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Heymann nephritis: Mechanisms of renal injury

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The Heymann nephritis (HN) models of rat membranous nephropathy are extraordinarily valuable tools to investigate the immunopathology of glomerular subepithelial immune deposit formation and the mechanism by which such deposits injure glomeruli. The immunopathology of HN is reviewed by Brentjens and Andres in this volume [1]. In this review we will largely confine ourselves to a discussion of the mediators, mechanisms and pathophysiological consequences of renal injury in rat membranous nephropathy.

The primary manifestation of glomerular injury in HN is proteinuria; however there are also subtle, but not trivial, changes in glomerular hemodynamics and tubular function, and alterations in renal hormone production. The alterations in glomerular permselectivity that lead to proteinuria closely resemble the defect in human membranous nephropathy, and the rat model has been used to study mechanisms of renal sodium retention, edema formation and metabolic abnormalities in nephrotic syndrome. Investigators have also used the HN models to study the impact of systemic hypertension on pre-existing glomerular disease, in terms of both altered glomerular function and progressive renal injury.

Mostly, the mechanisms and effects of glomerular injury in rat membranous nephropathy have been investigated in the standard active and passive (PHN) models of HN; however, both models have also been extensively modified to elucidate specific mechanisms or mediators. Such modifications, which include the use of the isolated perfused kidney, renal transplantation, partial renal ablation and, more recently, cell and tissue culture, will be discussed in context later in this review.

Active HN is induced by immunizing susceptible strains of rats (such as, Lewis, Fisher) with certain fractions of homologous or heterologous proximal tubular brush border and is characterized by granular glomerular capillary wall deposits of rat IgG and subepithelial electron-dense deposits after three to four weeks. Proteinuria develops in 30 to 80% of rats within eight to ten weeks of immunization. PHN is induced by a single intravenous injection of heterologous anti-brush border antiserum (anti-Fx1A) that produces heterologous IgG deposits which accumulate in glomeruli over hours and days. When an appropriate complement-fixing antiserum is used, proteinuria occurs in almost all animals within five days [2, 3]. This "heterologous phase" is then 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. HN more closely resembles human membranous nephropathy in its slow evolution than does PHN and it is truly an autoimmune disease. In those HN rats that become proteinuric, urine protein excretion and glomerular dysfunction tend to be more severe than in PHN; however, the variability between animals and the duration of onset make it a less suitable model than PHN for studying the mediators of injury.

Mediation of injury in PHN

Role of complement

There is now considerable evidence that implicates the C5b-9 membrane attack complex (MAC) in immunological injury to the glomerulus [reviewed in 4, 5], and most derives from the study of the PHN model of experimental membranous nephropathy in which the glomerular visceral epithelial cell (GEC) appears to be the primary target of injury. The first clue was provided by the observation that urine protein excretion in PHN is mediated by complement but is leukocyte-independent [6]. A single intravenous injection of anti-Fx1A results in binding of antibody to intrinsic GEC antigens and formation of subepithelial immune deposits in situ [7, 8]. Activation of the complement cascade by the subepithelial immune complexes leads to proteinuria four to five days after the administration of antibody (Table 1). Granular deposits of rat C3 are present in identical distribution to heterologous IgG, but without detectable leukocyte infiltration by either light or electron microscopy. Decomplementation of rats with cobra venom factor, before administration of antibody, does not affect glomerular antibody deposition, but abrogates the proteinuria (Table 1). In contrast, administration of anti-neutrophil serum prior to anti-Fx1A has no effect on protein excretion (Table 2).

Injury is mediated by similar mechanisms in the autologous phase of PHN [9]. Kidneys from rats passively immunized with a subnephritogenic dose of non-complement-fixing $\gamma 2$ sheep anti-Fx1A IgG ("planted" antigen) were transplanted into recipient rats that were either actively pre-immunized with sheep IgG or passively immunized with complement-fixing anti-sheep IgG, and both groups developed proteinuria. Proteinuria did not develop when rats, either actively or passively immunized, were decomplemented with cobra venom factor prior to transplantation. Neutrophil depletion of passively immunized animals did not inhibit the development of proteinuria.

In PHN, complement-depletion appears to be ineffective at abolishing proteinuria once it has already been established. Administration of cobra venom factor to rats with PHN during the naturally-occurring autologous phase of the disease (>8 days after injection of heterologous anti-Fx1A) does not dimin-

Table 1. Urine protein excretion in rats with PHN treated with cobra venom factor (CVF) or saline

Group	Serum C3, day 5 *% base line*	Urine protein, day 4–5 *mg/24 hr*
Saline	133 ± 121 (6)	70 ± 15 (16)
CVF	6 ± 4 (13)	4 ± 1 (16)

Values are mean ± SEM; number studied is in parentheses. From [7].

Table 2. Urine protein excretion in rats with PHN treated with anti-neutrophil globulin or nonimmune globulin

Group	Neutrophil count *cells/mm³*	Urine protein, day 3–4 *mg/24 hr*
Nonimmune	1100–5000 (6)[a]	25 ± 10 (6)[b]
Anti-neutrophil	0–200 (5)	34 ± 10 (5)

Number studied is in parentheses. From [7].
[a] Range of daily neutrophil counts
[b] Mean ± SEM

Table 3. Urine protein excretion in isolated rat kidneys with or without subepithelial antigen, perfused with antibody and plasma[a]

Group	CH_{50} *% normal*	Urine protein, 110 min *μg/min*
With antigen		
Normal plasma	50	4269 ± 1208[b] (8)
Heat-inactivated plasma	0	102 ± 57 (6)
C8-deficient plasma	0	236 ± 62 (6)
Without antigen		
Normal plasma	50	299 ± 122 (6)

[a] Human plasma: 50% vol/vol in buffer containing bovine albumin;
[b] Mean ± SEM; number studied is in parentheses. From [13].

ish protein excretion [6]. The reason for this is not established, but the findings suggest that additional mechanisms may be involved in the maintenance of glomerular injury after the initial complement-dependent insult.

These studies exclude a role for the chemotactic effects of complement, and when taken in conjunction with the findings in a rabbit model of membranous nephropathy [10], suggest that injury in PHN is mediated by the terminal C components that form the C5b-9 membrane attack complex (MAC). Activation of the terminal complement pathway [reviewed in 11] follows cleavage of C5 by the C5 convertase and leads to the formation of the C5b6 complex which remains associated with the C5 convertase and is able to bind C7. The C5b-7 complex acquires hydrophobic phospholipid-binding domains after binding C7 and can insert into a cell membrane but without deleterious effects on membrane integrity. It can, however, bind C8 and the resulting complex induces membrane damage in a process accelerated by the subsequent binding of C9. Thus, C8 is the component of the MAC which is essential for membranolysis. Depletion of C8 is difficult to achieve experimentally in vivo, and there are no C8-deficient strains of rats. Therefore, a cell-free isolated perfused kidney model was used to obtain definitive evidence for the pathogenic role of MAC in PHN [12]. As in the passive-autologous model in vivo, rats were injected with a subnephritogenic dose of the non-complement-fixing γ2 subclass of sheep anti-Fx1A IgG prior to kidney perfusion; this was done to provide a "planted" antigen. Kidneys were then removed and perfused in vitro with complement-fixing anti-sheep IgG together with various human plasmas as a source of complement. Perfusions with complement-replete normal plasma resulted in a marked increase in proteinuria (Table 3). In contrast, protein excretion remained at control values when kidneys were perfused C8-deficient plasma (Table 3) from an individual with a congenitally dysfunctional C8 molecule [13]. Reconstitution of the C8-deficient plasma with normal C8 restored proteinuria to the level seen with normal plasma. Glomerular antibody deposition was not affected by the absence of C8 [12]. Similar observations have also been made using a heterologous-phase model of PHN in the isolated perfused rat kidney [14]. Perfusion of kidneys containing complement-fixing γ1 anti-Fx1A IgG resulted in increased proteinuria when perfused simultaneously with complement-replete plasma, but not with C8-deficient human plasma or C6-deficient rabbit serum. Whereas the combined use of the C8- and C6-deficient reagents, whether simultaneously or in sequence, simulated the effect of normal plasma. It is reasonable to conclude that injury in PHN is mediated by the MAC as the only known function of C8 is its essential role in complement-mediated membrane injury.

This conclusion is supported by a study in which rats were depleted of C6 in vivo by injection of an IgG fraction or $F(ab')_2$ fragments of an antibody against rat C6 which reduced serum C6 hemolytic activity to <5% of normal [15]. These rats developed subepithelial immune deposits in amounts equal to those in complement-replete rats after administration of anti-Fx1A but proteinuria developed only in rats with an intact terminal complement pathway.

Formation of the MAC results in the development of MAC "neoantigens" [11]; antigenic determinants that are found on the assembled complex of C5b-9 but not on the individual complement molecules. Antibodies that recognize only the neoantigenic determinants have been used to demonstrate, by direct immunofluorescence [16] and by immunoelectron microscopy [17], the MAC complex in glomeruli of rats with PHN. Moreover, the presence of the MAC correlated with complement-mediated proteinuria [16]. In these models of PHN, glomerular injury develops relatively acutely, enabling the investigators to define a functional role for terminal complement components, either in vivo or in vitro. It is more difficult to show that the MAC has a functional role in active HN because long-term complement depletion is not technically feasible. Nevertheless, indirect evidence suggests that proteinuria is also complement-mediated in active HN. Immunization of Lewis rats, or other susceptible strains, with Fx1A, results in the development of subepithelial immune deposits in all rats after six weeks [18]. Of these, about 60% demonstrate glomerular C3 deposition by immunofluorescence microscopy and are proteinuric. The remainder do not have C3 deposits and excrete normal levels of protein but if given a booster injection of Fx1A, roughly 60% will develop glomerular deposits of C3 and proteinuria. Terminal complement components and MAC neoantigens can be identified in the glomeruli of rats with HN, and are distributed in a peripheral granular pattern resembling that of IgG and C3 [19]. Thus, the close association of proteinuria with glomerular complement deposits in the absence of inflammatory

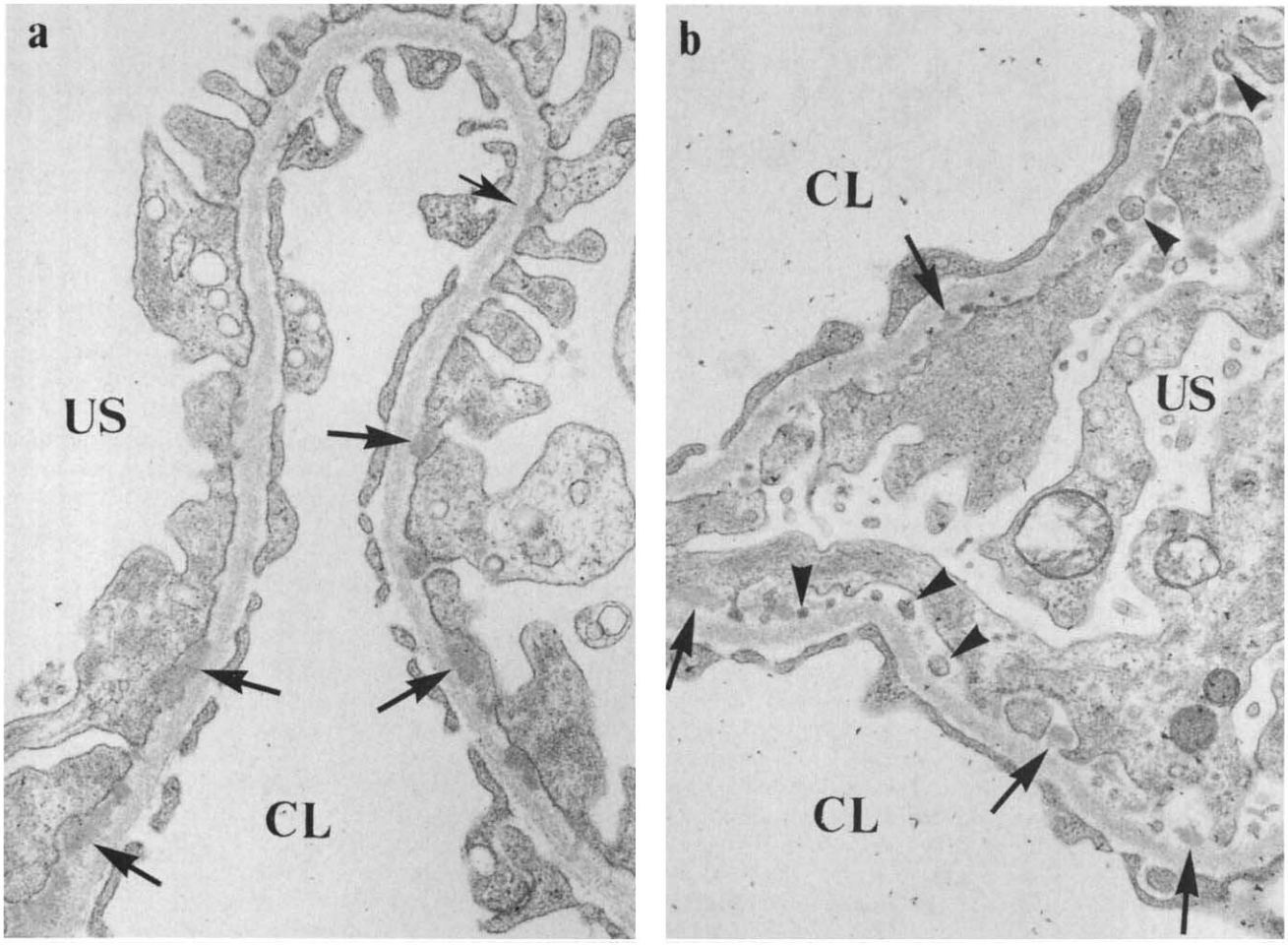

Fig. 1. *Glomerular epithelial cell lesions induced by antibody and complement in the isolated perfused kidney model of autologous-phase PHN.* (**A**) Kidney containing γ2 sheep anti-Fx1A, perfused with complement-fixing anti-sheep IgG and heat-inactivated plasma. Electron-dense deposits (arrows) are present in the subepithelial space. Epithelial morphology appears normal. Notice preservation of the diaphragms in epithelial slits (× 35,000). Similar morphology was observed in antigen-containing kidneys perfused with antibody and C8-deficient plasma. (**B**) Kidney containing γ2 sheep anti-Fx1A, perfused with anti-sheep IgG and complement-replete plasma. Glomerular capillary loop shows striking epithelial cell abnormalities, with effacement of foot processes, microvillous transformation of the plasma membrane and vacuolization. Electron-dense deposits (arrows) are present on the epithelial side of the GBM. Retraction of epithelium, disruption of filtration slit diaphragms and formation of small membrane vesicles (arrowheads) are seen in the vicinity of electron-dense deposits in the lamina rara externa (× 35,000). US, urinary space; CL, capillary lumen. Electron micrographs were kindly provided by Dr. Helmut Rennke, Brigham and Women's Hospital, Boston, MA, USA.

cells suggests that glomerular injury in HN is dependent on the formation of the MAC.

Target of injury

The studies reviewed in the previous section provide strong evidence that the MAC is present and active in inducing glomerular capillary wall injury in PHN. The late stages of both PHN and active HN are characterized by distortion and thickening of the glomerular basement membrane (GBM) around subepithelial immune deposits but it is not known whether the GBM is the primary site of damage. Moreover, in early PHN proteinuria occurs before ultrastructural changes in the GBM are apparent [2]. This does not preclude the possibility that alteration of the biochemical composition of the GBM might change its charge-selective and/or size-selective filtration properties. On the other hand, there are several reasons for believing that visceral glomerular epithelial cells (GEC) are the initial target of the antibody-directed, complement-mediated injury that is responsible for proteinuria. Effacement and displacement of epithelial cell foot processes, and microvillous transformation are found in PHN, and are accompanied by GEC dysfunction as demonstrated by the impaired clearance of protamine-heparin aggregates [20]. These GEC changes are not unique to PHN, and occur in other proteinuric disorders associated with epithelial injury, including aminonucleoside nephrosis [21]. More direct evidence for GEC injury in PHN has been obtained from the isolated perfused kidney model [12]. In these experiments, kidneys perfused in vitro with complement-fixing antibodies and heat-inactivated or C8-deficient human plasmas contained glomerular subepithelial electron-dense deposits but had normal GEC morphology (Fig. 1A). In contrast, kidneys perfused with the same antibody in the presence of complement developed severe GEC injury in the vicinity of subepithelial immune deposits (Fig. 1B); the changes included

extensive effacement of foot processes and villous transformation, and microvesiculation of the plasma membranes, and vacuolization and degeneration of some of the epithelial cells. In some capillary loops the epithelium detached to leave the GBM denuded (Fig. 1B). In the majority of loops, however, the GEC were abnormal but apparently viable. Such changes have not been demonstrated in isolated perfused kidneys made proteinuric by other methods which are complement-independent [22, 23]. This indicates that they are unlikely to be nonspecific effects of proteinuria but are likely to be the result of C5b-9 insertion into the plasma membranes. The severity of GEC injury seen in the perfused kidney model is probably facilitated by delivery of complement to the subepithelial space due to the intrinsic proteinuric nature of the model and by the use of heterologous complement components [14]. Nevertheless, these studies show conclusively that sublethal GEC injury can occur when MAC is assembled in the subepithelial space by complement-fixing antibodies. Moreover, the prompt onset of proteinuria coincident with the appearance of GEC injury, makes it unlikely that alterations in GBM composition are the cause of proteinuria.

Direct evidence of localization of the MAC in PHN has been provided by a recent immunoelectron microscopic study [17]. Using a monoclonal antibody to rat C5b-9 neoantigens, Kerjaschki et al showed that C5b-9 was localized in subepithelial immune deposits at the GBM-GEC interface, as well as in GEC multivesicular bodies, and was visible being exocytosed from GEC into the urinary space. The authors concluded that the MAC was assembled on the GEC membrane near the site of immune deposits, was selectively transported intracellularly, and was then extruded into the urinary space.

Schulze et al [24] have obtained additional evidence for C5b-9 assembly on the epithelial aspect of the glomerular capillary wall. Using an enzyme-linked immunoabsorbent assay for MAC neoantigens, they showed that the C5b-9 complex was shed into the urine of rats with PHN, but was confined to the circulation of rats made proteinuric with aminonucleoside of puromycin and infused with zymosan-activated serum, and of proteinuric rats in which immune deposits were formed on the endothelial aspect of the capillary wall. Thus, it was demonstrated that the MAC is too large to traverse the GBM despite heavy proteinuria, and that urinary excretion of the MAC may be a marker of complement-mediated GEC injury.

Secondary mediators of proteinuria

Nucleated cells are generally more resistant to complement attack than erythrocytes, and require multiple "hits" by the MAC for lysis to occur [25]. Furthermore, in nucleated cells, "sublytic" amounts of the MAC can alter plasma membrane integrity without causing cytolysis, and can activate cells to produce various soluble mediators, some of which have been implicated in disorders of glomerular permselectivity. Among these, the role of eicosanoids has been examined most closely, both in cultured GEC [26] and in PHN. Glomerular thromboxane production is increased in proteinuric rats with PHN [27, 28] and this increase is complement-dependent [27]. Cyclooxygenase inhibition with indomethacin reduces proteinuria by ~60% in active HN [29] and by 74% in the autologous phase of PHN [28]; in this latter study, there was a quantitatively similar decrease in urinary thromboxane excretion. The glomerular filtration rate was not substantially altered in either study. In the isolated perfused rat kidney model of PHN, inhibition of thromboxane synthetase with OKY-046, in doses sufficient to suppress renal thromboxane production, reduced complement-dependent proteinuria by ~75% without altering glomerular filtration rate or renal vascular resistance [30]. In contrast, in vivo treatment of PHN rats with the thromboxane synthetase inhibitor UK38485 inhibited glomerular thromboxane production by ~80% in the heterologous phase [27] and urinary thromboxane excretion by about 50% in the autologous phase [28], but in neither case did it reduce proteinuria. Thus, it appears that complement-mediated GEC injury can induce endogenous glomerular cells to increase their production of eicosanoids (particularly thromboxane A_2), but that the pathophysiological consequences of this phenomenon are, as yet, incompletely understood. Further studies will be required to determine whether augmented glomerular eicosanoid synthesis in PHN is incidental to cellular injury and the functional derangements, or not.

The administration of scavengers of reactive oxygen species to rats with PHN has suggested a role for reactive oxygen species in the pathogenesis of glomerular injury. By analogy to observations in cultured mesangial cells [31], complement-mediated injury of GEC in PHN could be associated with the production of reactive oxygen species, and thus be involved in the mediation of proteinuria. This has been investigated by administration of dimethylsulfoxide (DMSO), a scavenger of the hydroxyl radical, shortly after injection of heterologous anti-Fx1A, or later, when glomerular injury was already established. It resulted in a ~60% reduction in proteinuria during the autologous phase [32, 33]. In these studies, immunofluorescence staining for glomerular C3 suggested that less C3 was deposited in glomeruli of rats that were treated with DMSO. Therefore, while DMSO may have reduced proteinuria by inhibiting production of the hydroxyl radical, the authors suggested that DMSO may have acted by reducing glomerular complement deposition. Recently, Shah [34] demonstrated that dimethylthiourea, another scavenger of the hydroxyl radical, reduced heterologous phase proteinuria in PHN by >60%. In these experiments, quantitative studies of radiolabelled anti-Fx1A binding demonstrated that there was no reduction in antibody deposition in dimethylthiourea-treated rats compared to untreated controls, and immunofluorescence microscopy suggested that there was no difference in C3 deposition between the two groups. A second hydroxyl scavenger, sodium benzoate, also produced a large reduction in proteinuria as did the iron-chelating agent deferoxamine. This suggests that iron participates in the generation of hydroxyl radicals through the Haber-Weiss reaction. In contrast, superoxide dismutase, a superoxide scavenger, and catalase, a hydrogen peroxide scavenger do not affect proteinuria. While these latter observations suggest that the superoxide radical and hydrogen peroxide are not involved in the pathogenesis of proteinuria in PHN, it should be noted that superoxide dismutase and catalase are large molecules that may not be able to effectively permeate through the GBM to the GEC, the site of injury and likely site of oxygen radical generation. Nevertheless, these results implicate intracellularly generated hydroxyl radicals in the development of proteinuria in PHN. The mechanism by which the MAC

produces reactive oxygen species in GEC remains to be elucidated.

Pathophysiology of Heymann nephritis

Proteinuria, the principal functional abnormality in HN and PHN could arise in two possible ways. First, an increase in macromolecular flux across the glomerular capillary wall could occur purely on the basis of glomerular hemodynamic alterations. Second, the permselective properties of the filtration barrier could be altered by structural or compositional changes in the capillary wall.

Functional and morphological changes documented in the isolated perfused rat kidney model of experimental MN [12] point to a structural defect in the filtration barrier, and suggest that this defect is sufficient to cause proteinuria; however, additional data indicates that the magnitude of the defect is influenced by hemodynamic factors. Micropuncture studies in Munich Wistar rats have shown that glomerular function in PHN is characterized by marked proteinuria, elevated transcapillary hydraulic pressure difference, and depressed ultrafiltration coefficient; the net effect reduces single nephron glomerular filtration rate (SNGFR) [35–38]. In addition, using dextran clearance techniques, Yoshioka et al demonstrated that fractional clearance of low molecular weight uncharged dextrans (radius of 28 to 40Å) is similar in PHN and control rats, but that of large dextrans (radius >40Å) is elevated in PHN [38]. A theoretical model of macromolecule transfer that depicts the glomerular capillary wall as a size-selective isoporous membrane with a minor nonselective "shunt" pathway [39] revealed that the calculated mean pore radius is similar in PHN and control rats, but the fraction of filtrate permeating the shunt pathway in PHN is greater than in controls [38]. Acute blockade of angiotensin II in PHN lowers glomerular capillary pressure (and SNGFR) but has no effect on proteinuria (or ultrafiltration coefficient) [40]. In contrast, intra-aortic infusion of angiotensin II further elevates the transcapillary hydrostatic pressure difference and aggravates the fractional clearance of large dextrans, protein excretion, and fraction of filtrate permeating the shunt pathway [38]. Normalizing the transcapillary pressure difference of proteinuric PHN rats by intra-aortic infusion of acetylcholine brings about a partial parallel decline in the fractional clearance of large dextrans together with a similar reduction in urine protein excretion and the fraction of filtrate permeating the shunt pathway [38]. These results indicate that the size-selective defect in PHN is aggravated by glomerular hypertension and that the sieving abnormality is alleviated, but not abolished, by lowering glomerular capillary pressure. Parenthetically, the macromolecular clearance data and sieving defect in PHN are similar to those described in human MN by Shemesh et al [41].

It is still not known how nonlytic, complement-mediated GEC injury contributes to proteinuria, and one can only speculate on possible mechanisms. These include GEC shape alterations that might distort the GBM and lead to a loss of GBM permselectivity, or displacement and disruption of filtration slit-diaphragms causing defects in the epithelial barrier. The GEC is also partly responsible for the synthesis and maintenance of the GBM by synthesizing matrix components including type IV collagen, laminin and proteoglycans. Although disordered matrix synthesis and/or degradation may be responsible for long-term alterations in glomerular permeability and GBM thickening characteristic of advanced experimental [42] and human MN, it probably does not account for the rapid onset of complement-mediated proteinuria seen in PHN.

The role of complement in the hemodynamic alterations in PHN has not been fully elucidated. Recent data suggest that complement may be responsible for the elevated transcapillary pressure gradient and reduced ultrafiltration coefficient in PHN [37]. This effect could be due to the direct action of C5a [43] or secondary to vasoactive eicosanoids or other inflammatory mediators released from complement-injured glomerular cells [26–34].

The pathophysiology of glomerular and tubular function have also been studied in HN. In early HN, before the onset of proteinuria, GFR, SNGFR and renal plasma flow (RPF) are normal; however, after the onset of proteinuria, whole kidney GFR and RPF are reduced by about 40%, and proximal tubular dysfunction (including reduced glucose and fluid reabsorption and impaired organic anion secretion) is observed [44]. At this stage, SNGFR is uniformly depressed and the tubular disorders coincide with the filtration and binding of anti-brush border antibodies to proximal tubular cells together with morphological evidence of tubular injury (Brentjens, this volume, 1). Later in the course of HN, substantial heterogeneity of nephron function develops [35, 45]. Whole kidney GFR is persistently reduced by 30 to 40% but SNGFR is found to vary widely between nephrons, apparently due to variable closure of glomerular capillary loops [35, 45]. Despite this heterogeneity, glomerulotubular balance is well maintained and the absolute proximal tubular reabsorption of fluid and SNGFR are directly correlated in individual nephrons [35]. The variability of nephron function in HN has been ascribed to nonuniform distribution of IgG and C3 in different glomeruli [35, 45] but not all investigators agree [44].

The superimposed effect of pre-existing hypertension on proteinuria, blood pressure, renal function and renal morphology in HN has been studied in spontaneously hypertensive rats (SHR) [46, 47] and in rats made hypertensive with oral desoxycorticosterone acetate and 1% saline (so-called DOCA-salt hypertension) [48–50]. Blood pressure is usually normal in rats with HN and PHN [46, 49, 50]. DOCA-salt hypertension accelerates the onset and exacerbates the severity of proteinuria, reduces survival and causes glomerular sclerosis and tubulointerstitial and vascular damage in HN [49]. Similar effects have been observed in the autologous phase of PHN, associated with accentuated thickening of the GBM [50]. DOCA-salt hypertension is also more severe in rats with HN [48]. On the other hand, HN only modestly aggravates hypertension in SHR [46, 48], but there is a marked increase in proteinuria and profound effects on renal morphology; these include accelerated focal glomerular sclerosis and interstitial fibrosis, and proliferative and necrotizing arteriolitis reminiscent of malignant hypertension [47]. These findings in SHR have interesting pathophysiological implications. They suggest that, in contrast to the situation in non-nephritic SHR, the high systemic blood pressure is transmitted to some hyperperfused glomeruli in SHR with HN [35, 45]. This would be expected to exacerbate the defect in glomerular permselectivity [38] and might lead to focal glomerular sclerosis [51]. The necrotizing vascular lesions in HN-SHR are not seen in non-nephritic SHR

and have been attributed to intravascular thrombosis and hypercoagulability [47].

Heymann nephritis as a model of nephrotic syndrome

During the course of HN, affected animals develop biochemical abnormalities similar to those in human nephrotic syndrome: namely, hypoalbuminemia with decreased plasma colloid oncotic pressure, hyperlipidemia, and elevated plasma concentrations of fibrinogen and depressed plasma concentrations of antithrombin III [52, 53]. Despite the reduction in plasma colloid oncotic pressure and alterations in coagulation factors, edema and thromboembolic phenomena are unusual in HN [53]. The lack of edema in HN is reflected by a normal relationship between the plasma volume and extravascular sodium space [53]. However, when extracellular volume is expanded by inducing renal insufficiency with 7/8 nephrectomy, rats with HN develop edema and ascites [53]. This may be explained by the finding that rats with HN have a reduced ability to excrete a salt load during plasma volume expansion, an effect attributed to increased distal tubular reabsorption of sodium [54]. Kaysen, Kirkpatrick and Couser [55] have studied the effect of dietary protein on albumin metabolism in PHN. Regardless of diet, all animals had progressively declining serum albumin levels for 10 days following anti-Fx1A injection, at which time steady state hypoalbuminemia was reached despite continuing heavy proteinuria. In PHN rats fed a normal or high protein diet, this steady state was due to a combination of increased synthesis and decreased catabolism of albumin. Serum albumin was maintained constant in rats fed a low protein diet because of increased albumin synthesis.

Progressive PHN

Following the acute severe proteinuria of PHN, there is a period of stable, low-grade proteinuria lasting for 60 to 150 days [56]. After this so-called latent phase, proteinuria increases progressively and is associated with decreasing creatinine clearance, hypertension and eventually death. The morphological features of the chronic phase include thickened GBM with intramembranous electron dense deposits, and heterologous and autologous IgG deposits by immunofluorescence [56].

Progressive PHN with severe proteinuria after 16 weeks and circulating autoantibodies to brush border has been induced in rats already made proteinuric by pre-immunization with bovine serum albumin to induce serum sickness [57] or by injection of anti-GBM antiserum [58]. Progressive injury is also affected by uninephrectomy [42]; again proteinuria does not diminish after the acute phase of PHN, but increases progressively up to 29 weeks following anti-Fx1A injection. These animals developed mesangial sclerosis associated with deposition of type IV collagen and the MAC in sclerotic areas; they also develop glomerular crescents and interstitial fibrosis [42]. These results together with the effects of hypertension [46, 48, 50] emphasize the potential role of hemodynamic factors in determining the outcome of immunologic renal disease.

Complement induced injury of cultured glomerular epithelial cells

Improved techniques for stable GEC culture [59] have provided the opportunity to test whether nephritogenic antibodies, such as anti-Fx1A are capable of inducing complement-dependent cytotoxicity in vitro and are particularly suitable to investigate the sublytic cytotoxic effects of complement at a cellular level.

Anti-Fx1A and anti-gp 330 bind directly to the plasma membrane of cultured rat GEC [60, 61], and so we [62] and others [63], have assessed the ability of nephritogenic anti-Fx1A to induce cellular damage of primary and long-term cultures of rat GEC. GEC were sensitized with the complement-fixing γ1 subclass of sheep anti-Fx1A IgG and exposed to various sera serving as sources of complement [62]. Incubation of antibody-sensitized GEC in normal human serum resulted in a sigmoidal relationship between cytolysis and complement dose, consistent with the "multi-hit" requirement for cytolysis observed with other nucleated cell types [25]. Cytolysis only occurred when both anti-Fx1A and normal serum were present and not if nonimmune IgG or C8-deficient plasma were substituted; reconstitution of C8-deficient plasma with purified rat C8 restored cytolysis however. Therefore, the nephritogenic antibody of PHN directs insertion of the MAC into the GEC plasma membrane, resulting in cytotoxicity. Noncytolytic ("sublytic") injury was assessed using antibody-sensitized GEC that were preloaded with biscarboxyethyl carboxyfluorescein (BCECF; molecular wt ~520 daltons), a low molecular weight molecule that is largely retained by nonpermeabilized cells [62]. When sensitized cells were subsequently incubated with a series of limiting doses of complement, there was greater BCECF release than release of lactate dehydrogenase (molecular wt 140,000 daltons, a marker of cell death) at all serum concentrations (Fig. 2A), thus demonstrating cell membrane perturbation in the absence of cell death. Transmission electron microscopy of cultured rat GEC that were incubated with sublytic amounts of C5b-9 showed vesiculation of the apical and basolateral plasma membranes, and shedding of membrane vesicles (Fig. 2B, 2C) [62, 63]. C5b-9 neoantigens were localized by immunohistological techniques to the plasma membranes, and were shed in membrane vesicles (Fig. 2D) [63]. In addition, GEC that were sublytically injured by C5b-9 demonstrated loss of desmosomal junctions, suggesting that C5b-9 may have affected the intermediate filament structure (Fig. 2C) [62]. It should be noted that many of these ultrastructural findings in cultured rat GEC that were sublytically injured by the MAC are similar to the GEC ultrastructure in PHN in the perfused kidney model (discussed above).

Sublytic doses of complement, in addition to altering cell membrane integrity and morphology in nucleated cells, can stimulate phospholipid turnover with release of arachidonic acid and production of eicosanoids [64]. In studies using GEC outgrowths from explanted glomeruli, Hänsch et al [26] showed that C5b-9 stimulated a dose-dependent release of prostaglandin E and thromboxane B_2 within 5 to 10 minutes. Prostanoid release was stimulated less effectively by C5b-8, and not at all by C5b6. Prostaglandin E production was inhibited by both cyclooxygenase and phospholipase inhibitors. These findings are consistent with a complement-mediated increase in membrane phospholipid turnover and are consistent with the observations of stimulated glomerular prostanoid production in PHN (discussed above). However, the results require confirmation in a homogenous culture of GEC because the cell culture system employed does not exclude the possibility that the prostanoids

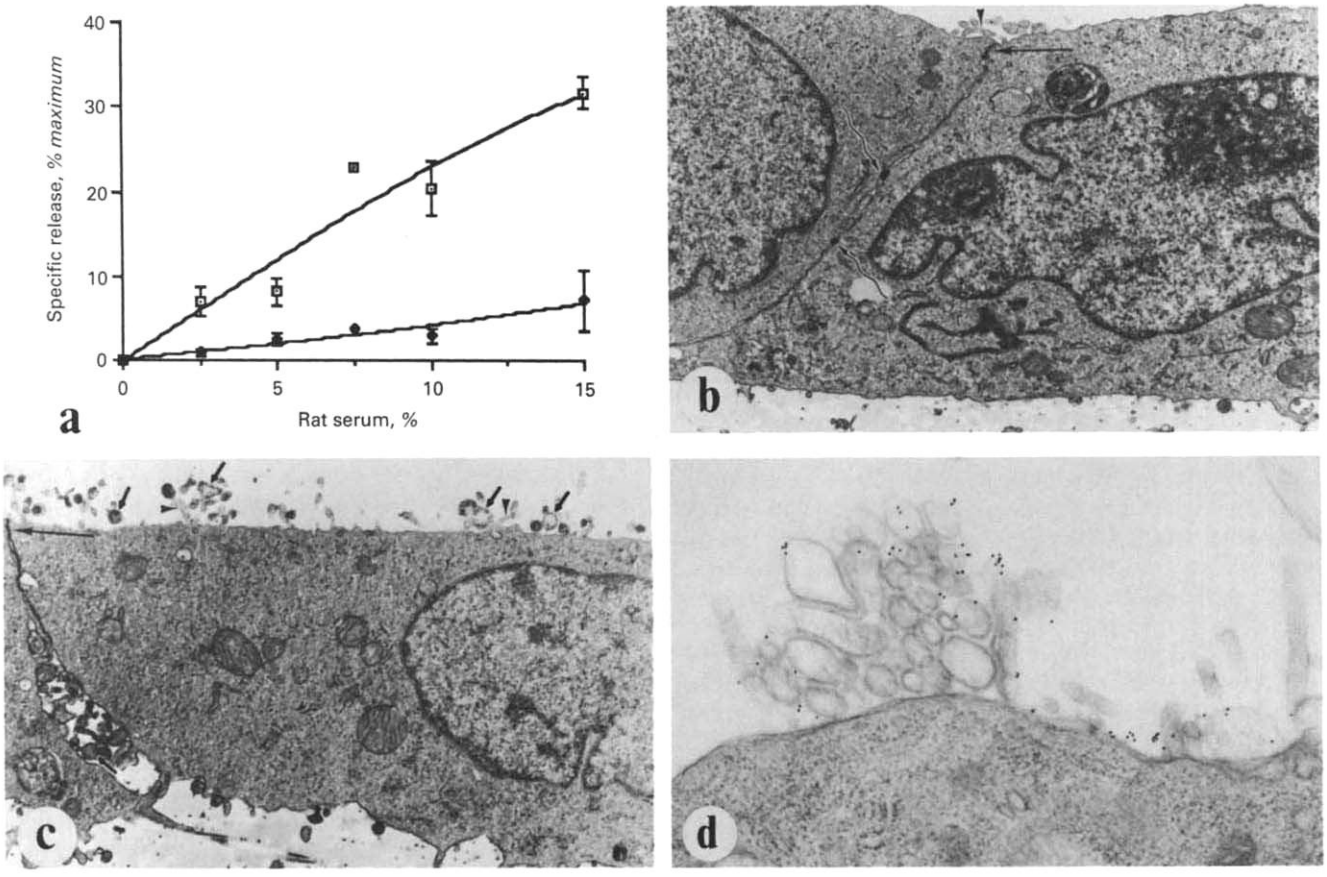

Fig. 2. *Complement-mediated sublytic cytotoxicity of glomerular epithelial cells (GEC) in culture.* (a) GEC on a collagen matrix were loaded with biscarboxyethyl carboxyfluorescein (BCECF; molecular wt ~520 daltons), incubated with anti-Fx1A IgG (1 mg/ml, 22°C, 30 min) and then various concentrations of rat serum as a source of complement (37°C, 40 min). Release of BCECF (□), an indicator of cell membrane damage, is greater at all serum concentrations than release of lactate dehydrogenase (LDH, ◆), an index of cell death. (b) Transmission electron micrograph of antibody-sensitized GEC exposed to buffer alone. Cells appear normal with apical microvilli (arrowhead), tight junctions (long straight arrow), and desmosomes (wavy arrows) (× 5400). (c) Transmission electron micrograph of antibody-sensitized GEC exposed to sublytic concentrations of rat serum (7.5%). Microvilli (arrowheads) and tight junctions (long straight arrow) are present as in controls, however desmosomes are absent. In addition, numerous vesicular structures are seen both on the apical surface and between cells (short arrows) (× 5400). (d) Immunoelectron microscopy of the MAC on GEC in culture. GEC were exposed to anti-brush border vesicle IgG (50 μg/ml) and rat serum (15% vol/vol) for 15 min at 37°C. The medium was changed and the incubation continued for 30 min. The cells were then fixed, and stained for rat C9 with colloidal gold-labelled antisera. C9 is associated with membrane shed from the cell and to membranes remaining attached to the cell surface (× 25,000). (Fig. 2D reprinted from reference 66 with permission.)

might be derived from complement-stimulated mesangial cells [65] within adherent glomeruli. Using homogeneous cultures of rat GEC, Cybulsky et al [66] recently demonstrated that C5b-9 rapidly increased cellular concentrations of 1,2-diacylglycerol and inositol trisphosphate, consistent with the activation of phospholipase C. This was associated with an increase in the intracellular free Ca^{2+} concentration due to release of Ca^{2+} from intracellular nonmitochondrial storage sites, as well as Ca^{2+} influx into cells. In addition, free arachidonic acid was elevated, suggesting that activation of phospholipase A_2 had occurred. These observations also suggest that C5b-9 might stimulate synthesis of eicosanoids in GEC. The pathways of arachidonic acid release and eicosanoid synthesis in GEC remain to be fully elucidated.

Thus, cell culture experiments have confirmed that GEC can be a target for sublytic complement-mediated injury, and are beginning to provide information on the alterations in cell function induced by such injury. Further studies are necessary to determine if C5b-9-induced phospholipase activation, elevation in intracellular free Ca^{2+} concentration, and release of eicosanoids lead to altered GEC morphology and impaired glomerular permselectivity in PHN.

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References

1. Brentjens JR, Andres G: Lesions of the kidney caused by the interaction of antibodies with antigens on the surface of renal cells. *Kidney Int* 35:954–968, 1989
2. Salant DJ, Belok S, Stilmant MM, Darby C, Couser WG: Determinants of glomerular localization of subepithelial immune deposits. Effects of altered antigen to antibody ratio, steroids, vasoactive amine antagonists, and aminonucleoside of puromycin on passive Heymann nephritis in rats. *Lab Invest* 41:89–99, 1979
3. Salant DJ, Darby C, Couser WG: Experimental membranous glomerulonephritis in rats: Quantitative studies of glomerular immune deposit formation in isolated glomeruli and whole animals. *J Clin Invest* 66:71–81, 1980
4. Couser WG, Baker PJ, Adler S: Complement and the direct mediation of immune glomerular injury: A new perspective. *Kidney Int* 28:879–890, 1985
5. Cybulsky AV, Quigg RJ, Salant DJ: Role of the complement membrane attack complex in glomerular injury, in *Contemporary Issues in Nephrology* (vol. 18), *Immunologic Mechanisms of Renal Disease*, edited by Wilson CG, Brenner BM, Stein JH, New York, Churchill Livingston 1988, p. 57
6. Salant DJ, Belok S, Madaio MP, Couser WG: A new role for complement in experimental membranous nephropathy in rats. *J Clin Invest* 66:1339–1350, 1980
7. Couser WG, Steinmuller DR, Stilmant MM, Salant DJ, Lowenstein LM: Experimental glomerulonephritis in the isolated perfused rat kidney. *J Clin Invest* 62:1275–1287, 1978
8. Van Damme BJC, Fleuren GJ, Bakker WW, Vernier RI, Hoedemaeker PhJ: Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis. *Lab Invest* 38:502–510, 1978
9. Adler S, Salant DJ, Dittmer JE, Rennke HG, Madaio MP, Couser WG: Mediation of proteinuria in membranous nephropathy due to a planted glomerular antigen. *Kidney Int* 23:807–815, 1983
10. Groggel GC, Adler S, Rennke HG, Couser WG, Salant DJ: Role of the terminal complement pathway in experimental membranous nephropathy in the rabbit. *J Clin Invest* 72:1948–1957, 1983
11. Müller-Eberhard HJ: The membrane attack complex, in *Complement*, edited by Müller-Eberhard HJ, Miescher PA, Berlin, Springer-Verlag, 1984, p. 228
12. Cybulsky AV, Rennke HG, Feintzeig ID, Salant DJ: Complement-induced glomerular epithelial cell injury. Role of the membrane attack complex in rat membranous nephropathy. *J Clin Invest* 77:1096–1107, 1986
13. Densen P, Brown EJ, O'Neil GJ, Tedesco F, Clark RA, Frank MM, Webb D, Myers J: Inherited deficiency of C8 in a patient with recurrent meningococcal infections: Further evidence for a dysfunctional C8 molecule and nonlinkage to the HLA system. *J Clin Immunol* 3:90–99, 1983
14. Cybulsky AV, Quigg RJ, Salant DJ: The membrane attack complex in complement-mediated glomerular epithelial cell injury: Formation and stability of C5b-9 and C5b-7 in rat membranous nephropathy. *J Immunol* 137:1511–1516, 1986
15. Baker PJ, Ochi RF, Adler S, Johnson RJ, Couser WG: C6 depletion abolishes proteinuria in experimental membranous nephropathy. (abstract) *Clin Res* 33:475, 1985
16. Perkinson DT, Baker PJ, Couser WG, Johnson RJ, Adler S: Membrane attack complex deposition in experimental glomerular injury. *Am J Pathol* 120:121–128, 1985
17. Kerjaschki D, Schulze M, Binder S, Couser WG: Localization and transport of C5b-9 by the glomerular epithelial cell in experimental membranous nephropathy. (abstract) *Kidney Int* 33:319, 1988
18. Noble B, Van Liew JB, Andres GA, Brentjens JR: Factors influencing susceptibility of LEW rats to Heymann nephritis. *Clin Immunol Immunopathol* 30:241–254, 1984
19. de Heer E, Daha MR, Bhakdi S, Bazin H, van Es LA: Possible involvement of terminal complement complex in active Heymann nephritis. *Kidney Int* 27:388–393, 1985
20. Sharon Z, Schwartz MM, Pauli BU, Lewis EJ: Impairment of glomerular clearance of macroaggregates in immune complex glomerulonephritis. *Kidney Int* 22:8–12, 1982
21. Ryan GB, Karnovsky MJ: An ultrastructural study of the mechanisms of proteinuria in aminonucleoside nephrosis. *Kidney Int* 8: 219–226, 1975
22. Sonnenburg-Hatzopoulos C, Assel E, Schurek HJ, Stolte H: Glomerular albumin leakage and morphology after neutralization of polyanions. II. Discrepancy of protamine induced albuminuria and fine structure of the glomerular filtration barrier. *J Submicroscop Cytol* 16:741–751, 1984
23. Couser WG, Darby C, Salant DJ, Adler S, Stilmant MM, Lowenstein LM: Anti-GBM antibody induced proteinuria in the isolated perfused rat kidney. *Am J Physiol* 249:F241–F250, 1985
24. Schulze M, Baker PJ, Perkinson DT, Johnson RJ, Ochi RF, Stahl RAK, Couser WG: Increased urinary excretion of C5b-9 distinguishes passive Heymann nephritis in the rat. *Kidney Int* 35: 60–68, 1989
25. Koski CL, Ramm LE, Hammer CH, Mayer MM, Shin ML: Cytolysis of nucleated cells by complement: Cell death displays multi-hit characteristics. *Proc Natl Acad Sci USA* 80:3816–3820, 1983
26. Hänsch GM, Betz M, Günther J, Rother KO, Sterzel B: The complement membrane attack complex stimulates the prostanoid production of cultured glomerular epithelial cells. *Int Arch Appl Immunol* 85:87–93, 1988
27. Stahl RAK, Adler S, Baker PJ, Chen YP, Pritzl PM, Couser WG: Enhanced glomerular prostaglandin formation in experimental membranous nephropathy. *Kidney Int* 31:1126–1131, 1987
28. Zoja C, Benigni A, Verroust P, Ronco P, Bertani T, Remuzzi G: Indomethacin reduces proteinuria in passive Heymann nephritis in rats. *Kidney Int* 31:1335–1343, 1987
29. Kirschenbaum MA, Liebross BA, Serros ER: Effect of indomethacin on proteinuria in rats with autologous immune complex nephropathy. *Prostaglandins* 30:295–303, 1985
30. Cybulsky AV, Lieberthal W, Quigg RJ, Rennke HG, Salant DJ: A role for thromboxane in complement-mediated glomerular injury. *Am J Pathol* 128:45–51, 1987
31. Adler S, Baker PJ, Johnson RJ, Ochi RF, Pritzl P, Couser WG: Complement membrane attack complex stimulates production of reactive oxygen metabolites by cultured rat mesangial cells. *J Clin Invest* 77:762–767, 1986
32. Lotan D, Kaplan BS, Fong JSC, Goodyer PR, DeChadarevian J-P: Reduction of protein excretion by dimethyl sulfoxide in rats with passive Heymann nephritis. *Kidney Int* 25:778–788, 1984
33. Kaplan BS, Milner LS, Lotan D, Mills M, Goodyer PR, Fong JSC: Interactions of dimethyl sulfoxide and nonsteroidal anti-inflammatory agents in passive Heymann's nephritis. *J Lab Clin Med* 107:425–430, 1986
34. Shah SV: Evidence suggesting a role for hydroxyl radical in passive Heymann nephritis in rats. *Am J Physiol* 254:F337–F344, 1988
35. Ichikawa I, Hoyer JR, Seiler MW, Brenner BM: Mechanism of glomerulotubular balance in the setting of heterogenous glomerular therapy. *J Clin Invest* 69:185–198, 1982
36. Gabbai FB, Gushwa LC, Wilson CB, Blantz RC: An evaluation of the development of experimental membranous nephropathy. *Kidney Int* 31:1267–1278, 1987
37. Gabbai RB, Mundy CA, Wilson CB, Blantz RC: An evaluation of the role of complement depletion in experimental membranous nephropathy in the rat. *Lab Invest* 58:539–548, 1988
38. Yoshioka T, Rennke HG, Salant DJ, Deen WM, Ichikawa I: Role of abnormally high transmural pressure in the permselectivity defect of glomerular capillary wall: A study in early passive Heymann nephritis. *Circ Res* 61:531–538, 1987
39. Deen WM, Bridges CR, Brenner BM, Myers BD: Heteroporous model of glomerular size selectivity: Application to normal and nephrotic humans. *Am J Physiol* 249:F374–F389, 1985
40. Gabbai FB, Wilson CB, Blantz RC: Role of angiotensin II in

experimental membranous nephropathy. *Am J Physiol* 23:F500–F506, 1988
41. Shemesh O, Ross JC, Deen WM, Grant GW, Meyers BD: Nature of the glomerular capillary injury in human membranous glomerulopathy. *J Clin Invest* 77:868–877, 1986
42. Adler S, Striker LJ, Striker GE, Perkinson DT, Hibbert J, Couser WG: Studies of progressive glomerular sclerosis in the rat. *Am J Pathol* 123:553–562, 1986
43. Pelayo JC, Chenowith DE, Hugli TE, Wilson CB, Blantz RC: Effects of the anaphylatoxin, C5a, on renal and glomerular hemodynamics in the rat. *Kidney Int* 30:62–67, 1986
44. Zamlauski-Tucker MJ, Van Liew JB, Noble B: Pathophysiology of the kidney in rats with Heymann nephritis. *Kidney Int* 28: 504–512, 1985
45. Allison MEM, Wilson CB, Gottschalk CW: Pathophysiology of experimental glomerulonephritis in rats. *J Clin Invest* 53:1402–1423, 1974
46. Okuda S, Onoyama K, Fujimi S, Oh Y, Nomoto K, Omae T: Influence of hypertension on the progression of experimental autologous immune complex nephritis. *J Lab Clin Med* 101:461–471, 1983
47. Okuda S, Onoyama K, Tsuruda H, Oh Y, Omae T: Necrotizing vascular lesions in spontaneously hypertensive rats with nephrotic syndrome: Hypercoagulability as a contributory factor. *J Lab Clin Med* 104:767–777, 1984
48. Tikkanen I, Fyrquist F: Immunological and endocrine features of a new experimental hypertension model: Heymann nephritis-DOCA-NaCl hypertension. *Acta Med Scand* 677(Suppl):130–133, 1983
49. Tikkanen I, Tornroth T, Miettinen A, Fyrquist F: Heymann nephritis-DOCA-NaCl hypertension in the rat. *Nephron* 28:90–95, 1981
50. Iverson BM, Ofstad J: Effects of hypertension on experimental glomerulonephritis in rats. *Lab Invest* 50:163–173, 1984
51. Brenner BM: Hemodynamically mediated glomerular injury and the progressive nature of kidney disease. *Kidney Int* 23:647–655, 1983
52. Girot R, Jaubert F, Leon M, Bellon B, Aiach M, Josso F, Lepelletier O, Beguin S, Monnet J-P: Albumin, fibrinogen, prothrombin and antithrombin III variations in blood, urines and liver in rat nephrotic syndrome (Heymann nephritis). *Thromb Haemostas* 49:13–17, 1983
53. Kaysen GA, Paukert TT, Menke DJ, Couser WG, Humphreys MH: Plasma volume expansion is necessary for edema formation in the rat with Heymann nephritis. *Am J Physiol* 248:F247–F253, 1985
54. Bernard DB, Alexander EA, Couser WG, Levinsky NG: Renal sodium retention during volume expansion in experimental nephrotic syndrome. *Kidney Int* 14:478–485, 1978
55. Kaysen GA, Kirkpatrick WG, Couser WG: Albumin homeostasis in the nephrotic rat: nutritional considerations. *Am J Physiol* 247:F192–F202, 1984
56. Iversen BM, Matre R, Ofstad J: The heterologous immune complex glomerulonephritis. A dose dependent glomerulonephritis with acute, latent and chronic phases in a long-term study. *Acta Pathol Microbiol Immunol Scand* 90:241–250, 1982
57. Barabas AZ, Lannigan R: Induction of an autologous immune-complex glomerulonephritis in the rat by intravenous injection of heterologous anti-rat kidney tubular antibody. *Br J Exp Pathol* 55: 47–55, 1974
58. Barabas AZ, Boyd N, Cornish J, Lannigan R: Progressive passive Heymann nephritis in the rat. *Lab Invest* 47:400–405, 1982
59. Harper PA, Robinson JM, Hoover RL, Wright TC, Karnovsky MJ: Improved methods for culturing rat glomerular cells. *Kidney Int* 26:875–880, 1984
60. Bertani T, Nolin L, Foidart J, Vandewalle A, Verroust P: The effect of puromycin on subepithelial deposits induced by antibodies directed against tubular antigens: A quantitative study. *Eur J Clin Invest* 9:465–472, 1979
61. Camussi G, Brentjens JR, Noble B, Kerjaschki D, Malavasi F, Roholt OA, Farquhar MG, Andres G: Antibody-induced redistribution of Heymann's antigen on the surface of cultuced glomerular visceral epithelial cells: Possible role in the pathogenesis of Heymann glomerulonephritis. *J Immunol* 135:2409–2416, 1985
62. Quigg RJ, Cybulsky AV, Jacobs JB, Salant DJ: Anti-Fx1A produces complement-dependent cytotoxicity of glomerular epithelial cells. *Kidney Int* 34:43–52, 1988
63. Camussi G, Salvidio G, Biesecker G, Brentjens J, Andres G: Heymann antibodies induce complement-dependent injury of rat glomerular visceral epithelial cells. *J Immunol* 139:2906–2914, 1987
64. Imagawa DK, Osifchin NE, Paznekas WA, Shin ML, Mayer MM: Consequences of cell membrane attack by complement: Release of arachidonate and formation of inflammatory derivatives. *Proc Natl Acad Sci USA* 80:6647–6651, 1983
65. Lovett DH, Haensch GM, Goppelt M, Resch K, Gemsa D: Activation of glomerular mesangial cells by the terminal membrane attack complex of complement. *J Immunol* 138:2473–2480, 1987
66. Cybulsky AV, Salant DJ, Quigg RJ, Badalamenti J, Bonventre JV: "Activation" of glomerular epithelial cells (GEC) by the membrane attack complex (MAC) of complement (C). (abstract) *Kidney Int* 33:153, 1988