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Detection of terminal complement components in experimental immune glomerular injury

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Detection of terminal complement components in experimental immune glomerular injury. Complement mediates glomerulonephritis by inflammatory cell-dependent and non-inflammatory cell-independent effects on glomerular permeability. The latter may involve terminal components of the complement system. We examined several models of immunologic renal injury in the rat by immunofluorescence (IF) for terminal complement components C5, C6, C7, and C8 in glomeruli using antisera to human C5-8, which cross-react with the analogous rat complement components. Rats with the heterologous and autologous phases of passive Heymann nephritis (PHN) had proteinuria and 1 to 2+ capillary wall deposits of heterologous or rat IgG, rat C3, and C5-8. Complement depletion with cobra venom factor (CVF) significantly decreased proteinuria in both models and prevented deposition of all complement components. Rats with active Heymann nephritis had similar deposits of rat IgG and C5-8. Rats with anti-GBM nephritis and aminonucleoside nephrosis had severe proteinuria which was not affected by CVF treatment and deposits of C5-8 were absent. The presence of terminal complement components in immune deposits in experimental glomerular disease correlates with a functional role for complement in mediating glomerular injury. These data support the hypothesis that the terminal complement pathway may be a major mediator of some types of immune glomerular injury.

Détection des constituants terminaux du complément au cours de lésions immunes glomérulaires expérimentales. Le complément est le médiateur d'une glomérulonéphrite par des effets inflammatoires cellule-dépendants, et non inflammatoires cellule-indépendants sur la perméabilité glomérulaire. Ces derniers pourraient mettre en jeu les constituants terminaux du système complémentaire. Nous avons examiné plusieurs modèles de lésions rénales immunologiques chez le rat par la immunofluorescence (IF) en ce qui concerne les constituants complémentaires terminaux C5, C6, C7, et C8 dans les glomérules en utilisant des antisérums contre C5-8 humain qui croisent avec les constituants complémentaires analogues du rat. Des rats dans les phases hétérologue et autologue d'une néphrite passive de Heymann (PHN) avaient une protéinurie et des dépôts sur les parois capillaires 1 à 2+ d'IgG hétérologue ou de rat, de C3 et de C5-8 de rat. Une déplétion complémentaire avec du facteur de venin de cobra (CVF) a diminué significativement la protéinurie dans les deux modèles et a prévenu le dépôt de tous les constituants complémentaires. Des rats atteints d'une nephrite active de Heymann avaient des dépôts identiques d'IgG et de C5-8 de rat. Des rats atteints de néphrite anti-GBM et de néphrose aux aminoglucosides avaient une protéinurie sévère non affectée par le traitement au CVF et les dépôts de C5-8 étaient absents. La présence des constituants terminaux de complément dans les dépôts immuns lors des glomérulopathies expérimentales est corrélée avec un rôle fonctionnel du complément dans la médiation des lésions glomérulaires. Ces données sont en faveur de l'hypothèse que la voie terminale du complément peut être un médiateur majeur de certains types de lésions glomérulaires immunes.

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The complement system is a well established mediator of glomerular capillary wall injury induced by antibody deposition in glomeruli [1–5]. Previous studies of nephrotoxic, or anti-glomerular basement membrane (GBM) nephritis, by several investigators [6–10] have established a correlation between glomerular C3 deposition, neutrophil infiltration, and proteinuria; they have shown that proteinuria can be markedly reduced by depletion of either complement or neutrophils and established that proteinuria can be restored in normocomplementemic, neutrophil-depleted animals by neutrophil replacement. Based on these findings, as well as the known chemotactic capacity of complement activation products, such as C5a, for neutrophils in vitro [11], and the capacity of neutrophil-derived enzymes to digest GBM in vitro [12], it has long been assumed that the major role for complement in the pathogenesis of immune renal injury was an indirect one mediated through inflammatory effector cells [6–8].

We have recently shown that proteinuria induced by the in situ formation of immune complexes in the subepithelial space due to antibody reacting with either fixed or planted glomerular antigens is highly complement-dependent but occurs independently of inflammatory cell participation, thereby demonstrating a direct effect of complement activation in the mediation of proteinuria in experimental membranous nephropathy [4, 5]. We speculated that this direct effect may result from a terminal complement mechanism, perhaps involving assembly of membrane attack complexes (MACs), with consequent injury to some component of the filtration barrier. Support for this hypothesis has been provided by our recent studies in which C6 deficient rabbits in the early phase of cationic-bovine serum albumin (BSA) induced chronic serum sickness and in the heterologous phase of nephrotoxic nephritis have a marked reduction in proteinuria compared to normo-complementemic controls despite equivalent amounts of glomerular antibody deposition [13, 14]. Additional indirect support for this hypothesis derives from several recent demonstrations of neoantigens of the MAC in glomerular immune deposits and elsewhere in a variety of human renal diseases [15–17] and in serum sickness in the rat [18]. However, in none of these studies were the lesions examined known to be complement-mediated. Moreover, the observation of MAC localization at sites of presumably non-

immune tissue injury, such as areas of sclerosis, vascular thickening, and interstitial disease, raised questions regarding whether terminal complement components and MAC formation represent a primary pathogenetic mechanism or a secondary manifestation of tissue injury from a variety of other causes.

To further explore the correlation between deposition of terminal complement components, glomerular injury manifested by proteinuria, and the role of complement in mediating tissue damage, we established the specificity of antibodies for terminal rat complement components C5, C6, C7, and C8. We then utilized these antibodies to study the deposition of terminal complement components in a variety of immune and non-immune glomerular lesions in the rat in which the participation or non-participation of complement in mediating the lesions could be established. Our findings demonstrate a close correlation between glomerular deposition of terminal complement components and complement-dependent glomerular injury and support the hypothesis that a terminal complement mechanism is an important primary mediator of immune glomerular disease.

Methods

Assay of rat complement components

Rat serum was assayed for C5, C6, C7, and C8 hemolytic activity using purified complement components and cellular intermediates obtained from Cordis Laboratories (Miami, Florida) using a modification of the manufacturer's method. The hemolytic assay was carried out in $GGVB^{++}$ buffer consisting of equal volumes of 5% glucose and 0.04 M veronal buffered saline, pH 7.3 with 0.1% gelatin, 0.25 mM Ca^{++}, and 0.5 mM Mg^{++}. EAClgp4hu, human C2, C3, C5, C6 and C7, and guinea pig C8 and C9 were used to measure C5, C6, and C7 activity. EAClgp4-7hu and human C9 were used to measure C8 activity. Serial dilutions of rat serum were made in $GGVB^{++}$ buffer. The reaction mixture consisted of 57 μl aliquots of indicator cells, serum dilutions, and all of the complement components except the one being assayed with a final volume of 235 μl. Following incubation at 37°C, 465 μl of saline were added and the absorption of the supernatants at 415 nm determined spectrophotometrically. The percentage of lysis was determined by comparison to an equal volume of cells lysed in distilled water. From a linear regression plot of -ln (1-% lysis) versus serum dilution, the dilution giving 50% lysis was calculated. The reciprocal of this value is the number of CH50 units per milliliter.

Antisera to rat complement components

Monospecific goat antisera to human C5, C6, and C7 were obtained from Cappel Laboratories (Cochraneville, Pennsylvania) and the fluorescein conjugated IgG fraction of goat antiserum to human C8 was obtained from Atlantic Antibodies (Scarborough, Maine). All antisera were heat-inactivated prior to use. Antisera were tested by micro-Ouchterlony immunodiffusion for reactivity against fresh normal human, sheep and rat serum, and IgG. To further insure non-reactivity with deposits of sheep or rat IgG present in glomeruli in the models studied, sheep IgG was fixed to rat tissue sections by incubating heat-inactivated (56°C, 30 min) sheep anti-FxlA antiserum with cryostat sections of normal rat kidney for 30 min at room temperature to produce brushborder deposits of sheep IgG [13]. Deposits of rat immunoglobulin G (IgG) were fixed to tissue sections in a similar distribution by incubating some of the sheep IgG containing sections with heat-inactivated rat anti-sheep IgG antiserum. Such sections stain positively for both sheep and rat IgG on tubular brush borders, but do not stain with antiserum to goat IgG. Sections containing deposits of sheep IgG alone or sheep and rat IgG without complement, were then stained with the goat antisera to complement components for 30 min followed by staining with fluorescein-conjugated sheep anti-goat IgG (Cappel Laboratories, Inc.). Antisera to complement components and goat IgG were used at the highest dilution which produced a precipitin line against undiluted normal human or goat serum in micro-Ouchterlony immunodiffusion. Controls included sections with positive staining for complement components stained directly, or following incubation with heat-inactivated normal goat serum, with fluorescein-conjugated sheep anti-goat IgG, and normal rat kidneys stained with the antisera to human complement components and sheep anti-goat IgG. Because purified rat complement components are not available, specific absorption studies were not carried out.

Because antisera to human complement components did not make precipitin lines against fresh rat serum in micro-Ouchterlony immunodiffusion, further studies were carried out to assess the capacity of each antiserum to specifically neutralize the functional activity of the corresponding individual rat complement component. For these studies, the IgG fraction of antiserum to human C6, C7, and C8 was isolated by ion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, New Jersey) in 0.0175 M sodium phosphate buffer, pH 7.4. IgG fractions of antiserum were concentrated to 2 mg/ml before use and were shown to lack complement component activity using the assay outlined above. To determine the dilution of rat serum to be used in neutralization studies, fresh normal rat serum was first assayed for C5, C6, C7, and C8 hemolytic activity as described above. Dilutions of rat serum which gave 90% hemolysis in the complement component assay were then mixed with 1:2 or 1:4 dilutions of antiserum or antibody IgG to the respective complement components and incubated at 25°C for 30 min. As controls, additional aliquots of the same dilutions of normal rat serum were incubated alone, or with equivalent concentrations of normal goat serum or IgG. Following incubation, the rat sera were assayed again for C5, C6, C7, and C8 hemolytic activity, respectively.

Models of glomerular disease

Passive Heymann nephritis (PHN-heterologous phase). PHN was induced in five male Sprague-Dawley rats (Tyler Laboratories, Inc., Bellevue, Washington) by injection of 1.25 ml i.v. of sheep anti-FxlA antiserum prepared as previously described [19, 20]. Sheep were hyperimmunized with rat proximal tubular epithelial cell brushborder antigen (FxlA) prepared according to the method of Edgington, Glassock, and Dixon [21]. Antisera were absorbed with rat red blood cells and plasma and heat-inactivated (56°C, 30 min) prior to use. Sheep anti-rat FxlA was not reactive with rat serum by micro-Ouchterlony immunodiffusion and immunoelectrophoresis (IEP), but made two precipitin lines against a suspension of crude rat FxlA (10 mg/ml) [21]. An additional four rats were

given the same dose of antiserum but were also depleted of circulating complement by injection of purified cobra venom factor (CVF, Cordis Laboratories) beginning the day prior to antiserum injection and continuing for 5 days as described below. Urine protein excretion was measured from days 4 to 5 following antiserum injection; tissue samples for immunofluorescence (IF) studies were obtained on day 5.

γ2-PHN (heterologous phase). To study animals with equivalent quantities of subepithelial sheep IgG deposits but without proteinuria or complement fixation, PHN was induced with the γ2 (non-complement fixing) IgG fraction of sheep anti-rat FxlA [4]. The γ2 fraction was isolated from a 50% ammonium sulfate precipitate of whole antiserum by ion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals) using 0.0175 M sodium phosphate buffer, pH 7.4 [4, 5]. The γ2 fraction made a single line in micro-Ouchterlony immunodiffusion and IEP against rabbit antiserum to whole sheep serum and sheep IgG (Cappel Laboratories, Inc.). Three rats were injected with 9.3 mg of γ2 anti-FxlA i.v. and studied 3 days later.

γ2-PHN (autologous phase). To obtain a model of complement-dependent proteinuria induced by antibody reacting with an exogenous, planted subepithelial antigen, we immunized four rats with sheep IgG, 1.0 mg in complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) to accelerate development of the autologous phase, and then we injected the rats 5 days later with 8.1 mg i.v. of γ2 anti-FxlA. This protocol produces subepithelial deposits of planted sheep IgG antigen, rat anti-sheep IgG antibody, and rat C3; proteinuria develops approximately 7 days following injection of the γ2 and anti-FxlA [5]. An additional three rats, treated as above, were depleted of circulating complement using CVF as described below. Urine protein excretion and tissue for IF studies was obtained on days 7 to 8 following antibody injection.

Autologous immune complex nephropathy (AICN). AICN was induced by immunizing five male Lewis rats, weighing 150 to 200 g, (Simonsen Laboratories Inc., Gilroy, California) in the rear foot pads with 5 mg of FxlA emulsified in complete Freund's adjuvant (Difco Laboratories) as described previously [22]. Urine protein excretion and IF studies were carried out 7 weeks following immunization.

Nephrotoxic nephritis (heterologous phase). Six rats were injected with 0.125 ml i.v. of sheep anti-rat GBM antiserum, prepared as described previously [23, 24]. Sheep were hyperimmunized with intact rat glomeruli prepared by differential sieving techniques [24], and antiserum was absorbed and heat-inactivated as described above for anti-FxlA. Protein excretion was measured during the 24-hr period following antiserum injection, and tissue for IF study was obtained by open renal biopsy 4 and 24 hr after antiserum injection. To assess the role of complement in mediating proteinuria in this model, six additional rats were treated with CVF as described below. Urine protein excretion was measured during the 24-hr period following antiserum injection and tissue for IF study was obtained by open renal biopsy 24 hr following antiserum injection.

γ2 anti-rat GBM nephritis (heterologous phase). To study another group of rats with equivalent amounts of anti-GBM antibody deposition without complement fixation, the γ2 (non-complement fixing) fraction of sheep anti-rat GBM IgG was isolated as described above for γ2 anti-FxlA. Three rats were each injected with 10 mg i.v. of γ2 anti-rat GBM IgG and studied as described for the group with nephrotoxic nephritis above.

Aminonucleoside nephrosis. To obtain a model of non-immune glomerular disease, we induced proteinuria in three rats by an intravenous injection of 100 mg/kg of aminonucleoside of puromycin (PA; ICM Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio) in normal saline as described previously [25]. Proteinuria was measured and renal tissue obtained for IF studies 9 days after PA injection.

Other procedures

Rats were depleted of circulating complement by injection of CVF (Cordis Laboratories, Inc.), 300 U/kg i.p. in four divided doses beginning the day prior to antibody injection and followed by daily injections of 100 U/kg i.p. for the duration of the experiment, following a protocol described by Cochrane, Muller-Eberhard, and Aikin [3]. Our previous studies demonstrate that this regimen reduces serum C3 levels to less than 10% of normal [4, 5].

Urine protein excretion was measured on 24-hr urine collections obtained in metabolic cages. Protein content was measured by the sulfosalicylic acid method [26] using a commercial standard (Lab-trol, Dade Diagnostics, Inc., Aguado, Puerto Rico). Normal 24-hr urine protein excretion in age-matched rats in our laboratory has been 2 to 6 mg.

Tissue samples for IF were obtained by open renal biopsy while the rats were under ether anesthesia or at sacrifice and snap-frozen in dry-ice-isopentane. Cryostat sections were cut at 4 μ, air-dried, and fixed in ether-alcohol as described elsewhere [25, 27]. In addition to the goat antisera to complement components and sheep anti-goat IgG described above, sections were also stained with the fluorescein-conjugated fraction of monospecific rabbit anti-sheep IgG and anti-rat C3 (Cappel Laboratories, Inc.). Stained sections were examined on a Zeiss photomicroscope III with an epifluorescence condenser and appropriate filters for FITC fluorescence (Carl Zeiss, Inc., Thornwood, New York). Sections were photographed on Kodak Ektachrome ED-135 film (Eastman Kodak Co., Rochester, New York) using an automatic exposure meter set at ASA-400. The intensity of fluorescent staining was reported semi-quantitatively on a 0 to 4+ scale, with 0, indistinguishable from control; trace, visible but cannot be photographed; 1+, definite but faint; 2+, definite; 3+, moderate intensity; and 4+, maximum intensity. Maximum intensity was taken to be the staining observed in rats injected with nephrotoxic serum or in AICN rats biopsied 7 weeks after immunization with FxlA.

Statistical analysis of data

All results of urine protein excretion determinations are expressed as mean ± SEM. The Mann-Whitney test was used to compare the results of protein excretion in different groups [28]. *P* values less than 0.05 are regarded as significant.

Results

Specificity of antisera and reactivity with rat complement components

In micro-Ouchterlony, antiserum to human C5, C6, C7, and C8 made single precipitin lines with fresh human serum. When

Table 1. Neutralization of rat hemolytic complement activity by antisera to human complement

	Residual complement activity *(CH50 U/ml)* following incubation with:		
	$GGVB^{++}$[a]	Normal serum or IgG	Antiserum or Ab IgG
C5	823	420	<4[b]
C6	10,400	10,500	1,100[c]
C7	36,200	48,600	2,523[c]
C8	346,000	412,000	54,700[c]

[a] For the makeup of $GGVB^{++}$, a complement assay buffer, see **Methods.**

[b] Whole antiserum to C5 was used.

[c] IgG fractions of antisera were used.

placed in adjacent wells, each antiserum made a line of non-identity with the lines made by antisera to the other complement components, demonstrating the specificity of the individual antibodies for the respective human complement components. None of the antisera made precipitin lines in micro-Ouchterlony or IEP against fresh rat serum. To test for the reactivity of these antisera with rat complement components, studies were carried out to assess the capacity of each antiserum to neutralize the functional activity of individual complement components in rat serum. Results of a typical neutralization experiment are presented in Table 1. Heat-inactivated antiserum to human C5, and the IgG fraction of antiserum to human C6, C7, and C8 had no significant residual hemolytic activity when tested for C5, C6, C7, and C8, respectively. When normal rat serum was incubated alone or with concentrations of normal goat serum or goat IgG comparable to those in the antibody preparations, no significant changes in levels of rat C5, C6, C7, or C8 were observed (Table 1). However, incubation with whole antiserum or IgG fractions of antisera to complement components under the conditions described resulted in a loss of over 90% of the hemolytic activity of rat C5, C6, and C7 and over 80% of the hemolytic activity of C8 (Table 1).

Studies to assess the possible cross-reactivity of these antisera with other serum proteins which might be present in glomerular deposits in the models studied, including sheep and rat IgG, revealed no reactivity of any of the anti-complement antisera with sheep or rat serum or IgG by micro-Ouchterlony. The antisera also did not react by indirect IF with sheep or rat IgG deposited on rat kidney sections. Similarly, the FITC-conjugated sheep anti-goat IgG used to detect the binding of antibody to complement components was not reactive with the sheep or rat serum in micro-Ouchterlony and did not stain sheep or rat IgG fixed to rat kidney sections. Deposit-containing sections incubated with normal goat serum and FITC-conjugated sheep anti-goat IgG demonstrated no non-specific binding of goat IgG to the deposits.

Presence of terminal rat complement components in glomerular disease models

Passive Heymann nephritis (heterologous phase). Five days after receiving an injection of sheep anti-rat FxlA antiserum, the rats exhibited 2 to 3+ finely granular deposits of sheep IgG and 1 to 2+ deposits of rat C3 in a subepithelial distribution in glomeruli as previously described [4, 20]. Faint deposits of IgG and C3 were present focally on tubular brush borders. Occasional deposits were also noted in a granular pattern along the basement membranes of proximal convoluted tubules. Urine protein excretion was 138 ± 27 mg/day. Staining for C5, C6, C7, and C8 revealed readily detectable (1 to 2+) deposits of each of the components in the glomerulus in a distribution similar to that of IgG (Table 2, Fig. 1). Terminal complement components were not detected along the tubule basement membranes or proximal tubular brush borders. Rats depleted of circulating complement components with CVF had similar deposits of sheep IgG but no deposits of rat C3, C5, C6, C7, or C8 (Fig. 2). Urine protein excretion in these animals was reduced to 8.1 ± 1.9 mg/day ($P < 0.05$ vs. controls; Table 2).

γ2 PHN (heterologous phase). Three days following injection of γ2 sheep anti-FxlA IgG, 1 to 2+ glomerular capillary wall deposits of sheep IgG were present in a distribution similar to that described above. No rat IgG was present. Previous studies demonstrate that urine protein excretion is normal in these animals at this time [5]. IF staining for rat C3, C5, C6, C7, and C8 was uniformly negative (Table 2).

γ2 PHN (autologous phase). Rats were immunized with sheep IgG prior to injection of γ2 anti-FxlA IgG to accelerate the development of the autologous phase. When these rats were studied 8 days following the injection of antibody, sheep IgG was still present in the glomerular capillary wall in a 1 to 2+ granular pattern and rat IgG was present in a similar distribution. IF staining revealed 1 to 2+ deposits of rat C3, C5, C6, C7, and C8 (Table 2). Urine protein excretion was 15.1 ± 7.2 mg/day. Treatment of three rats with CVF, beginning the day before antibody injection, did not alter deposits of sheep and rat IgG but abolished deposits of rat C3, C5, C6, C7, and C8. Urine protein excretion fell to 1.2 ± 0.2 mg/day (Table 2).

AICN. Seven weeks following immunization with FxlA, AICN rats had diffuse 3 to 4+ subepithelial deposits of rat IgG and 1 to 2+ deposits of rat C3, as described previously [22]. There were focal 1 to 2+ deposits of rat IgG on tubular brush borders, but tubular basement membrane staining was not detected. All rats had positive staining in their glomeruli for rat C5, C6, C7, and C8 in a distribution similar to that of rat IgG and C3 and appearing identical to that described under PHN above (Table 2). Urine protein excretion was 36.7 ± 11.3 mg/day. Because of the time period required to induce the disease, the role of complement in mediating proteinuria has not been studied but is presumed to be similar to that described in PHN [4, 29].

Nephrotoxic nephritis (heterologous phase). Four hours following injection of sheep anti-rat GBM antiserum, 3 to 4+ linear deposits of sheep IgG were present in all glomeruli. C3 was present in trace amounts in one animal but all others were negative at 4 hr. After 24 hr, sheep IgG staining was unchanged and trace-1+ deposits of rat C3, but not of C5, C6, C7, or C8 were detected along the GBM (Fig. 3). Urine protein excretion was 220 ± 38 mg/day (Table 2). Treatment of rats with CVF abolished deposition of C3 at 24 hr but did not alter urine protein excretion (308 ± 24 mg/day, $P > 0.05$ vs. controls).

γ2 Nephrotoxic nephritis (heterologous phase). Following injection of rats with γ2 anti-GBM IgG, the deposition of sheep IgG was similar to that of animals receiving whole antiserum at

Table 2. Results of IF and protein excretion[a]

	Sheep IgG	Rat IgG	Rat C3	C5	C6	C7	C8	24° Urine protein excretion, *mg*
Passive Heymann Nephritis (PHN)								
Heterologous phase (5 days; $N = 5$)	+	Tr(4/5)	+	+	+	+	+	138 ± 27
CVF-treated (5 days; $N = 4$)	+	+	−	−	−	−	−	8.1 ± 1.9
γ2 PHN								
Heterologous phase (3 days; $N = 3$)	+	−	−	−	−	−	−	NT
Autologous phase (8 days; $N = 4$)	+	+	+	+	+	+	Tr	15.1 ± 7.2
CVF-treated (8 days; $N = 3$)	+	+	−	−	−	−	−	1.2 ± 0.2
AICN (7 weeks; $N = 4$)	−	+	+	+	+	+	Tr	36.7 ± 11.3
Nephrotoxic nephritis (NTN)								
Heterologous phase (4 hr; $N = 6$)	+	−	−	−	−	−	−	NT
Heterologous phase (24 hr; $N = 6$)	+	−	+	−	−	−	−	220 ± 38
CVF-treated (24 hr; $N = 6$)	+	−	−	−	−	−	−	308 ± 24
γ2 NTN								
Heterologous phase (4 hr; $N = 3$)	+	−	−	−	−	−	−	NT
Heterologous phase (24 hr; $N = 3$)	+	−	−	−	−	−	−	9.0 ± 1.7
Aninonucleoside nephrosis (9 days; $N = 3$)	−	−	−	−	−	−	−	268 ± 7

Abbreviations and symbols: *N*, number tested; −, negative; +, present; Tr, trace; NT, not tested.
[a] Values are means ± SEM.

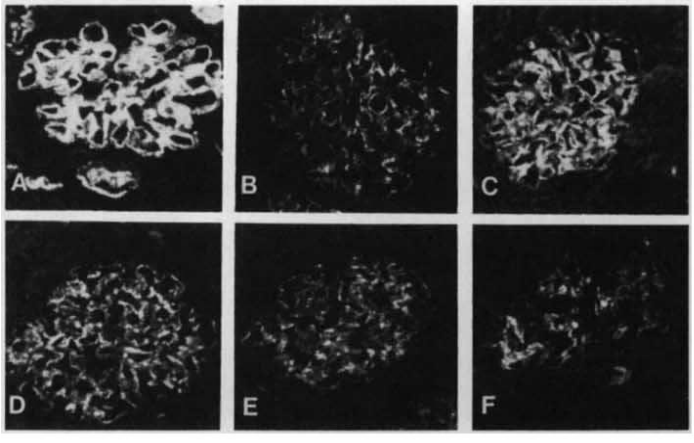

Fig. 1. *Immunofluorescence of kidney tissue removed 5 days following injection of 1.5 ml of sheep anti-rat FxlA antiserum.* Granular capillary wall deposits of sheep IgG (**A**), rat C3 (**B**), C5 (**C**), C6 (**D**), C7 (**E**), and C8 (**F**) are evident. (Original magnification, ×500)

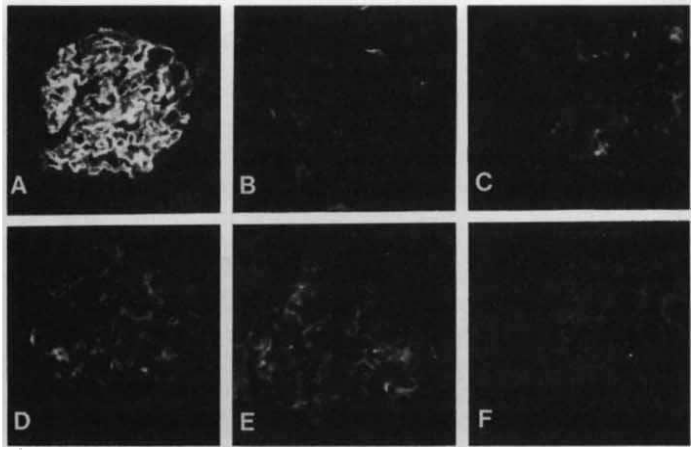

Fig. 2. *Immunofluorescence of kidney tissue removed 5 days following injection of 1.5 ml of sheep anti-rat FxlA antiserum in cobra venom factor-treated rats.* Granular capillary well deposits of sheep IgG (**A**) are present but no deposits of C3 (**B**), C5 (**C**), C6 (**D**), C7 (**E**), or C8 (**F**) are seen. (Original magnification, ×500)

4 and 24 hr, but no deposits of any complement components could be demonstrated (Table 2). Rats receiving γ2 nephrotoxic IgG developed a mild, heterologous phase proteinuria (9.0 ± 1.7 mg/day).

Aminonucleoside nephrosis. Nine days following injection of aminonucleoside, rats had 268 ± 7 mg/day of proteinuria. IF studies revealed focal 1+ mesangial deposits of rat IgG similar to normals, but no detectable glomerular deposition of any of the complement components (Table 2). Our previous laboratory studies demonstrate that treatment with CVF has no effect on the development of proteinuria induced by aminonucleoside [4].

Discussion

Several models of immunologic renal disease, in which different roles for complement have been proposed, were examined to correlate the presence of deposits of terminal complement components with the role of complement in causing glomerular injury, to support the hypothesis that fixation of terminal complement components in glomerular immune deposits may be a primary pathogenetic event. To do these studies, it was first necessary to verify that antisera against the terminal components of the human complement system reacted specifically with the same complement components in rat serum. Following incubation of rat serum with antibody to the component being evaluated, the hemolytic titer of that component in normal rat serum was markedly depressed, whereas incubation with assay buffer alone or normal goat serum or IgG led to no significant change in titer. Furthermore, comparison of each antiserum against all of the others in immunodiffusion versus normal human serum demonstrated no reactivity of any of the antisera with any of the components recognized by the other

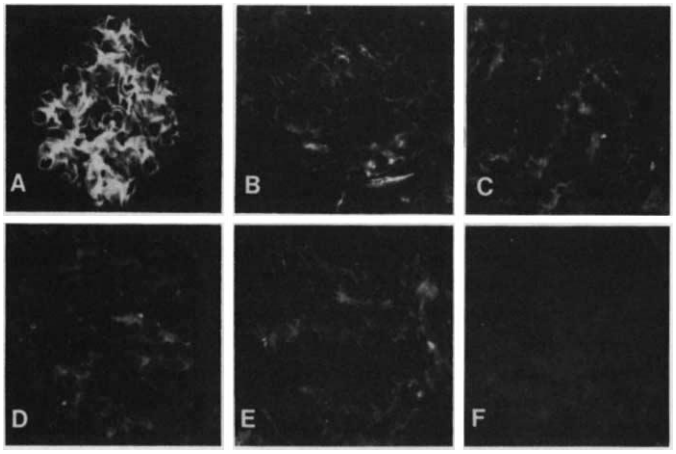

Fig. 3. *Immunofluorescence of kidney tissue removed 24 hr following injection of 0.15 ml of sheep anti-rat GBM antiserum.* Linear deposits of sheep IgG (**A**) are visible along the GBM. Faint deposit of rat C3 (**B**) are visible, but C5 (**C**), C6 (**D**), C7 (**E**), and C8 (**F**) are absent. (Original magnification, ×500)

antisera. Failure to demonstrate reactivity between the antisera and normal rat serum in micro-Ouchterlony immunodiffusion or IEP may have been due to low avidity of the antibodies used or to insufficient quantities of cross-reactive antigenic epitopes on the complement components in normal rat serum to produce visible precipitates.

Having demonstrated that these antisera react specifically with, and neutralize, rat C5, C6, C7, and C8, the antisera were then used in direct and indirect IF to examine renal tissue from rats with different types of immunologic renal disease which exhibit different roles for the complement system in the mediation of proteinuria. With respect to the membranous form of glomerular injury, three models were studied. In the heterologous phase of PHN, subepithelial deposits form as a consequence of antibody binding to an intrinsic glomerular antigen [19, 29], and proteinuria is complement-dependent and cell-independent [4]. In the present study, the complement-dependence of this lesion induced with unfractionated IgG was confirmed, and granular capillary wall deposits of C5, C6, C7, and C8 were demonstrated in the same distribution as deposits of rat C3 and sheep IgG in proteinuric animals. When rats were treated with CVF to deplete complement during deposit formation, or when deposits were induced with the non-complement fixing γ2 fraction of sheep IgG antibody, similar quantities of IgG were deposited in the glomerulus but deposition of terminal complement components was not seen and proteinuria did not occur. These findings suggest that antibody deposition alone does not result in non-specific localization of terminal components in immune deposits and that the terminal complement components may be involved in the pathogenesis of the functional lesion which leads to proteinuria. Similar results were observed in a second model of experimental membranous nephropathy induced by using the γ2 IgG as a planted subepithelial antigen to induce in situ formation of epimembranous immune complex deposits of sheep IgG and rat anti-sheep IgG. Again, deposits of rat C5, C6, C7, and C8 corresponded to the distribution of deposits of sheep IgG, rat IgG, and rat C3. CVF treatment abolished both deposition of terminal complement components and proteinuria. Finally, in the standard Heymann nephritis (AICN) model induced by active immunization with proximal tubule brushborder antigen, terminal complement components were seen in proteinuric animals in a distribution corresponding to that of rat IgG and C3. Although there is evidence that immune deposit formation in AICN probably occurs by an in situ mechanism similar to that demonstrated in PHN [30, 31], the role of complement in mediating this lesion has not been established because of the technical difficulties in carrying out complement depletion studies for the 8-week period required for proteinuria to develop in this model [22].

Because the antisera to C5-8 are directed against native complement components, the presence of a MAC can only be inferred. However, our data are consistent with our original hypothesis that the mechanism of the direct effect of complement on glomerular permeability that we have now demonstrated in several models of membranous nephropathy, probably involves participation of terminal complement components to form a MAC with a resulting structural lesion occurring somewhere in the distal portion of the filtration barrier [4, 32], although some contribution of C3 cleavage products cannot be ruled out. Further support for this hypothesis is provided by our recent finding that C6 is required for the development of proteinuria in the early phases of chronic serum sickness in rabbits induced with cationized BSA when the glomerular lesion is predominantly a membranous one [13], and by the observations of others that neoantigens of the MAC are present in glomerular immune deposits in both the idiopathic and lupus forms of membranous nephropathy in humans [15, 16].

In addition to the cytolytic role of complement in immune reactions, we have also shown that kidney cells damaged by a variety of mechanisms and incubated in autologous serum have the capacity to assemble a C3 cleaving enzyme on their surfaces, convert C3 by the classical and alternate pathways, consume C5, C7, and C8 hemolytic activity and bind C5, C6, C7, and C8 to their surfaces [33]. To further exclude the possibility that terminal complement component deposition may be a secondary response to the tissue injury which resulted in proteinuria, further studies were done in models of immune and non-immune glomerular disease induced by complement-independent mechanisms. Although others have described a role for complement and neutrophils in the heterologous phase of sheep anti-rabbit NTN [2, 3, 8], in our laboratory sheep antibody to rat GBM induces heavy proteinuria without significant C3 deposition or neutrophil infiltration, and complement depletion with CVF has no effect on proteinuria, a finding that agrees with the results of Pilia et al [34]. In this model of immunologically induced glomerular disease in which proteinuria equals or exceeds that in the models of membranous nephropathy, no terminal complement component deposition was observed in normocomplementemic or complement-depleted rats. Similar results were obtained in rats made proteinuric by injection of the aminonucleoside of puromycin, a lesion in which complement depletion has no effect on proteinuria [4]. Although our findings do not exclude the possibility that C5-9 assembly could be a secondary phenomenon due to in situ processing of immune complex deposits by complement, these findings suggest that deposition of terminal complement components is probably not a consequence of glomerular injury but rather a primary pathogenetic event. Taken together, these results are again consistent with the hypothesis that a MAC-

mediated form of injury may be an important aspect of complement participation in several forms of glomerular disease.

These results in animal models of immunologic glomerular injury are consonant with observations in human renal disease. Neoantigens of the MAC and poly-C9 have been found in glomerular deposits of patients with systemic lupus erythematosus, membranous nephropathy, anti-GBM disease and a variety of other types of non-immunologic renal injury [15–17]. The mechanism by which direct or indirect MAC damage to the glomerular capillary wall leads to increased permeability is unknown. The MAC is able to insert into some membranes and lipid bilayers to produce transmembrane channels that lead to cell lysis [35, 36]. It also appears to be able to produce lytic lesions in bacterial cell walls and certain virus particles [36]. An interaction with the cell membrane of the glomerular epithelial cell or slit diaphragm has been postulated but not demonstrated [4, 32]. More recently, binding of C9 to apparently cell-free alveolar basement membranes in the presence of antibody and normal serum has been demonstrated and donut-shaped lesions in the membrane, believed to represent the MAC inserted into the membrane, were also seen [37]. The mechanism by which terminal complement components participate in the alteration of glomerular permeability requires further investigation.

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