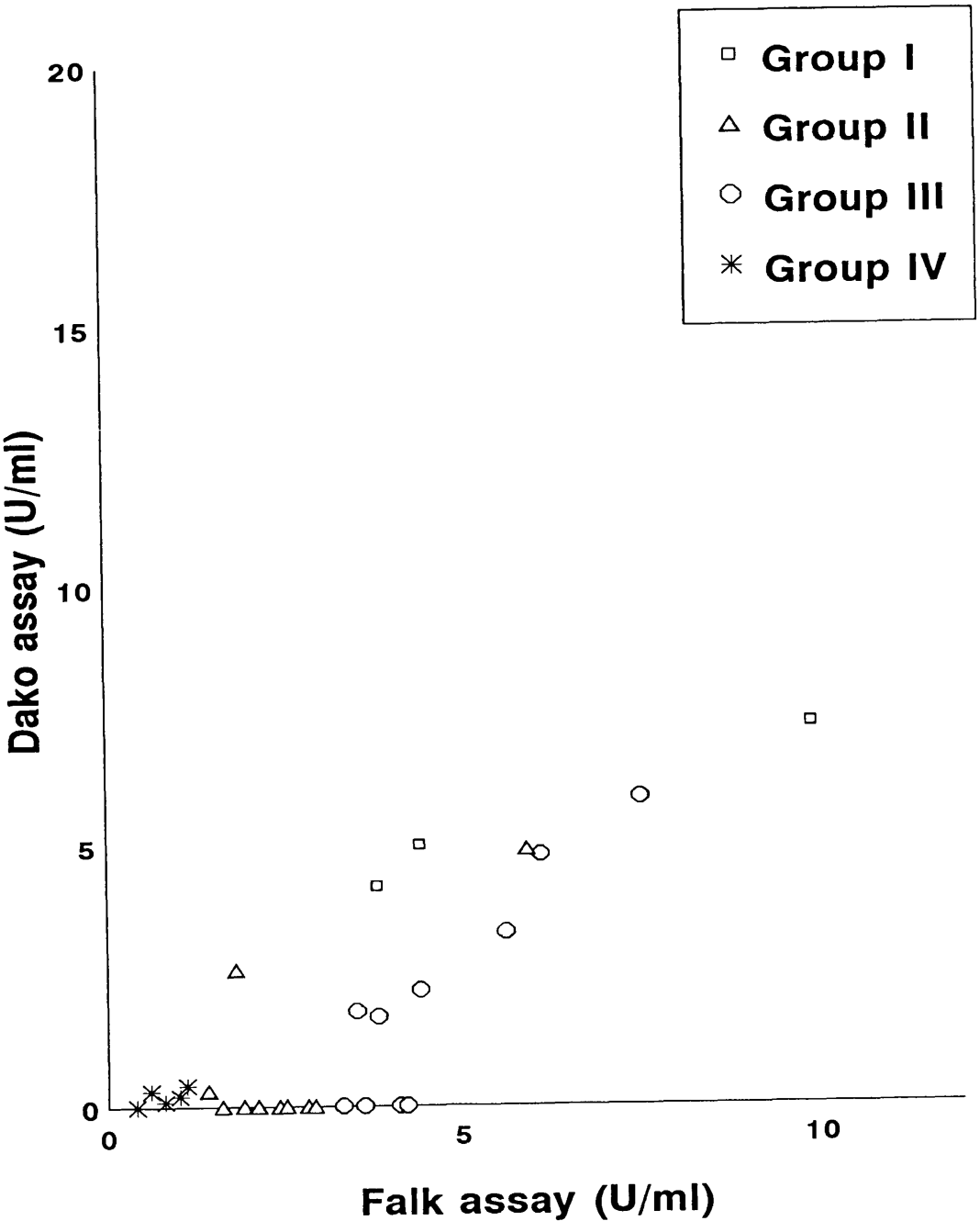

Fig. 9. Comparison of uC5b-9 levels in Falk versus Dako assay for all patient groups. Kendall correlation coefficient tau=0.90, $p<0.001$ ($n = 38$).
Group I - MN patients with high uC5b-9 measured by assay A
Group II - MN patients with no uC5b-9 measured by assay A
Group III - non MN patients as proteinuric controls
Group IV - healthy controls

(ii) Analysis of αC9 capture antibodies

Having noted the discrepancies, the role of the αC9 capture antibodies used in the different assays was investigated. Five mAbs which have been reported against a neoantigen of the C5b-9 complex were compared. Three of the mAbs, Falk, B7 and WU 7-2, were gifts from Drs RJ Falk, BP Morgan and R Wurzner respectively. Table 12 summarizes the characterisation of the mAbs as reported in the literature.

Table 12. Summary of the characterisation of monoclonal antibodies against the neoepitope of the C5b-9 complex.

	Native C9	Poly - C9	SC5b-9	MAC
Falk (Falk 1983)	-	+	+	+
Quidel (Kolb 1988)	±	+	+	+
Dako (Mollnes 1985)	-	+	+	+
B7 (Morgan 1988)	±	+	+	+
WU 7-2 (Wurzner 1991)	±	-	+	+

Poly - C9, polymerised C9.

The binding of the five mAbs to native C9, polymerised C9 (poly-C9), SC5b-9 complex and the MAC was assessed using ELISA assays. As immobilized C9 may be distorted conformationally to resemble poly-C9, the ELISA assay was repeated with a precapture layer of anti-C9 antibody.

Native C9 was isolated by gradient elution of plasma proteins from a Q-Sepharose column, followed by gradient elution from an hydroxyapatite column. The C9 positive fractions were pooled and concentrated and applied to a superose 12 column. C9 was eluted as the last peak (modification of the method by Biesecker 1980). Poly-C9 was produced from monomeric C9 by incubation in 20 mM Tris pH 8.5 and 5 mM Ca^{2+} at 37°C for four hours. The sample was then analyzed by superose 12 chromatography where the protein peak eluted at the void volume (modification of the method by Podack 1982). The SC5b-9 complex was prepared by incubating fresh NHS with 10 mg/ml zymosan at 37°C for four hours. The serum was clarified by centrifugation at 10,000g for 30 minutes and put through gel filtration chromatography, a modification of conventional procedures previously described (Kolb 1975a). The MAC was purified from a detergent extract of complement-lysed sheep erythrocytes and subjected to gel filtration as described under methods. The protein concentrations of the native C9, poly-C9, SC5b-9 and MAC were determined using the Bio-Rad DC protein assay (see methods).

The IgG concentration of the five mAbs was determined by ELISA using donkey anti-mouse antibody as the capture layer and peroxidase-conjugated anti-mouse IgG as the detection layer (both from Jackson Immunoresearch Labs). The dilutions of the five mAbs which gave an OD of 0.5 at absorbance 420 nm were taken to contain the same amount of IgG.

Each of the proteins - native C9, poly-C9, SC5b-9 and MAC, were coated at dilutions in the range from 2 - 20 μg/ml. The five mAbs were incubated at

concentrations containing the same amount of IgG as determined by the preliminary experiment above. Peroxidase-conjugated anti-mouse IgG was added and the plates were developed with ABTS. This ELISA assay was repeated with a precapture layer of goat anti-C9 antibody (ICN Biomedicals).

Native C9, SC5b-9 complex and MAC were subjected to SDS - PAGE followed by electrophoretic transfer to PVDF membrane. The five mAbs were compared by Western blotting. Bound proteins were detected by the mAbs followed by peroxidase-conjugated anti-mouse IgG and developed with DAB as described under methods. Poly-C9 was too large a molecule to enter the gel.

Inhibition of binding of the five mAbs to the SC5b-9 complex by NHS (as a source of native complement components containing 50 - 60 μg/ml of native C9) and normal urine was assessed by ELISA assays. The plates were coated with SC5b-9 at 1 mg/ml. The five mAbs, at a final concentration containing the same amount of IgG, were added to NHS and normal urine prior to the assay. The plates were then incubated overnight. Goat anti-C7 antibody and peroxidase-conjugated anti-goat IgG were used in succession and developed with ABTS, as detailed under methods - Manchester assay.

All the mAbs except the Dako antibody showed significant reactivity with native C9. However, this reactivity was abolished for the Falk antibody when native C9 was precaptured to prevent conformational distortion (Figure 10). Both the Falk and Dako mAbs showed higher reactivity to poly-C9 compared to precaptured

monomeric C9. All the mAbs reacted equally well with SC5b-9 and MAC (Figure 11).

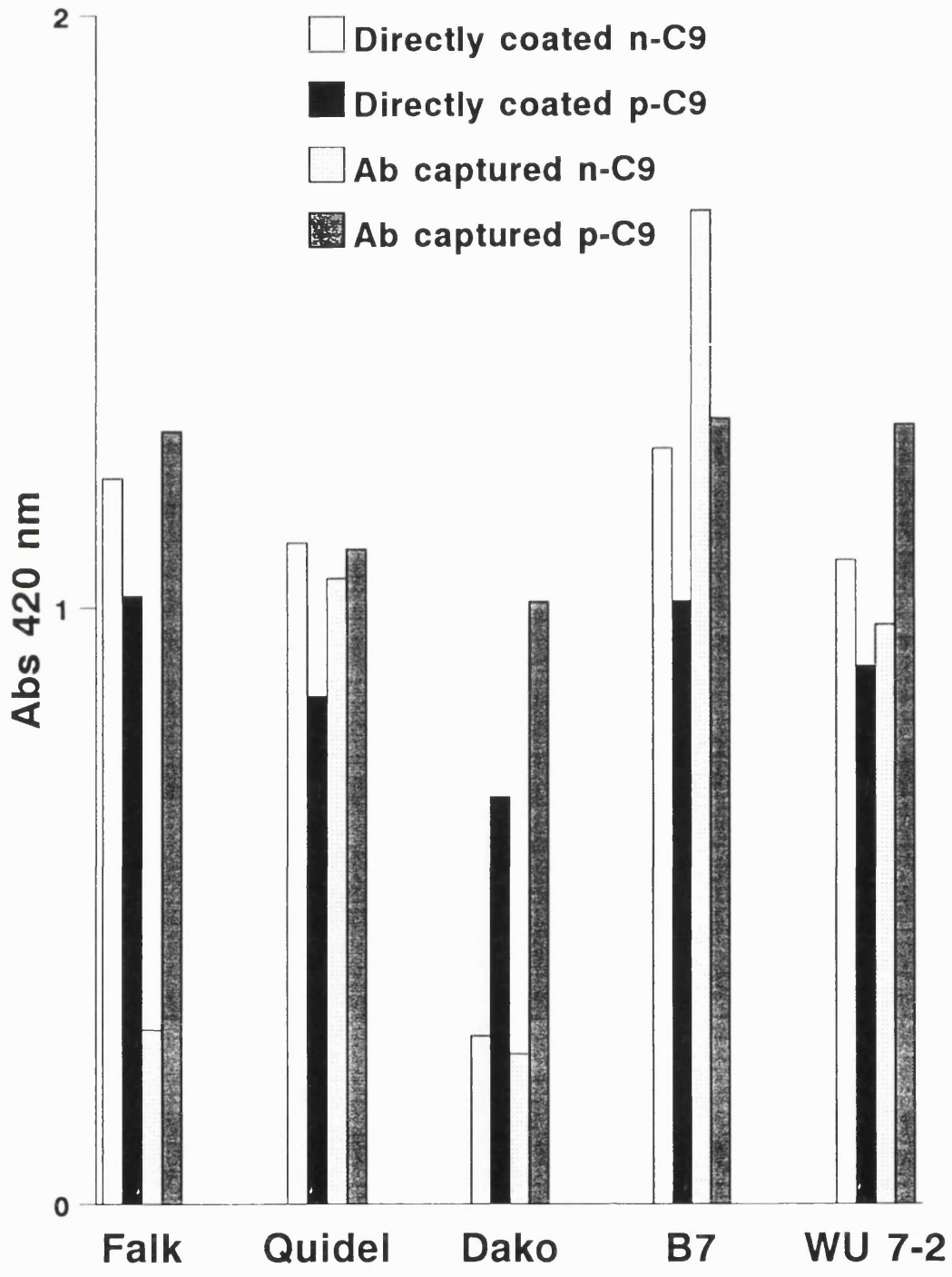

Fig. 10. Comparison of capture antibodies on native C9 (n-C9) and polymerised C9 (poly-C9) by ELISA, either directly coated or precaptured.

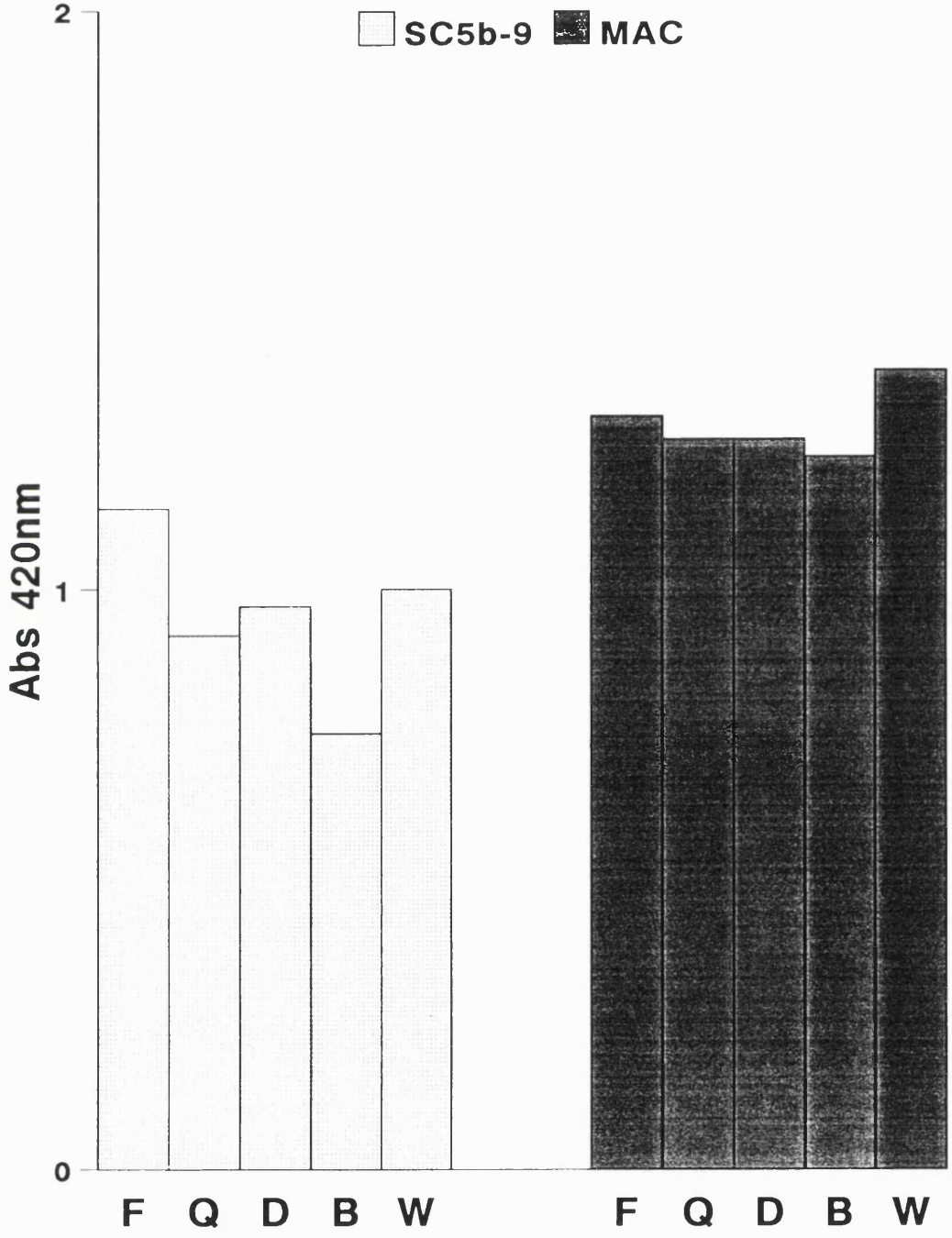

Fig. 11. Comparison of capture antibodies on SC5b-9 and MAC by ELISA. Five monoclonal antibodies. **F**, Falk; **Q**, Quidel; **D**, Dako; **B**, B7; **W**, WU 7-2.

Similarly, immunoblotting showed that only the Dako antibody had least reactivity on native C9 (M_r = 70,000), see Figure 12. They all reacted with SC5b-9 and MAC on immunoblotting (results not shown).

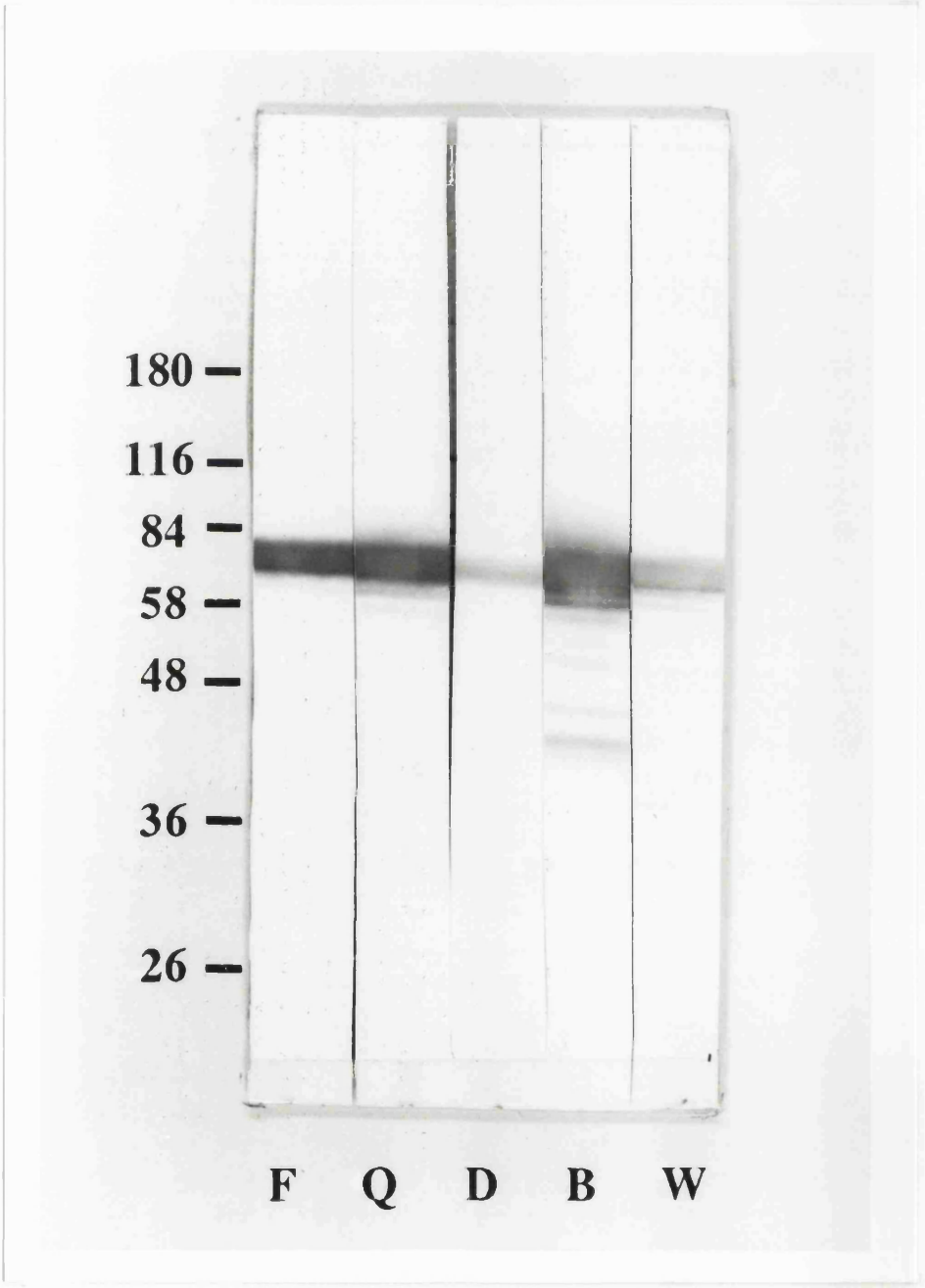

Fig. 12. Comparison of capture antibodies on native C9 (M_r = 70,000) by SDS-PAGE. Abbreviations are as in Figure 11.

NHS inhibited the binding of all the mAbs to SC5b-9, but no significant difference was noted amongst them. Normal urine inhibited binding to a lesser extent than NHS (Figure 13).

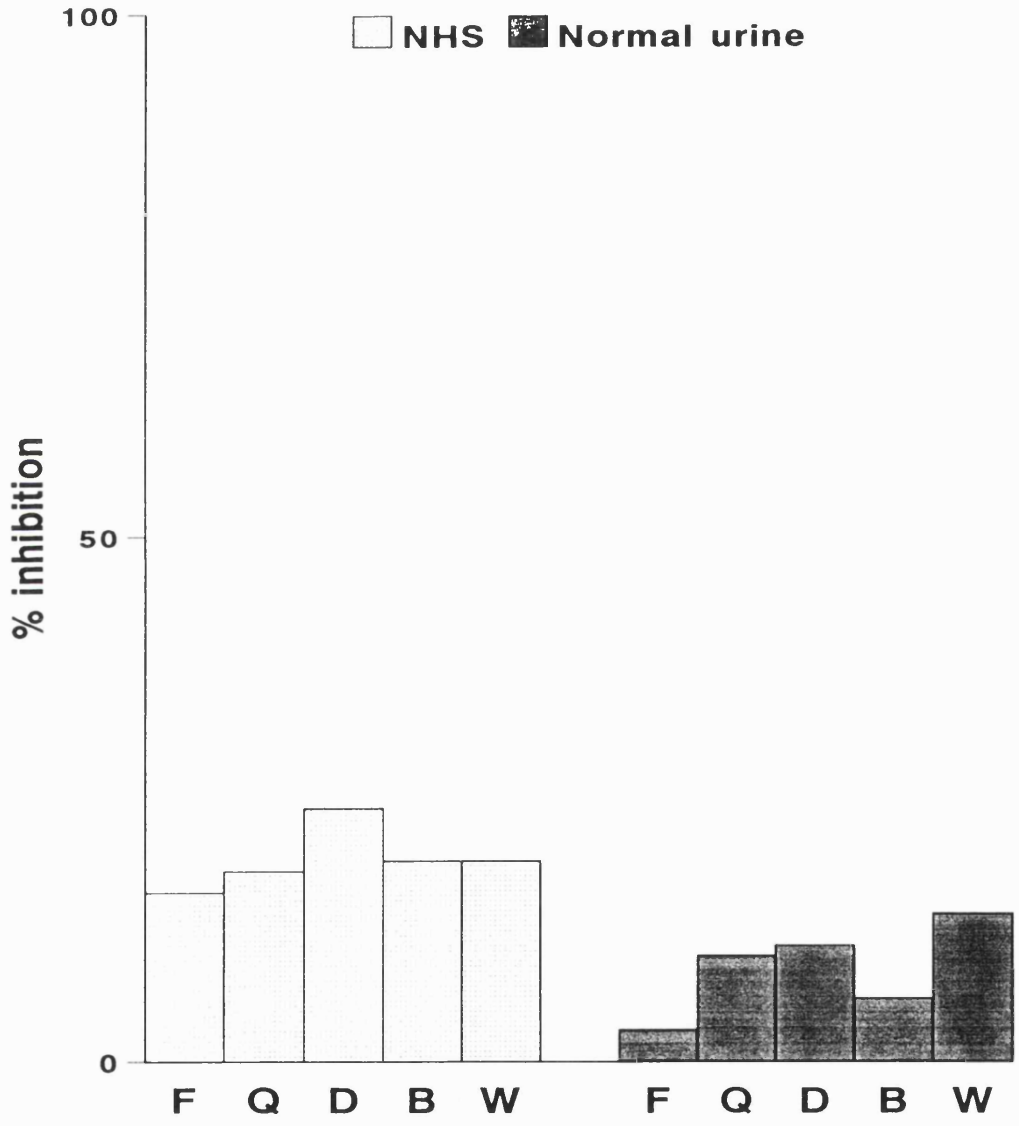

Fig. 13. Comparison of inhibition of antibodies to SC5b-9 by normal human serum (NHS) and normal urine by ELISA. Abbreviations are as in Figure 11.

(iii) Analysis of detection antibodies

Four detection antibodies were compared by ELISA assays - Quidel anti-C6 and anti-C7 serum (employed in the Quidel asssay), ICN Biomedicals anti-C6 serum and anti C-7 serum (employed in the Falk assay). The plates were coated with the five mAbs at 1 μg/ml. A urine sample, known to be positive for C5b-9 as measured by the Falk assay, was added to all the wells. The four detection antibodies at 1/500 dilution were added. The peroxidase-conjugated rabbit anti-goat IgG (Dako Ltd) at 1/2000 dilution was followed by peroxidase substrate and ABTS.

No difference was seen in the four detection antibodies (Figure 14). Results for anti-C6 serum are not shown.

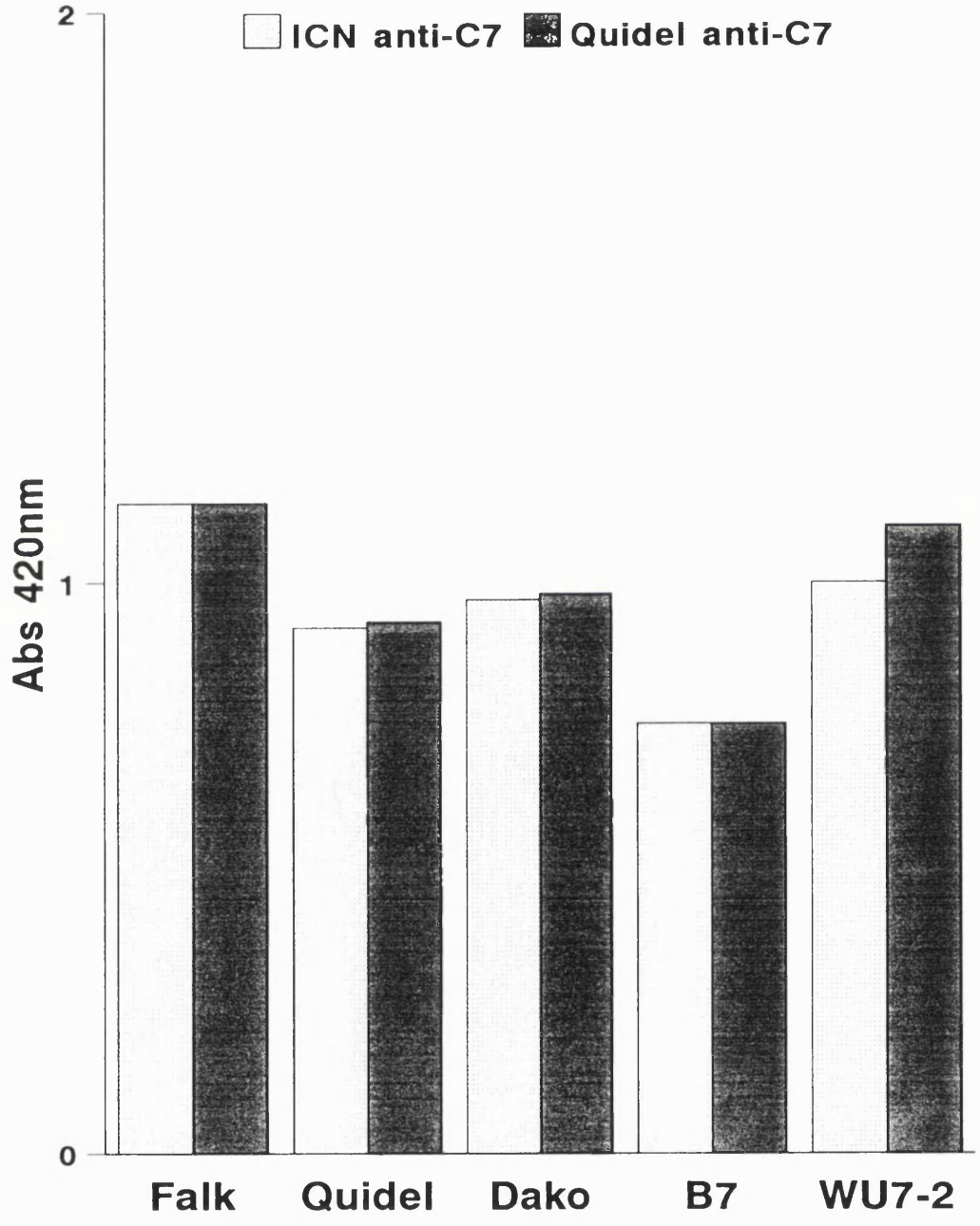

Fig. 14. Comparison of detection antibodies by ELISA.

(iv) Analysis of standards

The three standards supplied in the Quidel kit were run in the Falk assay. Similarly, the zymosan-activated serum containing SC5b-9 used in the Falk assay was run in the Quidel assay. Known quantities of purified SC5b-9 and MAC were measured in both the Falk and Quidel assays.

The working range in the Quidel assay was 0 - 146 ng/ml of SC5b-9. The working range for the Falk assay was much higher at 100 ng/ml - 10 μg/ml of SC5b-9. Similar values were obtained for MAC levels.

(v) Discussion

The results confirm the discrepancies in the detection of uC5b-9 between the assays. The role of the different reagents employed in these assays were then investigated - capture antibodies, detection antibodies and standards. This study showed that all five αC9 capture antibodies reacted with native C9 and this reactivity with the Falk antibody was reduced by precapturing native C9. Only the Falk and Dako antibodies showed enhanced reactivity with poly-C9. None of the five mAbs were able to distinguish SC5b-9 from MAC. No one antibody was preferentially inhibited by normal serum or urine. The difference in performance between the assays was not related to the detection layer. The Quidel kit has since been withdrawn from the market, so the differences in the reactivities of the capture antibodies could not be looked into further.

Neoepitopes, which are not present on native complement proteins, are generated

during C5b-9 assembly (Kolb 1975b). A variety of these "neoepitope-specific" mAbs are in use since such antibodies represent valuable immunological tools for the identification and quantification of C5b-9 in tissues and body fluids (Dalmasso 1989). None of the antibodies described so far is able to distinguish between fluid phase SC5b-9 and membrane bound C5b-9 (MAC). As S-protein is also able to integrate into the MAC after generation of C5b-9, this cannot be used to distinguish between the two forms (Bhakdi 1988). The binding regions of all neoepitope-specific mAbs to the C5b-9 complex described so far are located in the C9 moiety. The reaction of these mAbs with native C9 is meant to be much weaker. This probably reflects the marked conformational changes which C9 undergoes during C5b-9 assembly (Tschopp 1984). Poly-C9 activated by zinc results in a higher number of C9 molecules in the ring (Biesecker personal communication) compared to the poly-C9 activated by magnesium. It is possible that the number of C9 molecules in poly-C9 plays a major role in creating new epitopes. Access to neoepitopes probably present in both SC5b-9 and MAC may be inhibited by the many C9 molecules formed in the ring. These concealed neoepitopes may account for the different reactivities noted in the mAbs studied. Furthermore the C5b-8 to C9 ratio on membranes attacked is not constant (Podack 1982). This could also account for the differences noted.

C8α is structurally and functionally related to C9. This was confirmed by an anti-C9 neoepitope mAb which cross-reacted with an epitope exposed on C8α within the C5b-8 complex but not on monomeric C8 (Tshopp 1986). In the assembly of MAC, a heterogeneity of complexes are formed - C5b-6, C5b-7 and C5b-8. Hence the

mAbs could be detecting 'incomplete MAC' which is lacking C9.

Another possible explanation for the different reactivities in the mAbs studied is the absence or presence of S-protein in association with the C5b-9 complex. Access to neoepitopes present on the SC5b-9 complex may be inhibited by the S-protein.

In vivo complement activation is best assessed by C5b-9 levels. ELISAs to measure C5b-9 might be superior to assays quantitating C3 activation fragments or the measurement of the liberation of the anaphylatoxin C5a. Firstly, C5a has a very short half life due to immediate binding to its cell membrane receptor (Chenoweth 1978). Secondly, terminal complement activation can occur, in the absence of C3 activation or C5a liberation, by cleavage of C5 caused by enzymes released from injured tissue (Wetsel 1982). Therefore an ELISA for C5b-9 is the recommended assay for the assessment of terminal complement activation. The importance of the capture antibody (anti-C9 neoepitope) in determining the specificity of such an assay has been demonstrated in this study.

C. DEVELOPMENT OF THE MANCHESTER ASSAY

Another in-house immunoassay for C5b-9 using the mAb aE11 (Dako Ltd) was developed. It showed enhanced reactivity with poly-C9 (Figure 10), and correlated well with the Falk assay across all patient groups (Table 11). Furthermore this antibody was available commercially.

(i) Monoclonal and polyclonal antibodies

The mAb, aE11, was characterised by Mollnes (1985). It is reported to react with a C9 neoepitope on both SC5b-9 and C5b-9 forms. The mAb was supplied in tissue culture supernatant, so the IgG fraction was isolated in a 1 ml protein G column (Pharmacia).

The polyclonal goat anti-C7 serum (ICN Biomedicals) was used as the detection layer. Combination of the aE11 mAb with anti-C5, anti-C6, anti-C8 and anti-C9 were also evaluated, and the chosen combination produced the optimum signal to noise ratio when assaying partially purified C5b-9.

(ii) Sample collection and storage

Urine samples were collected in EDTA and left at 4°C and room temperature (20°C). Aliquots of the same urine were taken at various time points and stored at -70°C until time of analysis by the Manchester assay.

(iii) Assay technique

The optimal amounts of mAb, urine samples, polyclonal goat anti-C7 serum and peroxidase-conjugated donkey anti-goat IgG (Jackson Immunoresearch Labs) were determined in preliminary experiments. The assay technique is as detailed under methods (see Manchester assay).

(iv) Standardisation and quality control

The standard containing SC5b-9 was prepared by incubating fresh NHS with zymosan at 37°C for 4 hours. The serum was clarified by centrifugation at 10,000g for 30 minutes and stored in aliquots at -70°C. Dilutions of the standard were used to construct a linear standard curve over an OD range of 0.2 to 1.0 (background 0.1) from which arbitrary units of C5b-9 were read. The concentration of C5b-9 was expressed as a ratio of units per mg of creatinine in the freshly voided urine samples. Known quantities of SC5b-9 and MAC were run in the assay. Two internal controls consisting of pooled urines known to be C5b-9 positive were used to monitor any drift of the standard curve. One arbitrary unit of C5b-9 is equivalent to 20 ng/ml of SC5b-9. The conversion factor is similar for MAC.

(v) Results

The usual working range was 25 ng/ml to 2 μg/ml of SC5b-9 and a similar range for MAC. The background value, defined as the negative control as a percentage of the top standard, was originally as high as 40%. This was discovered to be due to the cross-reactivity of the donkey anti-goat IgG to the mouse mAb. This high background was reduced to below 10% by absorption of the donkey antiserum with mouse serum prior to its addition to the assay.

Figure 15 shows the results from serial aliquots taken from urine allowed to stand at 4°C and at room temperature for various lengths of time. No significant difference in uC5b-9 levels was observed between 0 and 7 days.

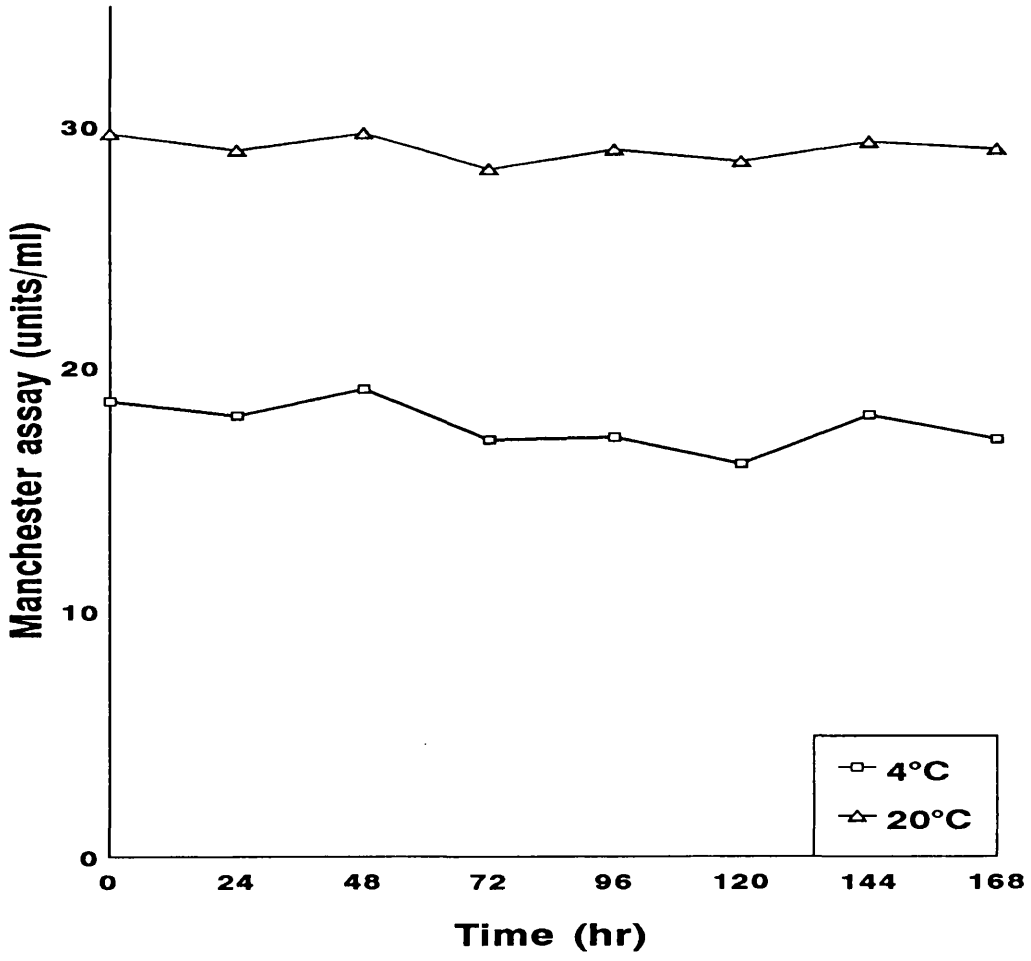

Fig. 15. Urinary C5b-9 levels when allowed to stand at 4°C and at room temperature for varying lengths of time.

No C5b-9 was detected in urines from a control group of 25 laboratory workers. Urine C5b-9 was analyzed in 45 urines from patients with other nephropathies with significant levels of proteinuria (mean = 5.8 ± 3.6 g/24hr, range = 2 to 19 g/24hr). Only two out of the 45 patients (one with minimal change and one with chronic glomerulonephritis) were positive for uC5b-9. No correlation between proteinuria and uC5b-9 levels was found in this proteinuric control group (Spearman rank correlation coefficient $r_s = 0.25$, $n = 45$, $p > 0.05$). Urine C5b-9 levels showed no correlation with a similar range of proteinuria in membranous patients (Spearman rank correlation coefficient $r_s = 0.24$, $n = 40$, $p > 0.05$).

The inter-assay coefficient of variation for the ELISA was determined by including 2 internal controls of pooled urine in ten separate assays. The intra-assay coefficient of variation was determined by including twenty replicates of a single sample at random points within an assay plate. The coefficients of variation for the assay was 12% inter-assay and 5% intra-assay respectively.

(vi) Discussion

This monoclonal based ELISA assay for C5b-9 detection was chosen because the mAb has been fully characterised. It was based on a commercially available antibody. There was good correlation with the Falk assay and would therefore be useful clinically to identify patients with an ongoing immunological insult. Furthermore twelve urine samples from membranous patients, representing a range of uC5b-9 levels as measured in the Manchester assay, were run by Dr B.P.Morgan (see methods - under B7). There was good correlation of uC5b-9 levels between the

Manchester assay and Dr Morgan's assay (Spearman rank correlation coefficient r_s = 0.89, n = 12, p <0.01). The ability of this assay to measure uC5b-9 reproducibly was then used to assess the clinical usefulness of uC5b-9 in HMN.

CHAPTER 4

CLINICAL USE OF URINARY C5b-9 IN IDIOPATHIC HUMAN MEMBRANOUS NEPHROPATHY

A. URINARY C5b-9 AS A MARKER OF DISEASE ACTIVITY

(i) Patients and clinical features

One hundred patients fulfilled the selection criteria. They followed one of two clinical course (definitions as in chapter 2):

(a) Stable renal function - defined by a maintenance of serum creatinine at a normal ($\leq$ 110 μmol/l) or impaired level, or

(b) Declining renal function - defined by a sustained $>30\%$ rise in serum creatinine during the previous twelve month period.

Sixty-three patients maintained stable renal function: six of them were treated with immunosuppressive drugs for their nephrotic state. They were excluded from the study as the data analysis for response to treatment was based only on patients with declining renal function. Thirty-seven patients had declining renal function during the study period: nineteen received no immunomodulating treatment whilst the remaining eighteen patients did.

Thus, the patients were categorized into three groups defined by clinical progress and therapeutic regimens :

Group A — Patients who maintained stable renal function during the study period (n=57),

Group B — Patients with declining renal function who did not receive immunoactive drugs (n=19),

Group C — Patients with declining renal function who received immunosuppression treatment (n=18).
This group will be discussed in section B (see below).

Table 13 shows the clinical features of groups A and B. The ages shown are at the time of the first available uC5b-9 sample. The observation period was taken from the date of the first to the last available uC5b-9 sample. There was no significant difference in the age (54 yr vs 56 yr) or the observation period (4.4 yr vs 5.0 yr) between the two groups. However, there were more males in group B. The nineteen patients with declining renal function (group B) did not receive immunoactive drugs because of clinical contraindications: two reached ESRF soon after diagnosis, two had recently undergone coronary bypass grafting, three had acute myocardial infarctions, one had recurrent peptic ulceration, one had a stroke, two were more than 70 years of age, two had benign prostate hyperplasia and required surgical intervention, one had moved away from Manchester, two were deemed non-compliant, one died of non-renal cause whilst under consideration and for two the reason was uncertain.

Table 13. Clinical features of group A and group B.

	Group A n = 57	Group B n = 19	p
Age (yr)	54 ± 14.5	56 ± 12.1	>0.05 ns
Sex (M:F)	39 : 18 (68%: 32%)	16 : 3 (84%: 16%)	<0.05
Observation period (yr)	4.4 ± 3.0	5.0 ± 3.4	>0.05 ns

All data, mean ± SD; **p,** group A vs group B (Student's t-test for age and observation period, and chi square test for sex).

(ii) Laboratory data

The laboratory features of groups A and B are shown in Table 14. The data were expressed in median values as the distribution of serum creatinine and proteinuria were skewed. In group A, there was no significant change in serum creatinine (98 μmol/l vs 96 μmol/l) but there was a significant fall in proteinuria by the end of the study period. In group B, there was a significant increase in serum creatinine (129 μmol/l vs 240 μmol/l) without a significant fall in proteinuria (6.2 g/24hr vs 5.0 g/24hr) by the end of the observation period.

Table 14. Serum creatinine and proteinuria at the beginning and at the end of the observation period.

Median values		Group A n = 57	Group B n = 19
SCreat (μmol/l)	Initial	98	129
	Final	96	240
	p	>0.05 ns	<0.001
Proteinuria (g/24 hr)	Initial	3.0	6.2
	Final	1.2	5.0
	p	<0.001	>0.05 ns

SCreat, serum creatinine; **p,** laboratory value at the beginning vs the end of the observation period (Wilcoxon test).

(iii) Urinary C5b-9 results

The number of samples tested was expressed in median values because of the skewed distribution. The median numbers of samples tested per patient and the median

numbers of positive results for uC5b-9 (>3 units / mg creatinine) are presented in Table 15. More samples per patient were tested for group B compared to group A (16 vs 9) as patients with declining renal function were seen more frequently. Statistical analysis using a general linear modelling programme (Payne 1986) confirmed that patients with declining renal function (group B) excreted significantly more C5b-9 than did patients following a stable clinical course (group A), $p < 0.001$. Taking this binomial logit model further to correct for patient-to-patient variation, the Multilevel Models Project (1993) statistical analysis was used. This difference was still significant.

Table 15. Urinary C5b-9 positive samples in group A and group B.

	Median no of samples tested per patient	Median no of positive samples per patient	Positive samples (%)
Group A	9	0	0%*
Group B	16	6	38%*

* $p < 0.001$ number of positive urinary C5b-9 samples in group A vs group B (General linear model programme).

B. THE EFFECT OF DIFFERENT IMMUNOSUPPRESSIVE REGIMENS ON URINARY C5b-9

(i) Patients and clinical features

Group C consists of eighteen patients with declining renal function who received immunosuppressive treatment. Three of these patients received more than one

treatment regimen because of clinical relapse, hence twenty-two treatments were available for analysis. Group C was further divided into three groups according to the immunosuppressive treatment received:

Group I Prednisolone alone (n=13),

Group II Prednisolone and chlorambucil (n=4),

Group III Prednisolone and cyclophosphamide (n=5).

Table 16 shows the clinical features of the three groups. The ages shown were at the start of treatment. The observation period was taken from the date of the first to the last available uC5b-9 sample. There was no significant difference in the age, sex and the observation period between the three groups. There was only one withdrawal in this study. The patient did not complete the course of prednisolone and chlorambucil because of nausea and vomiting. Observation was continued and the study was analyzed on an intention-to-treat basis.

Table 16. Clinical features of groups I, II and III.

	Group I n = 13	Group II n = 4*	Group III n = 5	p
Age (yr)	51 ± 14.1	49 ± 14.6	52 ± 16.3	>0.05 ns
Sex (M:F)	9 : 4 (56%: 44%)	3 : 1 (67%: 33%)	4 : 1 (75%: 25%)	>0.05 ns
Observ. period (yr)	4.1 ± 2.7	3.2 ± 3.5	5.1 ± 3.0	>0.05 ns
No. of samples tested per patient	17 ± 10	17 ± 7	26 ± 15	>0.05 ns

* One patient did not complete the course of treatment but observation continued and the study was analyzed on an intention-to-treat basis; **All data,** mean ± SD; **p,** groups I vs II vs III (Student's t-test for age, observation period and number of samples tested per patient, and chi square test for sex); **observ,** observation.

(ii) Laboratory data

Values for serum creatinine and urinary protein for each group are presented in Table 17. Values were taken before and after treatment, respectively, which best reflects the effect of treatment. In group I, the serum creatinine improved significantly from a pretreatment value of 350 μmol/l to a post-treatment value of 180 μmol/l with a decrease in proteinuria from 15 g/24hr to 5 g/24hr. Two patients progressed to ESRF despite treatment. Their pre- and post-treatment serum creatinine and proteinuria values were therefore excluded from the statistical analysis of group I in Table 17. In group II, the serum creatinine improved from a

pretreatment value of 220 μmol/l to a post-treatment value of 150 μmol/l with a decrease in proteinuria from 17 g/24hr to 9 g/24hr. In group III, the serum creatinine improved from a pretreatment value of 330 μmol/l to a post-treatment value of 260 μmol/l with a decrease in proteinuria from 9 g/24hr to 7 g/24hr. The data in groups II and III were not subjected to statistical analysis because of the small numbers in these two groups.

Table 17. Serum creatinine and proteinuria before and after treatment.

Median values		Group I n = 11*	Group II n = 4	Group III n = 5
SCreat (μmol/l)	Before Rx	350	220	330
	After Rx	180	150	260
	p	<0.05	ND	ND
Protein-uria (g/24hr)	Before Rx	15	17	9
	After Rx	5	9	7
	p	<0.05	ND	ND

* 2 patients excluded from analysis as they reached end-stage renal failure; **SCreat,** serum creatinine; **Rx,** treatment; **p,** laboratory value before treatment vs after treatment (Wilcoxon test); **ND,** not done.

(iii) Urinary C5b-9 results

The concentration of uC5b-9 was expressed in median values as the distribution was skewed. Treatment effected a fall in the concentration of uC5b-9 in all three groups (Table 18). This decline was significant in group I ($p<0.05$). The data in groups II and III were not subjected to statistical analysis because of the small numbers in each group. However, a downward trend in uC5b-9 was observed in these two

treatment groups as well.

Table 18. Concentration of urinary C5b-9 before and after treatment.

Median values	Group I n = 13	Group II n = 4	Group III n = 5
uC5b-9 (units/mg creatinine)			
Before Rx	19 (6 - 30)	10 (0 - 43)	3 (2 - 52)
After Rx	0 (0 - 15)	0 (0 - 8)	0 (0 - 28)
p	<0.05	ND	ND

Data in brackets ( ), interquartile; **Rx,** treatment; **p,** uC5b-9 levels before treatment vs after treatment (Wilcoxon test), **ND,** not done.

The clinical outcome of the patients in group C was analyzed in association with the uC5b-9 status. Patients had one of three clinical outcomes (definitions as in chapter 2):

(a) Response to treatment - defined by an improvement in renal function or stabilisation of serum creatinine so that the value did not continue to rise $>30\%$ by the end of the study period, or

(b) Relapse - defined by a sustained $>30\%$ rise in serum creatinine over twelve months despite treatment, or

(c) ESRF - defined by a serum creatinine >500 μmol/l or dialysis dependent.

In group I, twelve out of the thirteen patients were excreting C5b-9 before treatment. After treatment, eight patients were negative for C5b-9: one of these progressed to ESRF and another relapsed, the other six patients responded to treatment . Five patients continued to excrete C5b-9 after treatment: one progressed to ESRF and four patients relapsed. In group II, two patients were excreting C5b-9 before treatment: one continued to excrete C5b-9 after treatment and had a clinical relapse, the other patient who was negative for C5b-9 after treatment also had a relapse. In group III, four patients were excreting C5b-9 before treatment: one continued to excrete C5b-9 after treatment and relapsed subsequently, one patient who was negative for C5b-9 after treatment progressed to ESRF despite treatment.

Table 19 shows the clinical outcome of all eighteen patients (drawn from all three treatment groups) who were excreting C5b-9 before treatment. Seven patients continued to excrete C5b-9 after treatment: they all relapsed or progressed to ESRF. Eleven patients were negative for C5b-9 after treatment: nine showed clinical response to treatment, one went on to ESRF and the other relapsed.

Table 19. Clinical outcome of patients in group C who were excreting C5b-9 before treatment (n= 18).

Clinical outcome	Urinary C5b-9 status after treatment positive	negative
Number of patients		
Response to treatment	0	9
Relapse / ESRF	7	2
	7	11

ESRF, end-stage renal failure; **p** <0.005 Fisher test.

The clinical outcome of patients in group B (patients with declining renal function who did not receive immunoactive drugs) was also analyzed in association with the uC5b-9 status. Remission was defined as stabilisation of serum creatinine so that the value did not continue to rise $>30\%$ by the end of the study period (see chapter 2). Table 20 shows the clinical outcome of the seventeen patients in group B who were excreting C5b-9 at the beginning of the observation period. Ten patients continued to excrete C5b-9 at the end of the observation period: one relapsed and four progressed to ESRF, five went into spontaneous remission and followed a stable clinical course subsequently although the serum creatinine settled at a higher value. Seven patients were negative for uC5b-9 at the end of the observation period: all these patients subsequently followed a stable clinical course, albeit the serum creatinine settling at a higher level compared to baseline. The level of renal impairment in group B at the end of the study period (240 μmol/l) was lower than

the level in group C before the commencement of treament (300 μmol/l).

Table 20. Clinical outcome of patients in group B who were excreting C5b-9 at the beginning of the observation period (n=17).

Clinical outcome	Urinary C5b-9 status at the end of observation period positive	negative
Number of patients		
Remission	5	7
Relapse / ESRF	5	0
	10	7

ESRF, end-stage renal failure; **p** <0.05 Fisher test.

C. HLA TYPING

HLA DR3 data were available on sixty-four out of the one hundred patients who fulfilled the selection criteria (courtesy of Dr P.Dyer, St Mary's Hospital, Manchester). These patients were divided into two groups:

(i) Patients who never excreted C5b-9 (n=30),

(ii) Patients who excreted C5b-9 at some point during their observation period (n=34).

No correlation was shown between HLA DR3 and the urinary excretion of C5b-9 (Table 21).

Table 21. Effect of MHC tissue typing (HLA DR3) on C5b-9 excretor status of patients (n=64).

C5b-9	HLA DR3 Positive	HLA DR3 negative
Number of patients		
Excretors	17	13
Non-excretors	26	8
	43	21

$p > 0.05$ chi square test.

D. DISCUSSION

A number of points have to be considered when addressing questions which involve progression or treatment of IMN. First, the clinical course of patients with IMN is variable. Perhaps only 25%, although in some series up to 50% of patients develop ESRF (Honkanen 1992). Secondly, the results of treatment with various therapeutic regimens remain contentious as discussed in the introduction. Thirdly, once progressive decline in GFR ensues, in the absence of treatable or self-limiting causes, renal function invariably proceeds to ESRF (Davidson 1984).

Thus there is a need to identify patients in whom there is evidence of ongoing immunological insult which may indicate current or potential progression. Prior to the current studies on urinary complement components, the only putative markers of ongoing glomerular injury were measurements of GFR and proteinuria. Complement activation products have been detected in body fluids and correlated

with activity in other disease states eg. systemic lupus erythematosus and rheumatoid arthritis (Falk 1985, Morgan 1988). A number of studies have documented the detection of complement activation products in plasma and urine in human glomerular disease. In IgA nephropathy, plasma C3d fragments correlated with markers of active disease (O'Donoghue 1990), which may reflect complement activation in the mesangium as opposed to a subepithelial site in MN. Brenchley (1992b) has previously described a correlation between C5b-9 excretion and clinical course in cross-sectional analyses. Elevated uC5b-9 in a subset of patients with IMN or lupus MN has been reported (Schulze 1991), while others have correlated uC5b-9 with proteinuria (Ogrodowski 1991, Kusunoki 1991) and the intensity of immunofluorescence on biopsy (Hasunuma 1990). In this study, C5b-9 excretion does not correlate with proteinuria. Furthermore in a group of 45 proteinuric controls, only two patients excreted C5b-9 (discussed in chapter 3).

Ninety-four adult patients were included in this study. Seventy-six, who did not receive immunosuppressive treatment, were identified, followed and assessed retrospectively at the end of the study period. The temporal relationship between uC5b-9 and clinical parameters in these 67 patients with IMN is demonstrated. The patients were classified into groups defined by clinical outcome. Excretion of C5b-9 in patients following a stable clinical course was relatively rare regardless of proteinuria. Patients with declining renal function were positive for uC5b-9 on most dates tested.

Treatment was given to eighteen patients with any degree of persistent proteinuria

who developed renal impairment. This non-randomised prospective trial was to assess the short and medium-term effect of three commonly used treatment regimens on the clinical course and the excretion of C5b-9. Focusing on these patients with a poor long-term prognosis facilitated the statistical demonstration of effective treatment and also provided adequate justification for the use of potentially toxic therapy. To avoid the problems of disease variability, patients served as their own controls. To qualify for treatment, each patient had to have had at least six months of documented progressive renal dysfunction. This selection process eliminated those patients who might have remitted spontaneously as well as those who might not have had progressive renal dysfunction. Furthermore, there was a control group with declining renal function who did not receive immunoactive drugs because of clinical contraindications. Patients with uC5b-9 had a better clinical outcome if treatment abolished excretion of C5b-9. Patients with declining renal function were more likely to stabilise and enter spontaneous remission if they stopped excreting C5b-9. A late response to immunosuppressive treatment may be possible as suggested by this data. However, a policy of waiting for two to three years for those with declining renal function to identify themselves carries with it the risk that early events, which might determine prognosis irrevocably, will be missed. C5b-9 excretion appears to be a dynamic marker of ongoing immunological insult in IMN and will help identify such patients.

It is likely that patients with evidence of current progression are the ones who may benefit most from immunomodulating drugs. The question arises then of what treatment to use. All three treatment regimens were able to abolish the excretion of

C5b-9 in a manner which correlated with clinical course. The addition of either one of the alkylating agents (chlorambucil or cyclophosphamide) did not appear to be more efficient in abolishing the excretion of C5b-9. The abolishment of C5b-9 excretion would seem to be a useful end-point in determining the efficacy of treatment. Individual patients respond differently to a standard regimen. The efficacy of the chosen treatment may be judged by the abolishment of C5b-9 excretion. If C5b-9 excretion was not abolished, more intense or more prolonged therapy or the addition or substitution of a drug might be more effective. Therapy could therefore be tailored to the individual patient in a more rational manner.

The mechanisms by which these immunomodulating drugs suppress or abolish the excretion of C5b-9 are unknown. They do not appear to block the complement cascade nor suppress the production of the human equivalent of the "Heymann autoantibody". These drugs may affect the cell biology of the GEC, so that the GEC can eliminate MAC by one of several ways as demonstrated in the animal model. The rat GEC has been shown in vitro to exocytose MAC following vesiculation of the MAC (Camussi 1987). The rat GEC in vivo was able to endocytose glomerular deposits of C5b-9 and these were transported transcellularly to be exocytosed into the urinary space (Kerjaschki 1989).

Short (1983) showed that the major histocompatibility complex (MHC) type may play a role in the progression of IMN, but this was not confirmed by the Medical Research Council trial (Cameron 1990). Since patients who excrete C5b-9 had a worse clinical outcome, the HLA DR3 status was examined. It did not correlate with

the C5b-9 excretor status of the patients.

This study shows that uC5b-9 is a marker of ongoing immunological insult in IMN and is important in the selection of patients for treatment and in monitoring the efficacy of treatment. The treatment regimen chosen should aim to abolish excretion of C5b-9.

CHAPTER 5

ISOLATION AND CHARACTERISATION OF URINARY MEMBRANE ATTACK COMPLEXES

A. RESULTS

(i) Isolation of urinary membrane attack complexes (MAC)

Twenty-four hour urine specimens were collected in six patients with IMN who were known to excrete C5b-9 by the Manchester assay. The pelleted urine solubilised in Zwitt was subjected to gel filtration in the presence of the same detergent. Gel filtration profiles were heterogenous in all six patients examined. The fractions collected were assayed for C5b-9 by ELISA. The fractions which eluted at void volume contained the major proportion of the MAC. These fractions were pooled and analyzed further (Figure 16). This fraction was subsequently called urinary MAC, whilst the urine after ultracentrifugation was called supernatant urine .

The individual steps in the urinary MAC purification procedure were analyzed by discontinuous SDS-PAGE system. The gel filtration step resulted in substantial purification of the urinary MAC (Figure 17, lane 4).

The purified urinary MAC from the six patients demonstrated similar reactivity with the mAb aE11 (Dako) known to detect the C9 neoepitope on C5b-9 when examined by SDS-PAGE (Figure 18, lanes 1-6).

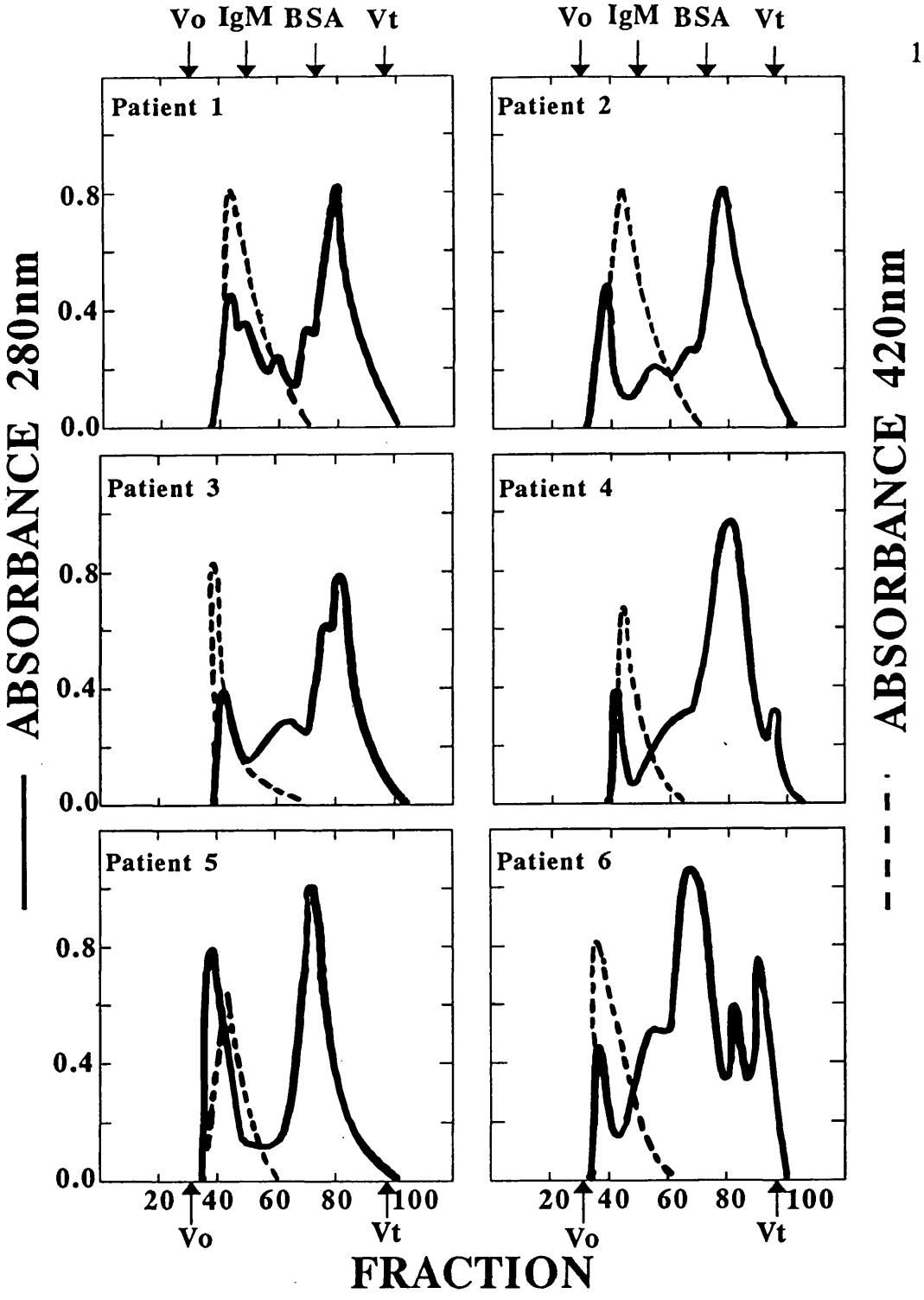

Fig. 16. Gel filtration of solubilised urine pellets using the superose 6 column and C5b-9 levels of FPLC fractions collected. Samples were applied to the column at a flow rate of 0.5 ml/min and 300 μl fractions were collected and assayed for C5b-9 by ELISA at absorbance 420 nm. Proteins eluting from the column were monitored by absorbance at 280 nm. The void volume (V_o) and total volume (V_t) for the column are indicated. Fractions eluted at the void volume containing most of the C5b-9 were pooled and analyzed further. Molecular weight markers employed were IgM 900,000 and BSA 67,000 (other mw markers not shown).
___ eluted protein; ----- C5b-9 level.

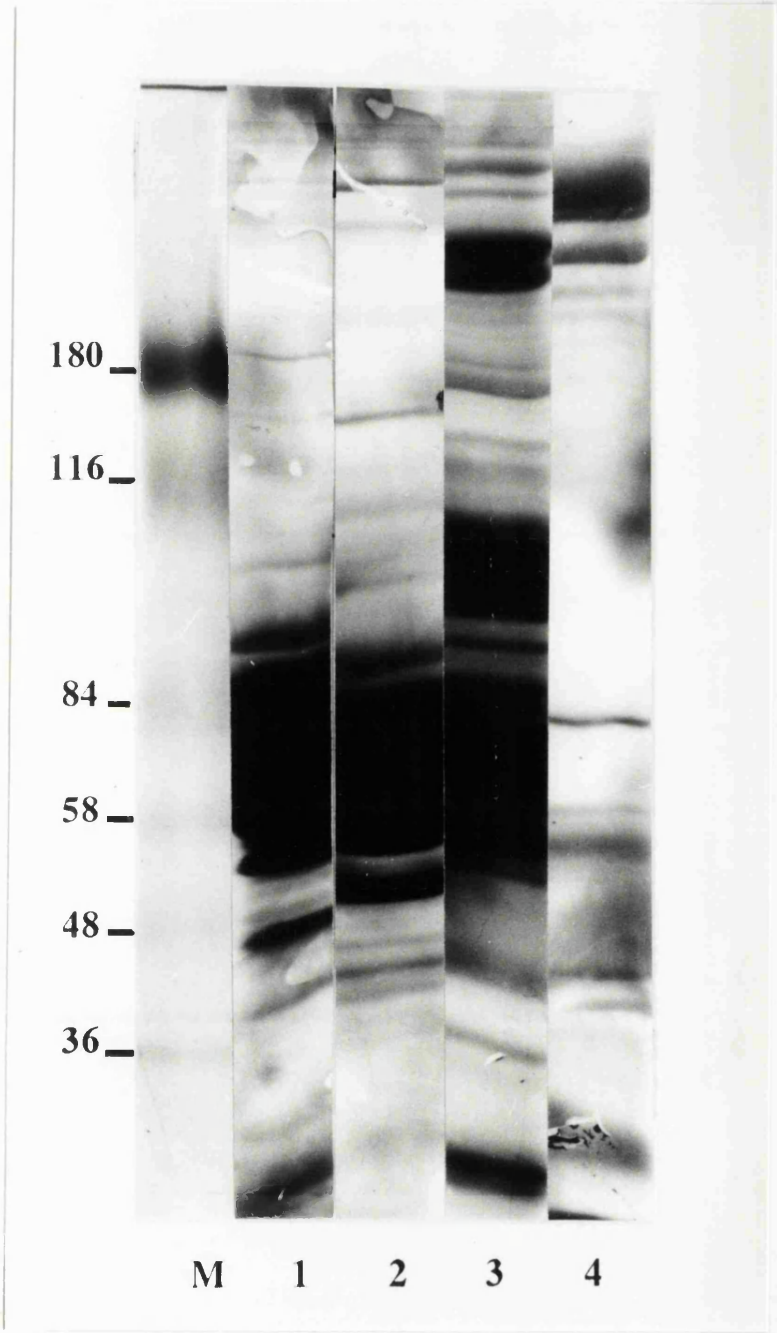

Fig. 17. Silver-stained 7.5% SDS-PAGE analysis of urinary MAC of patient 1 at different stages of purification. **Lane 1,** urine of patient 1; **lane 2,** supernatant urine after centrifugation of urine at 25,000g for ten hours; **lane 3,** urine pellet solubilised at 1% Zwitt; **lane 4,** solubilised urine pellet after purification by gel filtration; **lane M,** prestained molecular weight markers (kD). All samples were run non-reduced.

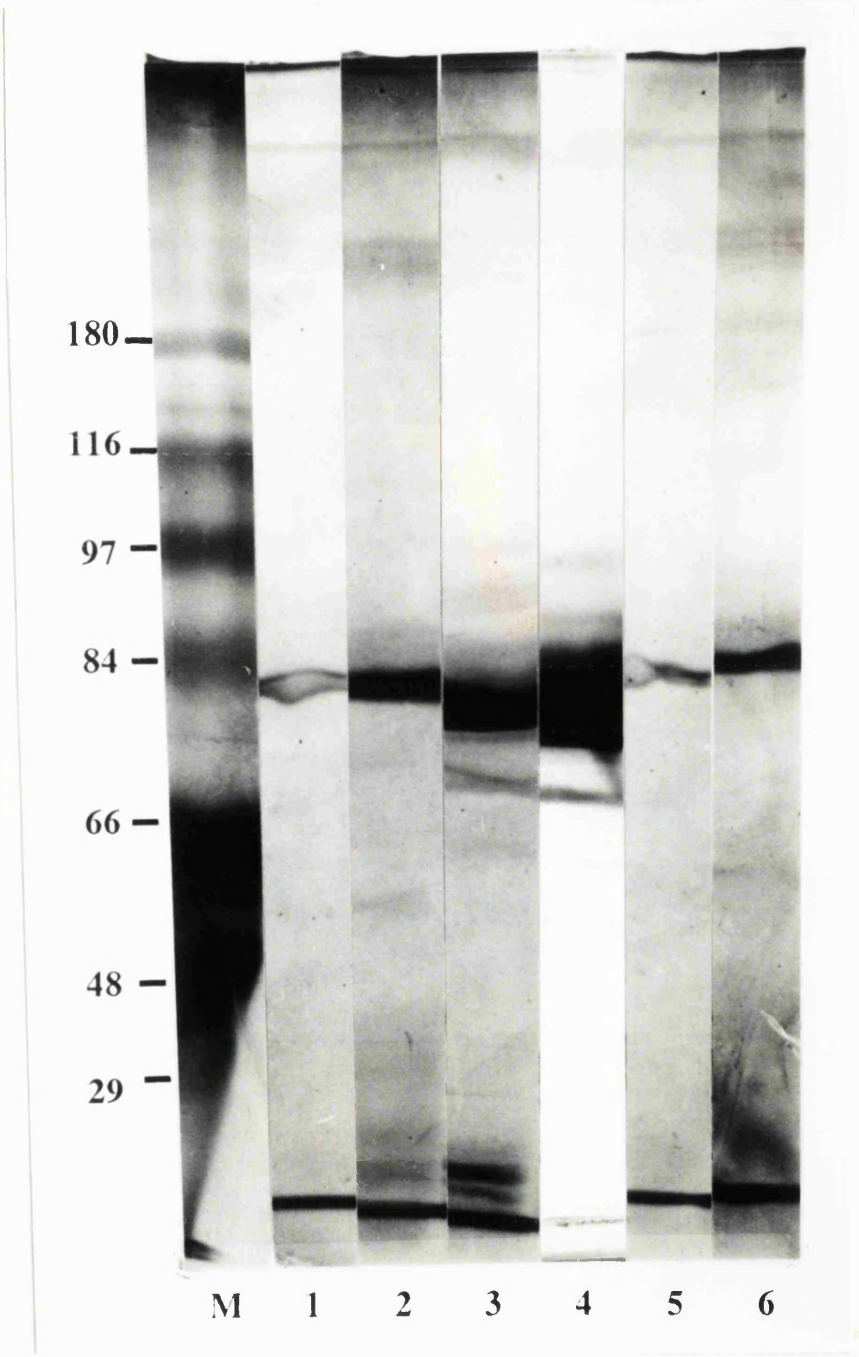

Fig. 18 Western blot of 7.5% SDS-PAGE of urinary MAC of six patients following gel filtration stained with mAb (aE11, Dako) which reacts with C9 neoepitope of C5b-9. **Lanes 1-6,** urinary MAC of the six patients; **lane M,** prestained molecular weight markers (kD). All samples were run non-reduced.

(ii) Characterisation of urinary membrane attack complexes (MAC)

Kerjaschki (1989) showed the transcytotic transport of subepithelial deposits of C5b-9 by the rat GEC in vivo. These cell receptors for C5b-9 could be recycled or exocytosed into the urinary space together with C5b-9. Cell receptors present in the rat GEC of the PHN model have been described - CD10 (Jongeneel 1989) and CD26 (Natori 1987) antigens. These two cell receptors were looked for in association with the purified human urinary MAC and with the supernatant urine.

Variability in the clinical course of IMN may be partly accounted for by the underlying immune mechanism, reflected by the C5b-9 excretor status of the patients (see chapter 4). MAC formation is controlled by fluid-phase and membrane-associated inhibitors so that cell lysis does not occur. To investigate the role of these inhibitors, urines from six patients known to excrete C5b-9 were analyzed. The membrane-bound MAC inhibitory proteins studied were CD35, CD45, CD55 and CD59 antigens (see table 2, page 30). The fluid-phase inhibitor, S-protein, and its receptor, vitronectin receptor $\alpha_v\beta_3$ or CD51, were studied.

Clathrin was also studied. In the PHN model, the best characterised antigen, gp330, was located in the clathrin-coated pits of the GEC (Kerjaschki 1982). A similar mechanism may be operative in HMN.

In HMN there are changes in the GEC shape with loss of interdigitating foot processes (Heptinstall 1983). Cells maintain close contact with their surrounding ECM via specialised cell surface receptors called integrins (Hynes 1987). The

integrins, $ß_1$ and $\alpha_v ß_3$, were studied.

Possible components associated with the purified urinary MAC and with the supernatant urine were screened using the two-site Manchester ELISA assay. Antibodies to components which gave positive results were then used in immunoblotting.

The following antibodies gave a negative result by ELISA:- $ß_1$ integrin, clathrin, CD10 (CALLA), CD26 (DPP IV), CD35 (CR1), CD46 (MCP), CD51 (vitronectin receptor $\alpha_v ß_3$) and CD55 (DAF). These antibodies were not investigated further by immunoblotting.

The complement components in the purified urinary MAC were identified by subjecting the samples to SDS-PAGE and immunoblotting with the antibodies to the five complement components of MAC - C5, C6, C7, C8 and C9. All the five complement components were identified in the urinary MAC in the six patients (Figure 19 - patient 1 shown).

Similarly S-protein in the urinary MAC and the supernatant urine was identified by subjecting the samples to SDS-PAGE and immunoblotting with rabbit anti S-protein antibody. S-protein was found in the urinary MAC and the supernatant urine of all six patients (Figure 20 - patient 1 shown).

CD59 antigen was identified in the urinary MAC (Figure 21) and in the supernatant

urine (Figure 22) by subjecting the samples to SDS-PAGE and immunoblotting with the anti-CD59 mAb. In Western blot analysis, the CD59 antigen was not detected by the less efficient colorimetric system (DAB). The sensitivity of the ECL system was able to reveal this protein. There was a high mw component which reacted with the anti-CD59 antibody in four out of six urinary MAC samples and in all the supernatant urines. In the gel filtered urinary MAC samples, only patient 3 (Figure 21 - lane 3) contained a 20kD band. Patients 2, 4 and 6 had a similar signal in the supernatant urine (Figure 22 - lanes 2, 4 and 6). The absence of a 20kD band in the supernatant urine of patient 3 suggests that the prominent 20kD band in the urinary MAC is not simply a carry over contamination , but rather it is a component of the C5b-9 complex in this patient.

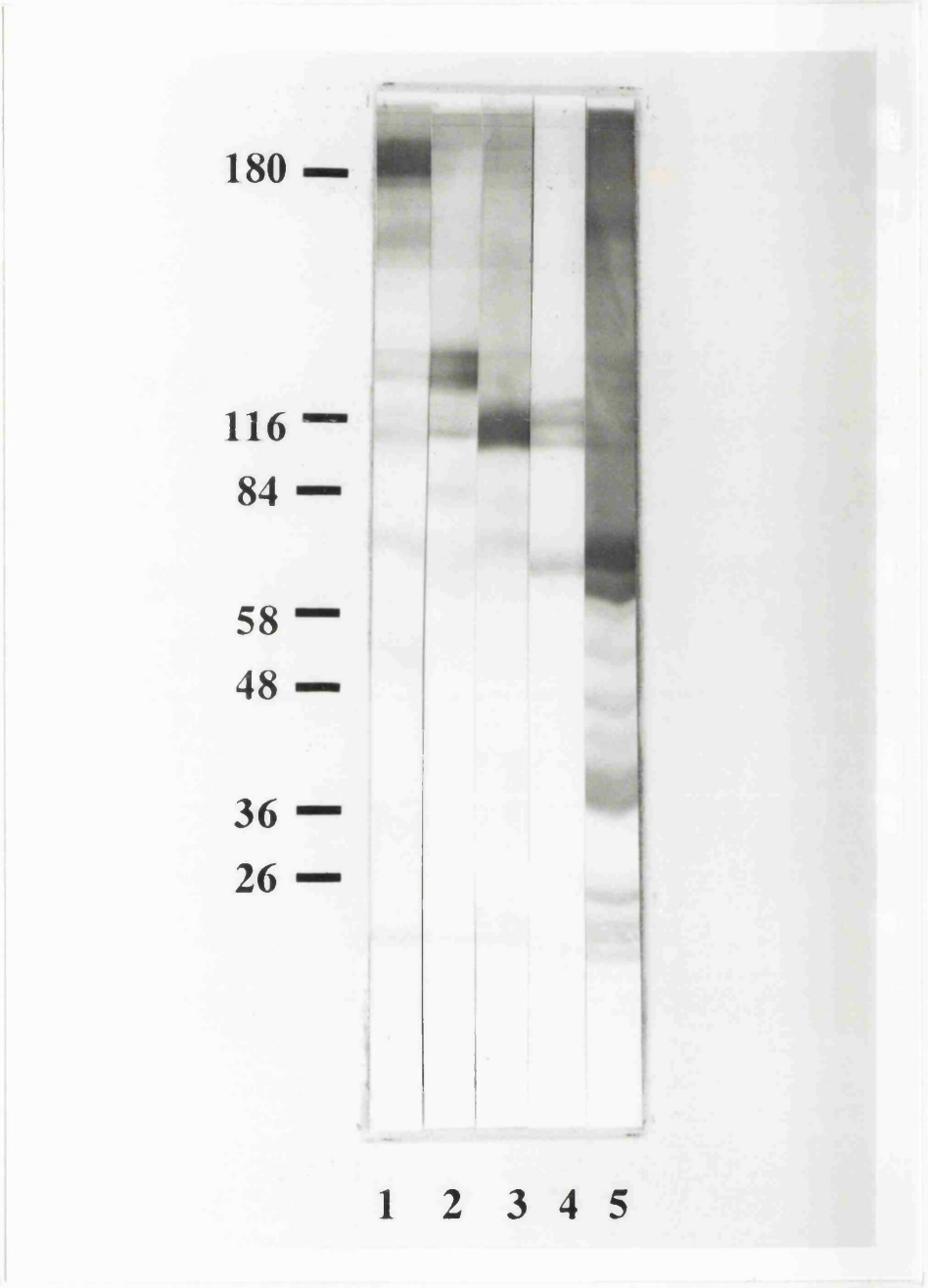

Fig. 19. Western blot of 7.5% SDS-PAGE stained with the 5 polyclonal antibodies to complement components in urinary MAC of patient 1. M_r of C5b **(lane 1)** = 180,000, C6 **(lane 2)** = 120,000, C7 **(lane 3)** = 110,000, C8 **(lane 4)** = 87,000 and 64,000 (2 subunits), C9 **(lane 5)** = 71,000. **Left hand lane ,** molecular weight markers (kD). All samples were run non-reduced.

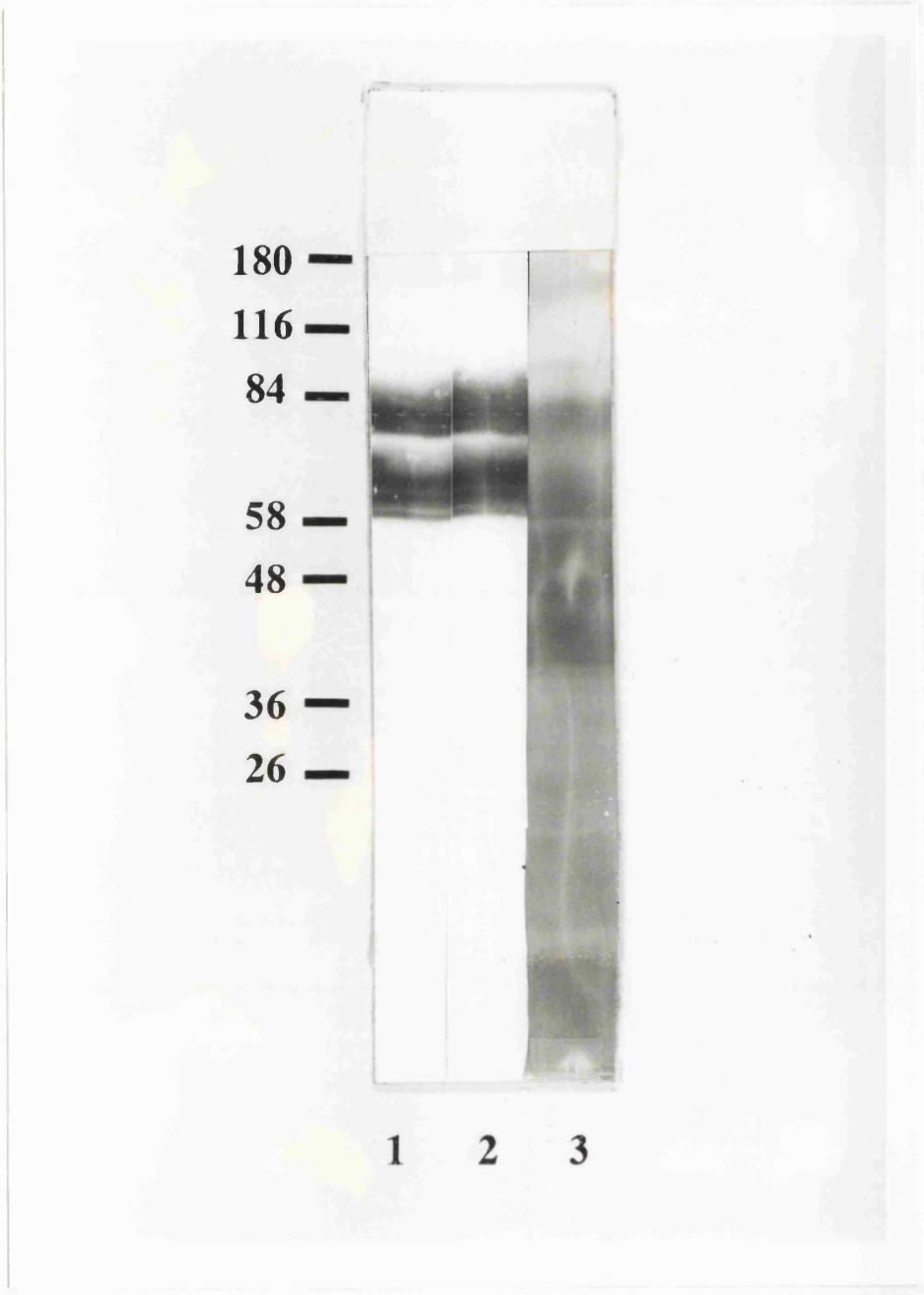

Fig. 20. Western blot of 7.5% SDS-PAGE stained with rabbit anti S-protein. **Lane 1**, urinary MAC of patient 1; **lane 2,** supernatant urine after centrifugation of urine at 25,000g for ten hours; **lane 3**, normal human serum as positive control for S-protein; **left hand lane,** molecular weight markers (kD). M_r of S-protein = 85,000 with 2 subunits 75,000 and 65,000. All samples were run non-reduced.

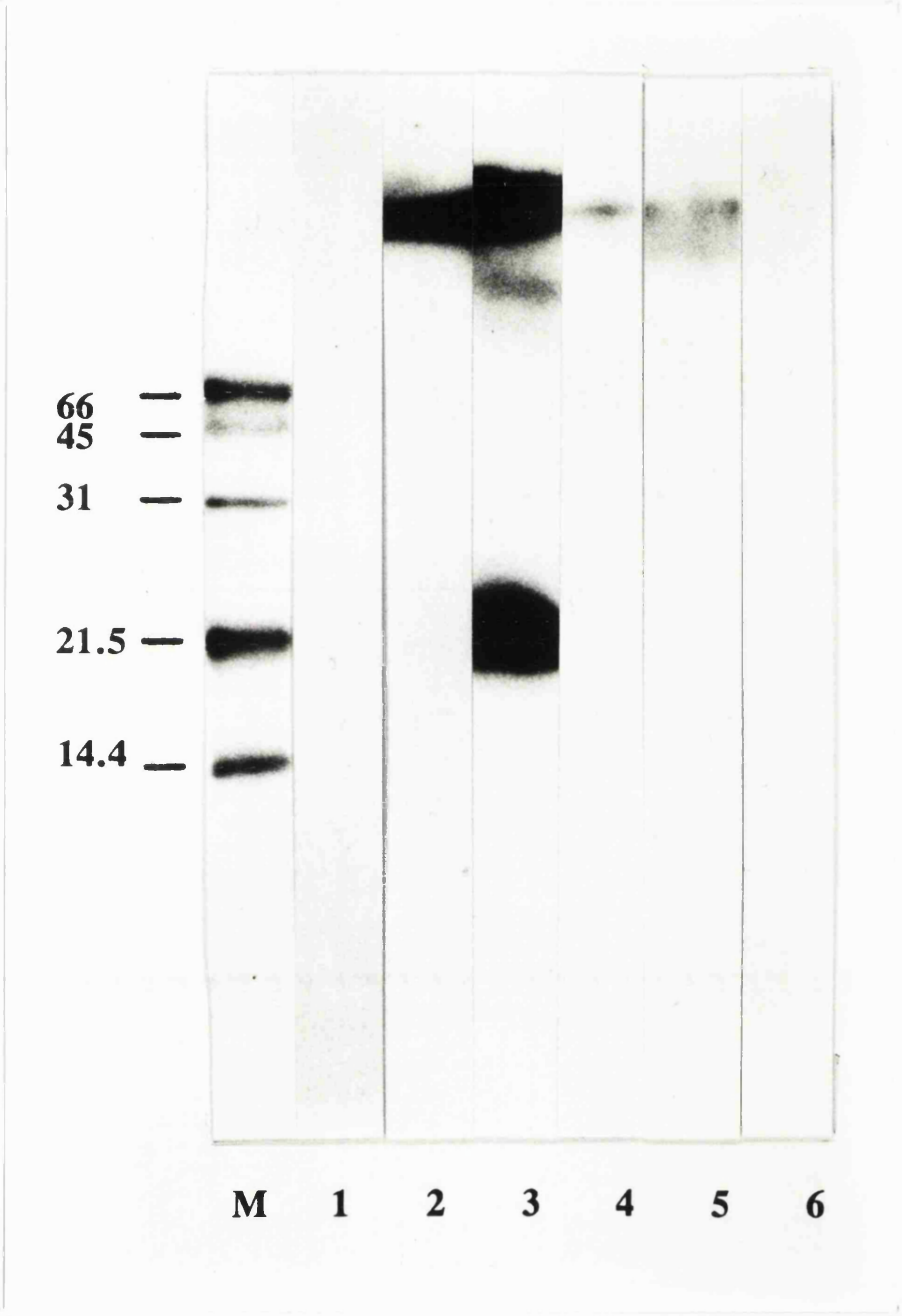

Fig. 21. Western blot of 15% SDS-PAGE stained with anti-CD59 mAb. **Lanes 1 - 6,** urinary MAC of six patients; **lane M,** molecular weight markers (kD). M_r of CD59 = 20,000. All samples were run non-reduced.

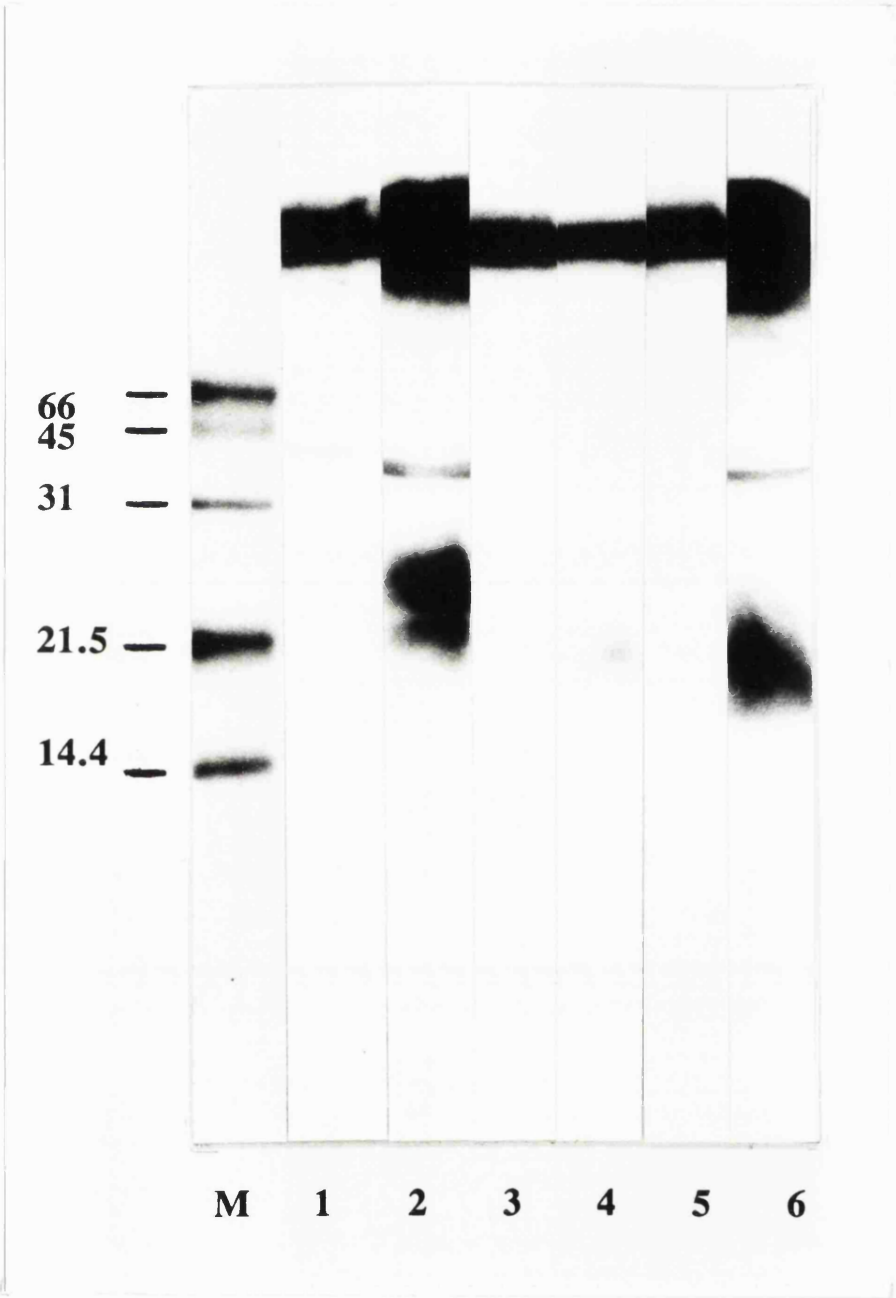

Fig. 22. Western blot of 15% SDS-PAGE stained with anti-CD59 mAb. **Lanes 1 - 6,** supernatant urine of six patients after centrifugation of urine at 25,000g for ten hours; **lane M,** molecular weight markers (kD). M_r of CD59 = 20,000. All samples were run non-reduced.

B. DISCUSSION

The one step gel filtration procedure for MAC isolation was used, having solubilised the membranes of urine pellets in Zwitt detergent. The M_r of the MAC which eluted close to the void volume was $> 1x10^6$. Western blotting of the detergent solubilised urine membranes showed that the detergent had no demonstrable effect on complex composition as all the five complement components were identifiable after gel filtration. The gel filtration profiles of the six patients were different. This may represent a heterogenous population of oligomeric MAC solubilised from urine membranes. The structural composition of the various complex populations (eg. MAC monomers, dimers, trimers etc) was not investigated further. Different oligomers of MAC have been reported in MAC solubilised from complement lysed antibody-coated sheep erythrocytes (Ware 1981). The physical heterogeneity of the MAC extracted from solubilised urine membranes may pertain to the pore size heterogeneity of the functional complement lesions. Boyle (1979) suggested that the ratio of C9 to C8 in the MAC complex is the major determinant of pore size, the higher the C9 ratio to C8 ratio favoured larger channels. This difference in pore size due to the heterogenous population of MAC may cause variable damage on the GEC, which may in turn be reflected in the variability of proteinuric states in HMN.

Data from Kerjaschki (1989) suggested that in PHN a fraction of C5b-9 inserted in the GEC was removed by endocytosis, packaged intracellularly in multivesicular bodies and then disposed of by dumping into the urinary space. There is no current evidence that such a mechanism is operative in HMN. Ogrodowski (1991) and Kusunoki (1991) found that all proteinuric patients excreted detectable amounts of

C5b-9 which correlated with proteinuria. This suggests that proteinuric patients excrete detectable amounts of C5b-9 regardless of their underlying glomerular disease and in diseases where glomerular complement deposits are not detected by immunofluorescence. The size of the C5b-9 complex ($M_r > 1x10^6$) suggests that filtration is not possible. This may imply that a substantial amount of C5b-9 formation occurs in the postglomerular proteinuric urine. Attempts were made in this study to distinguish between the glomerular or tubular origin of the uC5b-9 detected in HMN. If uC5b-9 was of glomerular origin, the implication of such a finding is that the pathogenesis of MN in humans, at least in a subset of patients, involves an autoimmune mechanism. However, this would not exclude the possibility that most, or even all, patients with IMN have a similar mechanism operative, but that antibody deposition was not ongoing at the time of study in many patients. The observation that patients with elevated C5b-9 excretion had a worse clinical outcome (discussed in chapter 4) would be consistent with the possibility that the pathogenesis of IMN may be similar to that defined in the HN models in rats.

Electron microscopy results did not reveal the presence of pores or rings in the pelleted membranes of the urine (courtesy of Dr I Roberts, Dept of Cell and Structural Biology, University of Manchester). There was too much debris in the pelleted membranes of the urine. The urine pellets could be enriched further for the membrane fractions by subjecting the urine pellets to a density gradient. Such an enriched membrane fraction could then be analyzed by electron microscopy.

CD antigens known to be present on the GEC (Ronco 1992), CD10 (CALLA) and

CD26 (DPP IV), could not be detected in association with the urinary MAC. This study could not distinguish between the glomerular or tubular origin of the uC5b-9 detected in HMN.

S-protein is present in the circulation as well as in the urine (Shaffer 1984). S-protein was found colocalised with the C5b-9 complex in the subepithelial deposits in HMN (Bariety 1989). One of the many functions of S-protein is to occupy the site of the precursor complex C5b-7 such that the formed water-soluble SC5b-7 macromolecule is unable to insert into cell membranes. Although the SC5b-7 complex may take up further C8 and C9 molecules to form SC5b-8 and SC5b-9 complexes respectively (Bhakdi 1983), the latter complex is non-lytic. This study attempted to compare the expression of S-protein in the six patients on the assumption that S-protein is unavailable or less available to bind the precursor C5b-7 complex. This would result in MAC formation and excretion of C5b-9 in HMN. However, S-protein was demonstrated in both the supernatant urine and the urinary MAC of all the six patients who were known to excrete C5b-9. S-protein is able to integrate into the membrane bound complex after generation of C5b-9 (Bhakdi 1988). This may account for its presence in both the soluble and membrane-bound forms of C5b-9.

Another important function of S-protein is cellular adhesion. Adhesion to ECM may directly or indirectly modulate proliferative responses to cells to polypeptide growth factors and maintain differentiated function (Kleinham 1981). The cell attachment activity of S-protein resides in the versatile Arg-Gly-Asp (RGD) epitope (Ruoslahti 1986). A variety of cell types, including the GEC (Cybulsky 1992), express these

receptors. These receptors, called integrins, are composed of non-related α and ß subunits. Interaction of cells with ECM are mediated primarily by the $ß_1$ integrin subfamily. Anti-Fx1A, the nephritogenic antibody in PHN, recognises an $\alpha_3ß_1$ integrin in cultured rat GEC and inhibits cell adhesion (Adler 1992). It is possible that in PHN, the anti-Fx1A may be interacting with the GEC integrins, leading to cell detachment from GBM and abnormal glomerular permeability (Cybulsky 1992). Integrins have been shown to control cell shape, motility and adhesion to other cells and to the ECM (Burridge 1988). The vitronectin receptor (CD51, $\alpha_v ß_3$), a member of the $ß_3$-integrin family of cell adhesion glycoproteins, has been demonstrated on the GEC (Kerjaschki 1990). Its role is presumed to be the disposal of the S-protein complexed, inactivated C5b-9. However, both the $ß_1$ and the $\alpha_v ß_3$ (CD51) integrins were not identified in association with the urinary MAC.

In PHN, subepithelial immune deposits of IgG were found within the clathrin-coated pits where the gp330 is located on the GEC (Kerjaschki 1982). These clathrin-coated pits on the membranes are known to mediate the efficient uptake into the cell of macromolecules, which are first bound by specific receptors. The most thoroughly characterised receptor that concentrates on membrane clathrin-coated pits is the low-density lipoprotein (LDL) receptor (Anderson 1977). A cDNA clone, named C14, was prepared from mRNA with IgG eluted from glomeruli of PHN rats. It encoded a polypeptide which contained a pathogenic epitope of PHN responsible for the initiation of the disease (Pietromonaco 1990). Subsequently, another protein, α_2-macroglobulin receptor associated protein (α_2-MRAP), which is a subunit of the human α_2-macroglobulin receptor and LDL receptor, showed extensive homology to

the C14 protein. This polypeptide of M_r ~ 40,000 maybe a protein separate from gp330 but binds in a similar manner as it binds to the α_2-macroglobulin receptor. α_2-MRAP has been shown to bind to gp330 in vitro (Orlando 1992). The LDL receptor has been noted in a clone believed to be derived from gp330 (Raychowdhury 1989). As the LDL receptor is concentrated in the clathrin-coated pits, identification of clathrin in association with the urinary MAC may throw some light in the identification of the Heymann-like antigen in HMN. This study did not show clathrin in association with the urinary MAC. The search for an antigen in HMN comparable to gp330 in rats may prove unfruitful as HMN is not a homogenous disease.

Besides fluid phase inhibitors of MAC formation like S-protein, membrane-bound MAC inhibitory proteins have been identified. Most MACs formed by homologous complement in nucleated cells are therefore inactivated by these inhibitory proteins prior to their removal from the cell surface. Such mechanisms protect self against complement mediated injury and limits the damage of MAC in cells. The resultant sublytic doses of MAC may then be responsible for the release of inflammatory mediators which could alter the GEC function so that proteinuria ensues. CD35 (CR1) has been demonstrated on the GEC and recent development in the construction of soluble CR1 has led to its potential use as a therapeutic agent for the treatment of diseases that involve complement activation (Couser 1992). CD35 was not identified in this study.

Both CD55 (DAF) and CD59 (MIRL) are glycosyl phosphatidylinositol (GPI)-anchored membrane proteins (Medof 1986). They have been shown to protect the

human amniotic epithelial cells from complement mediated lysis (Rooney 1990). Furthermore amniotic fluid contained a soluble form of CD59, (without its GPI anchor), implying that the human amniotic epithelial cells secrete into the amniotic fluid a form of CD59 antigen which retains its inhibitory activity (Rooney 1992). CD55 and CD59 antigens have been isolated from urine (Davies 1989). The urine CD55 and CD59 lack the membrane anchor compared to the membrane-bound CD55 and CD59 (Medof 1986). This study did not identify CD55 in either the supernatant urine or the urinary MAC. However, the CD59 antigen was identified in urinary MAC and supernatant urines of some patients. This suggests that the CD59 antigen is incorporated into the MAC on the GEC or is released spontaneously from the GEC into the urine. CD59 expression may be upregulated in response to complement injury and the variation in the expression of the CD59 antigen may depend on genetic polymorphism. The gene for CD59 has been cloned (Sugita 1989), but no genetic polymorphism has been described for the CD59 antigen so far. However, the other membrane-bound inhibitory proteins, CD35, CD46 and CD55, have been shown to display genetic polymorphism (Carroll 1988, Lublin 1988).

MAC inhibitory proteins warrant further investigation. Cells may be partially deficient in these regulatory proteins. Distinct clinical syndromes have been described with deficiencies - paroxysmal nocturnal haemoglobinuria is probably the best understood (Holguin 1989). The polymorphism of the membrane-bound control proteins may provide a reason for the variability in the clinical course of HMN.

CHAPTER 6

CYTOKINES IN IDIOPATHIC HUMAN MEMBRANOUS NEPHROPATHY

A. RESULTS

Twenty patients with IMN were studied. Paired plasma and urine samples were collected on dates when patients were excreting C5b-9, and again when they were not.

(i) Tumour necrosis factor - α (TNF-α)

TNF-α was not detected in any of the plasma or urine samples irrespective of the patients uC5b-9 status. TNF-α was not present in the plasma and urines of healthy controls. The working range this assay was 4 pg - 1.0 ng/ml.

(ii) Interleukin -1ß (IL-1ß)

Seven out of the twenty patients had detectable plasma IL-1ß. IL-1ß was not present in the plasma of healthy controls. This was significantly elevated in the IMN group compared to healthy controls (Fisher test $p < 0.05$), but it did not correlate with uC5b-9. Urines of healthy controls contained IL-1ß, ranging from 30 - 100 pg/ml. IL-1ß was present in the majority of the urine samples of the patients, the range being similar to that of the healthy controls. The working range for this assay was 30 pg - 2 ng/ml.

(iii) Interleukin - 6 (IL-6)

Five out of the twenty patients had detectable plasma IL-6 levels but these were within the normal range. The detection limit for this assay was 30 pg/ml, and the normal range in healthy controls was < 60 pg/ml. Urinary IL-6 was detected in nine patients, of whom five were above the normal range. This was significantly elevated

in the IMN group compared to healthy controls (Fisher test $p < 0.05$), but it did not correlate with uC5b-9.

(iv) Transforming growth factor - $ß_1$ (TGF-$ß_1$)

Plasma TGF-$ß_1$ was not detected in these patients or in healthy controls, the detection limit of the assay being 100 pg/ml. Urines of healthy controls contained TGF-$ß_1$, ranging from 1 - 35 ng/ml. TGF-$ß_1$ was found in the majority of the urine samples of the patients, the range being similar to that of the healthy controls. The working range for this assay was 100 pg - 50 ng/ml.

B. DISCUSSION

Urinary C5b-9 remains pivotal in the induction of proteinuria in PHN. However, the subsequent effector mechanisms for induction of proteinuria following complement activation remain speculative. The roles of TNF-α, IL-1ß, IL-6 and TGF-$ß_1$ were studied as potential mediators of glomerular injury induced by C5b-9.

Sublytic doses of C5b-9 have been shown to induce production of TNF-α and IL-1 by mesangial cells (Lovett 1987). Most nucleated cells including the GEC have receptors for TNF-α and IL-1. These two cytokines have been demonstrated, both in vitro and in vivo, to induce the synthesis of several inflammatory mediators which are potentially damaging in glomerulonephritis - ROM (Radeke 1990), prostaglandins (Topley 1989), other cytokines (Zoja 1991) and also adhesion molecules (Brennan 1990). Their many activities indicate that they are central mediators of inflammation. However, this study could not detect raised levels of

TNF-α in plasma or urine. Plasma IL-1ß was detected in seven out of twenty patients with IMN, but this did not correlate with the uC5b-9 status of the patients. It is unlikely that this cytokine could be a possible effector of proteinuria. IL-1ß was found in the urines of healthy controls and IMN patients within the same range. TNF-α and IL-1 are close associates and frequent companions. The major source of the two cytokines is activated macrophages. Cellular infiltrate is not a feature in MN although cytokines can be produced by the GEC as well as by inflammatory cells. There was no evidence that such a mechanism was operative in HMN.

Following its original description in monocytes, IL-6 has been shown to be synthesized by mesangial cells and acts as an autocrine growth factor (Kishimoto 1989). Moreover, IL-6 was demonstrated in the proliferative mesangial cells of diseased glomeruli and in the urine of patients with mesangial proliferative glomerulonephritis (Horii 1989), suggesting that it has an important role in the pathogenesis of renal disease. Since both TNF-α and IL-1 are known to stimulate IL-6 production (Zoja 1991), plasma and urinary IL-6 levels were measured. Urine from healthy controls did not contain measurable IL-6 levels. Urinary IL-6 was detected in nine out of twenty patients with IMN. This proportion is much higher than that reported by Horii (1989) where urinary IL-6 was detected in only two out of twenty-seven patients with MN. No IL-6 was detected in the glomeruli from patients with MN using indirect immunofluorescence with a mAb anti-human IL-6, but it was strongly expressed by damaged tubules (Fukatsu 1991). It has also been observed that urinary IL-6 was markedly increased in patients with urinary tract infection (Ohta 1992). The renal biopsies in the group of patients studied here were

not scored for tubular atrophy. Although there was no clinical evidence of urinary tract infection in this study, urine microscopy and urine cultures were not done routinely on the dates of urine collection. There is no evidence in this study to indicate the source of the raised urinary IL-6, whether it be derived from the GEC participating in the inflammatory reaction in HMN or from the renal tubular cells or from both sources.

Pathological accumulation of ECM is a central feature of progressive glomerular disease. Such accumulation appears to be the structural basis for the development of glomerulosclerosis and ESRF. TGF-ß has been recognised to mediate the pathological increase of ECM in progressive glomerular disease (Roberts 1986). It is able to stimulate the expression of protease inhibitors so that levels of enzymes like collagenases are decreased (Edwards 1987). TGF-ß has also been shown to upregulate integrin expression leading to a loss of adhesion to the ECM (Ignotz 1989). Most cell types are capable of producing and responding to TGF-ß. The GEC is known to express receptors for TGF-ß (MacKay 1989). ECM accumulation could lead to thickened GBM, whilst loss of cell adhesion could lead to detachment of the GEC from the GBM. To investigate whether circulating TGF-$ß_1$ could be detected in IMN, plasma and urine samples of patients with IMN were assayed for TGF-$ß_1$ levels. TGF-$ß_1$ was not detected in any of the plasma samples, irrespective of the patients uC5b-9 status. TGF-$ß_1$ was found in the urines of healthy controls and IMN patients within the same range. In a rabbit model of anti-GBM disease, urinary TGF-ß correlated with the degree of glomerulosclerosis (Coimbra 1990). Urinary TGF-ß does not appear to be disease-related in man in this study.

The studies here do not support a role for either TNF-α or TGF-β_1 in glomerular epithelial cell injury in IMN. To understand the potential role of IL-1ß and IL-6, it is important to appreciate that they function in the context of a cytokine network which is inherently complex and interdependent upon both inhibitory and stimulatory components. A possible reason for the lack of evidence of cytokine involvement may be due to the natural history of IMN. Cytokines are early mediators of inflammation and if these were measured in the early stages of IMN, the cytokine profile could be entirely different. However, the diagnosis of IMN is seldom made before the onset of proteinuria. When proteinuria is present, the condition is established and the cytokine profile would be unremarkable at this stage. To further aid our understanding of the operative mechanisms in the glomerular injury of HMN, other inflammatory mediators should be investigated in addition to the cytokine network.

CHAPTER 7

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The immunopathogenic mechanisms which mediate IMN in man are unknown. Because of the striking similarities in the clinical, histologic and immunopathologic findings between IMN and the PHN model in rats, the disease mechanisms may be similar. In PHN, the subepithelial deposition of IgG reacting with antigens on the GEC membrane resulted in proteinuria. C5b-9 has been shown to be the principal mediator of proteinuria. Increased uC5b-9 excretion was found in PHN before the onset of proteinuria and uC5b-9 was not seen in other forms of experimental models of proteinuric glomerular disease. Furthermore, uC5b-9 excretion accompanied ongoing glomerular immune deposit formation and uC5b-9 excretion resolved when the IgG antibody disappeared despite persisting proteinuria. This animal model suggests that uC5b-9 reflects the presence of complement fixing antibodies to the GEC and represents a sensitive, dynamic marker of active immune disease and ongoing subepithelial immune deposit formation. The primary aim of the project reported here was to determine the reliability of uC5b-9 as a marker of active immunological injury in human IMN.

An ELISA was developed for the detection of C5b-9 in urine using a monoclonal antibody to the human C5b-9 neoantigen and a polyclonal antibody to C7. The usual working range was 25 ng/ml to 2 μg/ml of SC5b-9 and a similar range for MAC. No correlation between proteinuria and uC5b-9 levels was found in a proteinuric control group (mean proteinuria = 5.8 $\pm$ 3.6 g/24hr, $n=45$, $p>0.05$). Furthermore, uC5b-9 levels showed no correlation with a similar range of proteinuria in membranous patients ($n= 40$, $p>0.05$). The coefficients of variation

for the assay were 12% inter-assay and 5% intra-assay, respectively.

Urinary C5b-9 was shown to be a stable molecule with respect to temperature, storage time and repeated freeze-thaw. Some studies have found that uC5b-9 correlated with proteinuria. If the highest uC5b-9 levels are seen in patients with heavy proteinuria, this would invalidate the clinical usefulness of uC5b-9 in determining immunological activity in IMN. This apparent discrepancy was confirmed by parallel immunoassays and the reagents employed in these assays were investigated in an attempt to provide an answer to the discrepancies noted. It was shown to be due to the specificity of the anti-C9 neoepitope capture antibody used.

Urinary C5b-9 was measured serially in seventy-six patients with IMN who did not receive immunosuppressive treatment : fifty-seven patients maintained stable renal function over an observation period of 4.4 years and nineteen patients had declining renal function over an observation period of 5 years. Patients with declining renal function excreted significantly more C5b-9 than did patients following a stable clinical course ($p < 0.001$). Patients with declining renal function were more likely to stabilise and enter spontaneous remission if they stopped excreting C5b-9 ($p < 0.05$).

Treatment was given to eighteen patients with any degree of proteinuria who developed renal impairment. Three patients received more than one treatment regimen because of clinical relapse. Hence, twenty-two treatments were available for analysis. This non-randomised prospective trial was to assess the short and medium-

term effect of three commonly used treatment regimens on the clinical course and excretion of C5b-9. The three immunosuppressive treatment regimens used were : prednisolone alone (n = 13), prednisolone and chlorambucil (n = 4), and prednisolone and cyclophosphamide (n = 5). Patients with uC5b-9 had a better clinical outcome if treatment abolished excretion of C5b-9 ($p<0.005$). All three treatment regimens were able to abolish the excretion of C5b-9 in a manner which correlated with the clinical course. The addition of either one of the alkylating agents did not appear to be more efficient in abolishing the excretion of C5b-9.

This study strongly suggests that uC5b-9 is a marker of ongoing immunological insult in IMN and should be regarded as important in the selection of patients who may benefit from treatment and in monitoring the efficacy of treatment. As different individuals respond variably to a standard regimen, the efficacy of the chosen treatment may be judged by the abolishment of C5b-9 excretion. Since this study suggests that the aim of the treatment regimen chosen should be to abolish the excretion of C5b-9, therapy could therefore be tailored to the individual patient.

While it has been suggested that the MHC type may play a role in the progression of IMN, the HLA DR3 status did not correlate with the C5b-9 excretor status of the patients.

MAC formation is controlled by fluid-phase and membrane-associated inhibitors to prevent cell lysis. To investigate the role of these inhibitors in IMN, urines from six patients known to excrete C5b-9 were analyzed. The membranes in the urines were

isolated by ultracentrifugation, solubilised in 1% Zwitt and urine MAC was fractionated by gel filtration. Both the membrane component and the supernatant were subjected to ELISA and SDS-PAGE and Western blotting for different control proteins. S-protein, a fluid-phase MAC inhibitor, was found both in the membrane and the supernatant. Of the membrane-bound MAC inhibitory proteins studied, CD59 was the only antigen detected in the membrane component and in the supernatant. The presence of the CD59 antigen in C5b-9 complexes may be a marker for the membrane assembled form as opposed to the S-protein soluble complexes. The results of this study have suggested that CD59 expression may be upregulated in response to complement injury to prevent epithelial cell lysis but not sufficient to prevent MAC-induced proteinuria.

C5b-9 plays a pivotal role in the induction of proteinuria in PHN. However, the subsequent effector mechanisms for induction of proteinuria following complement activation remain speculative. Non-lethal doses of C5b-9 have been shown to induce cytokine production in glomerular cells. Paired plasma and urine samples were collected on dates when twenty IMN patients were excreting C5b-9 and again when they were not. The cytokines IL-1ß, IL-6, TNF-α and TGF-$ß_1$ were assayed by ELISA. TNF-α were not detected in plasma and urine. TGF-$ß_1$ was not detected in plasma but was found in the majority of urine samples including healthy controls. IL-1ß was significantly raised in the plasma of IMN patients compared to healthy controls ($p < 0.05$), but there was no correlation with the uC5b-9 status of the patients. Urinary IL-1ß was detected in both patients and healthy controls. Plasma IL-6 was detected in five patients within the normal range. However, urinary IL-6

was significantly elevated in the IMN group compared to healthy controls ($p < 0.05$), but it did not correlate with uC5b-9. C5b-9 does not appear to release cytokines as inflammatory mediators in the glomerular injury of IMN. The inflammatory mediators of proteinuria in IMN remain unresolved. These findings suggest that the role of other inflammatory mediators such as eicosanoids and reactive oxygen metabolites should be investigated in addition to the cytokine network in future studies.

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