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Depletion of C6 Prevents Development of Proteinuria in Experimental Membranous Nephropathy in Rats

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To study the possible role of the complement membrane attack complex, C5b-9, in an experimental rat model that is morphologically indistinguishable from membranous nephropathy in man (passive Heymann nephritis [PHN]), an antibody to rat C6 was used to deplete C6 levels to less than 5% of pretreatment values (C6D) during disease development. C3, C7, C8, and C9 levels were not different in C6D and control rats. After injection of nephritogenic quantities of ^{125}I-anti-Fx1A antibody, the kinetics of disappearance of labeled IgG from the blood were identical in the complement deficient and sufficient groups, and glomerlar deposition of ^{125}I-antibody was the same in both groups at 5 days. Glomerular deposits of sheep IgG and C3 were also similar in C6D and controls, but glomerular deposits of C6 and C5b-9 neoantigens were markedly reduced or absent in C6 depleted rats. However, despite equivalent antibody deposits, proteinuria was abolished in C6D rats compared with normocomplementemic controls. Similar results were obtained when F(ab')$_2$ anti-rat C6 IgG was used to deplete C6 during development of PHN. These results demonstrate that C6 is required for the development of the increased glomerular permeability that occurs in PHN, presumably because C6 is required for formation of C5b-9. We conclude that glomerular injury in the PHN model of membranous nephropathy in the rat is mediated by C5b-9. (Am J Pathol 1989, 135:185–194)

The role of complement in the mediation of immune glomerular injury is well established.[1,2] More than 20 years ago Cochrane et al demonstrated that renal injury in nephrotoxic (anti-glomerular basement membrane [GBM]) nephritis was abolished by complement depletion[3–6] and found that a similar effect was achievable with depletion of neutrophils that were histologically prominent in the glomeruli.[5,7] Based on these studies, the role of complement in antibody-mediated glomerular disease was long regarded as an indirect one involving neutrophil-mediated tissue injury resulting from a chemotactic response to C5a, immune adherence to C3b opsonized membranes, or both.[8] In 1980, however, we defined a new neutrophil independent direct role for complement in immune glomerular disease using the passive Heymann nephritis (PHN) model of membranous nephropathy in rats, in which subepithelial immune deposits form *in situ* by heterologous antibody binding to a glycoprotein antigen expressed on the membrane of the podocyte of the glomerular epithelial cell (GEC).[9–12] Proteinuria was not associated with neutrophil infiltrates and was not reduced by neutrophil depletion, but was abolished by cobra venom factor depletion of C3-C9. The possible mechanism of this complement-dependent, inflammatory cell-independent complement effect has since been clarified by the establishment of a functional role for C6 and presumably, therefore, for C5b-9 in mediating proteinuria in both chronic serum sickness and anti-GBM nephritis models using rabbits genetically deficient in plasma C6.[13,14]

In the rat, C5b-9 was shown to be deposited in glomeruli in several different disease models[15–18] and was demonstrated to be capable of mediating injury in studies in which subepithelial immune complex deposits are formed *ex vivo* in isolated rat kidneys perfused with C6- or C8-deficient serum.[19,20] Recently, we have documented GEC membrane insertion of C5b-9 in PHN in the intact animal and have described a unique mechanism of endocytosis, transcellular transport, and exocytosis into urine of membrane-inserted C5b-9 complexes.[21] However, no studies have yet looked directly at the requirement for C5b-9 in the production of antibody-mediated glomerular injury *in*

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vivo in PHN. To test the hypothesis that the glomerular injury in the PHN model *in vivo* is mediated by C5b-9, we used antibody-to-rat C6 to deplete PHN rats of C6 (C6D) over a 4-day period from antibody injection until proteinuria developed. Our findings provide the first evidence that C6 and C5b-9 assembly are required to mediate glomerular injury in this model.

Materials and Methods

Production of Passive Heymann Nephritis (PHN)

PHN was induced in male Sprague Dawley rats weighing 100 to 110 g (Tyler Laboratories Inc., Bellevue, WA) by intravenous injection of antibody to rat proximal tubular brush border antigen (Fx1A).[22] Anti-Fx1A was produced by hyperimmunization of sheep with rat Fx1A prepared as described elsewhere.[9] In the studies described here, functional evidence of disease was documented by measurement of urine protein excretion from day 3 to 4 (or day 4 to 5) after administration of anti-Fx1A antibody (see below). In animals subjected to complement depletion and controls, PHN was induced with ^{125}I-labeled anti-Fx1A IgG to permit comparison of plasma disappearance and glomerular binding of antibody in C6D and control rats (see below).

Isolation of Rat C6

Our previous studies showed that goat anti-human C6 antibodies cross react with the same component in rat serum as shown by *in vitro* neutralization assays.[15] Goat anti-human C6 IgG (Cappel Laboratories, Cochransville, PA) was isolated according to the method of Wetzel et al[23] and coupled to Cn-Br activated Sepharose 4B for use as an anti-C6 matrix. Fresh frozen (−70 C) normal rat plasma containing 0.04 M ethylenediaminetetraacetic acid (EDTA) was applied to the column. The column was washed with phosphate-buffered saline containing 0.01 M EDTA (PBS-EDTA) followed by 1% Triton X-100 and then PBS-EDTA containing 2 M NaCl. The C6 was removed with 5 M urea and immediately dialyzed against veronal-buffered saline. After concentration of the eluate 20-fold, it was tested by micro-Ouchterlony against monospecific antibodies to whole-rat serum, human C6, rat C3, and rat IgG. The only contaminant detected was a small amount of rat IgG, which was removed by affinity chromatography using anti-rat IgG (Cappel Laboratories, West Chester, PA) bound to Cn-Br Sepharose 4B. The final C6 antigen preparation had C6 activity in a standard C6 hemolytic assay (see below).

Production and Characterization of Goat Anti-rat C6

Goats were immunized at multiple sites on the back and flanks with 60 to 100 μg rat C6 antigen in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) at 1 week and 3 weeks and in incomplete Freund's adjuvant at 6 and 9 weeks. The capacity of goat anti-rat C6 to reduce rat C6 hemolytic activity *in vitro* (neutralization) was tested as previously described.[15] After 10 to 12 weeks of immunization, immune goat plasma neutralized 50% of rat C6 hemolytic activity at a titer of 1:6 to 1:1000. The IgG fraction was isolated by precipitation with 5% caprylic acid (1 hour at 22 C), the precipitate was filtered, and the IgG-containing supernatant was dialyzed extensively. Although no rat C3 was detected in the C6 immunogen by Ouchterlony, the IgG fraction of the anti-rat C6 antibody had slight anti-rat C3 activity in a neutralization assay. Anti-rat C3 activity was removed by affinity chromatography using purified human C3 bound to Cn-Br Sepharose 4B. An immunoblot was made using this absorbed anti-rat C6 against normal rat serum.[21] A monoclonal antibody prepared in this laboratory that recognizes rat C6, and a commercial antisera to human C6 (Cytotech, San Diego, CA) were used as anti-C6 standards. To verify the capacity of purified antibody to deplete C6 *in vivo,* varying doses of the antibody were administered intraperitoneally (IP), and C6 levels were measured at multiple time points thereafter.

Preparation of $F(ab')_2$ Fragments of Goat Anti-rat C6 IgG

$F(ab')_2$ fragments of IgG were prepared according to the methods of Nelson and Manning.[24] Normal goat IgG or anti-C6 IgG (60 mg/ml) was dialyzed against 0.1 M acetate buffer, pH 4.3. An equal volume of pepsin (1.71 mg/ml; Sigma Chemical Co., St. Louis, MO) in acetate buffer was added and incubated with shaking for 18 hours at 37 C. The mixture was then dialyzed against 0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.2, and concentrated on a YM-10 membrane (Amicon, Danvers, MA). $F(ab')_2$ were separated from Fc pepsin digestion fragments by chromatography on Sephacryl S-200 (Pharmacia, Inc., Piscataway, NJ) equilibrated in pH 7.2 phosphate buffer. $F(ab')_2$-containing fractions were pooled and concentrated on a YM-10 membrane before use in depletion studies.

Effect of C6 Depletion with Anti-C6 IgG on the Development of Glomerular Injury

Baseline C6 levels were first measured in a group of 12 100-g Sprague Dawley rats that were then divided into

two groups of six with equivalent mean baseline C6 levels. C6 depletion was initiated in one group (C6D) by IP injection of 100-mg goat anti-rat C6 IgG. The control group received 100 mg of normal goat IgG. C6 depletion was maintained by injections of 45 mg of anti-rat C6 IgG at 10-hour intervals, and control rats received equivalent amounts of normal goat IgG. To document the maintenance of C6 depletion, blood samples were taken 10 hours after the initial antibody injection and before administration of anti-Fx1A IgG and then at daily intervals for measurement of C3, C5, C6, C7, C8, and C9 activity. Daily blood samples were drawn immediately before a maintenance injection of anti-C6 IgG to insure that the maximal C6 levels present during the experiment would be measured.

Three hours after the first maintenance dose of anti-rat C6 IgG was given, when C6 levels were less than 5% of baseline, PHN was induced by the administration of a standard nephritogenic quantity of sheep anti-Fx1A antibody trace labeled with ^{125}I-Fx1A IgG.[25] Urine protein excretion was determined in C6D and control animals on 24 hour collections obtained on day 3 to 4 after antibody injection. At the end of the experiments, biopsies were taken for microscopy. To measure glomerular antibody binding, the animals were sacrificed by ether anesthesia, kidneys were perfused with 60 ml of heparinized PBS, pH 7.2, and glomeruli were isolated by differential sieving techniques.[25] The quantities of glomerular bound ^{125}I-anti-Fx1A IgG were determined by counting in a Packard gamma counter (Downer's Grove, IL) and corrected for background and decay. Values were expressed as μg $\times 10^{-3}$ of antibody bound per 76,000 glomeruli.[25]

C6 Depletion with F(ab')$_2$ Anti-C6 IgG

In separate experiments after obtaining an initial blood sample for baseline levels of C6 and C3, rats were injected with 65 mg F(ab')$_2$ anti-C6 IgG or F(ab')$_2$ normal goat IgG. Two hours later, blood samples were obtained. PHN was then induced by giving nephritogenic amounts of sheep anti-Fx1A. C6 depletion was maintained by injection of 25 mg F(ab')$_2$ anti-C6 IgG every 8 hours. Urine protein excretion was determined on 24-hour collections obtained from day 4 to 5 after anti-Fx1A injection. Biopsies were taken for immunofluorescent studies.

Other Measurements

For complement component determinations, blood was collected from the retroorbital sinus into 40 mM EDTA. The plasma was separated and immediately frozen at −70 C until assayed. C6 was measured according to the recommendations of Diamedix Corp. (Miami, FL) except that 50 μl quantities of reagents were used because of the small amounts of rat blood available. To measure C6, EAC1gp4hu were used with an excess of Diamedix C2, C3, C5, C7 (human), and C8 and C9 (guinea pig). Measurements of C5 and C7 were made identically to those of C6 except excess Diamedix C6 was added and either Diamedix C5 or C7 was deleted. To measure C8 or C9, EAC 1 to 7 were used with dilutions of the test sera as the source of C8 or C9, with excess Diamedix C9 or C8. Results are expressed as the percentage of baseline C6 hemolytic titers remaining in the serum at the time of assay. The titer for each sample is the reciprocal of the dilution resulting in lysis of 50% of the cells as calculated from a linear regression plot of $-1n$ (1 − % lysis) *vs.* serum dilution. Plasma C3 levels were measured in all samples by Mancini single radial immunodiffusion[26] and expressed as a percentage of baseline C3 level in each animal measured before administration of anti-rat C6 or normal rat IgG.

Anti-Fx1A disappearance kinetics in C6D and control animals were determined from blood samples that were taken at 1 hour, 24 hours, and then daily. Radioactivity was measured in 100 μl of rat blood and expressed as the percentage of the initial concentration remaining. The concentration at time 0 was calculated based on the dose administered and the assumption that it was distributed in the total blood volume that was assumed to be 7% of body weight.[27]

Protein excretion was determined on 24-hour urine collections from rats housed in individual metabolic cages with free access to water. Protein was measured by the sulfosalicylic acid method[28] using a commercial whole serum standard (Lab-Trol, Dade Division, American Hospital Supply Corp., Miami, FL). Renal function was assessed by measurements of serum creatinine on day 4 of the study in animals depleted of C6 and controls.

Renal biopsies obtained at the time of sacrifice at 4 days were divided in three parts. One portion was fixed for light microscopic study in 10% neutral formalin. A second portion was snap frozen in isopentane in dry ice and stored at −70 C for immunofluorescent studies. Fluorescein conjugated antibodies to sheep IgG (Litton Biometics, Charleston, SC), rat IgG, goat IgG, and rat C3 (Cappel Laboratories) were employed. Indirect immunofluorescence was carried out using a goat anti-human C6 antibody known to be cross reactive with rat C6 (Atlantic Antibodies, Scarborough, ME), followed by rabbit anti-goat IgG[14]; staining for rat C5b-9 neoantigens used an antibody prepared in our laboratory.[18] Results were graded semiquantitatively on a scale of 0 to 4$^+$. Tissue for electron microscopy was immersion-fixed in glutaraldehyde paraformaldehyde in cacodylate buffer, and postfixed in osmium tetroxide before embedding. Thin sections were

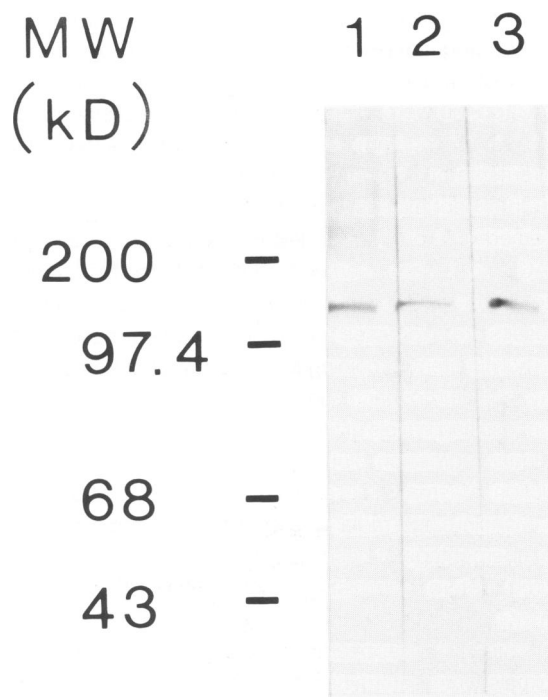

Figure 1. *Immunoblot of normal rat serum stained with goat anti-rat C6 (lane 1). In addition to staining rat C6 (middle), the antisera can be seen to be mildly reactive with other serum components. The normal rat serum was also stained with polyclonal goat anti-human C6 (lane 2) and monoclonal mouse anti rat C6 (lane 3).*

stained with uranyl and lead and examined in a Phillips 40 (Phillips, Amsterdam) transmission electron microscope.[15]

The Student's *t*-test for unpaired data was used to analyze results of the complement assays, urinary protein excretion and creatinine, and glomerular antibody binding.[29] Differences were regarded as significant when $P < 0.05$.

Results

Goat Anti-rat C6 Serum

Figure 1 is an immunoblot using the human C3 absorbed goat anti-rat C6 to stain separated normal rat serum proteins. The rat C6 band is identified near the center of the blot by the polyclonal goat anti-rat C6 (lane 1), the polyclonal cross reacting anti-human C6 (lane 2), and by monoclonal mouse anti-rat C6 (lane 3). The goat anti-rat C6 reacts primarily with C6 but also interacts slightly with several proteins over 200,000 kd.

C6 Depletion by Anti-rat C6 IgG

Baseline C6 levels were 93,772 ± 10,335 (SD) hemolytic activity units in six control rats and 92,899 ± 10,597 in experimental rats later subjected to C6 depletion. Three hours before administration of anti-Fx1A IgG (and 12 hours after the initial injection of anti-rat C6 IgG), C6 levels in the C6D group were 13.5 ± 7.9% (SEM) of the original titers, with four of six animals having values less than 1% compared with 111 ± 18.8% for the control group (Figure 2). At this point, the first maintenance dose of 45 mg of anti-C6 IgG was administered followed in 3 hours by administration of anti-Fx1A IgG. C6 levels, measured daily thereafter immediately before a maintenance injection of anti-C6 IgG, remained less than 4% of initial values in 25 of 30 serial measurements done over 4 days in the C6D group (Figure 2). The mean daily C6 levels in the control animals were always greater than 100% of baseline and exceeded 200% on 3 of the 4 days studied (Figure 2).

Effect of C6 Depletion on C3, C5, C7, C8, and C9 Levels

The mean levels of C3 10 hours after the initial injection of anti-C6 IgG and 3 hours before administration of anti-Fx1A were 87% ± 5.6% in the control group and 67% ± 13.4% in the C6D group ($P > 0.1$, Table 1). Subsequent measurements of C3 levels throughout the rest of the study revealed levels consistently above 55%; no significant difference between control and C6D animals was seen (Table 1). It was not possible to assay C5 hemolytically in rat plasma obtained after initiation of the experiment. The anti-C6 in the plasma samples neutralized the added excess Diamedix C6 making reaction mixtures C6 deficient. C7, C8, and C9 titers on representative C6D

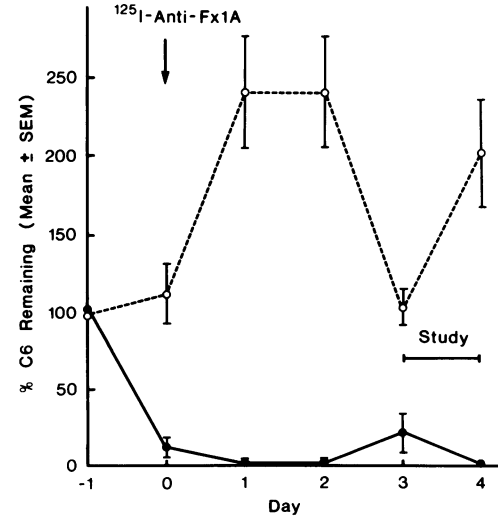

Figure 2. *Plasma C6 hemolytic activities were measured for 5 days after C6 depletion. PHN was induced 24 hours after initial C6 depletion and urinary protein excretion was measured at day 3 to 4.* ○, *controls;* ●, *C6D.*

Table 1. *Effect of C6 Depletion on C3 Levels in Rats Developing PHN*

Treatment	Baseline value (%)*				
	Day 0	Day 1	Day 2	Day 3	Day 4
Anti-C6 IgG (N = 6)	67 ± 13.3	94 ± 9.2	108 ± 17.3	84 ± 15.8	55 ± 7.8
Normal IgG (N = 6)	87 ± 5.6	98 ± 11.5	112 ± 11.5	97 ± 8.67	67 ± 5.6
$F(ab')_2$ anti-C6 (N = 3)	106 ± 7.6	80 ± 0.3	80 ± 2	121 ± 16.2	86 ± 9.8
$F(ab')_2$ Normal IgG (N = 4)	106 ± 11.8	115 ± 13	120 ± 20	128 ± 18	118 ± 7

* Values (±SEM) are based on the percentage of component activity compared with baseline plasma sample collected before the initial injection of goat anti-C6 or normal goat IgG or $F(ab')_2$ fragments. Day 0 values represent component activity before induction of PHN with sheep anti-Fx1A. All other samples were taken just before a maintenance injection of anti-C6 or normal IgG.

animals were equal to or greater than those of plasma samples drawn before initiation of the experiment at all times tested (data not shown).

Effect of C6 Depletion on Disappearance Kinetics of Anti-Fx1A IgG

The disappearance kinetics of radiolabeled anti-Fx1A IgG in C6D and control animals were similar to those described previously.[25] There was no difference in the rate of disappearance of radiolabeled antibody between the C6D animals and controls (data not shown).

Glomerular Antibody Binding

Values obtained for the binding of anti-Fx1A IgG in glomeruli in control and experimental animals measured 4 days after antibody injection are shown in Figure 3. C6D animals bound a mean of $5.7 \pm 0.7 \times 10^{-3}$ μg of ^{125}I-anti-Fx1A IgG/76,000 glomeruli (two kidneys) compared with $4.8 \pm 0.7 \times 10^{-3}$ μg in the controls. Although the experimental animals had more antibody deposition than the controls, the difference was not statistically significant ($P > 0.05$).

Effect of C6 Depletion on Proteinuria

The values for 24-hour urine protein excretion in C6D and control animals are also shown in Figure 3. Animals treated with normal goat IgG only had a mean protein excretion of 13.7 ± 4.5 mg per 24 hours. Proteinuria was totally abolished in the C6D group (1.5 ± 0.16 mg/24 hours; $P < 0.025$). On day 4, serum creatinine values were normal in both the C6D and control groups (0.69 ± 0.14 [SD] *vs.* 0.84 ± 0.22 mg [%]).

The Effect of C6 Depletion Using $F(ab')_2$ Anti-rat C6 on PHN

Results similar to those obtained with intact IgG were obtained when rats were depleted of C6 using the $F(ab')_2$ fragments of anti-C6 IgG. Three hours before injection of anti-Fx1A $F(ab')_2$ IgG, C6 titers were 17.3% ± 3.9% of baseline in the C6D group and 103% ± 26% in the control

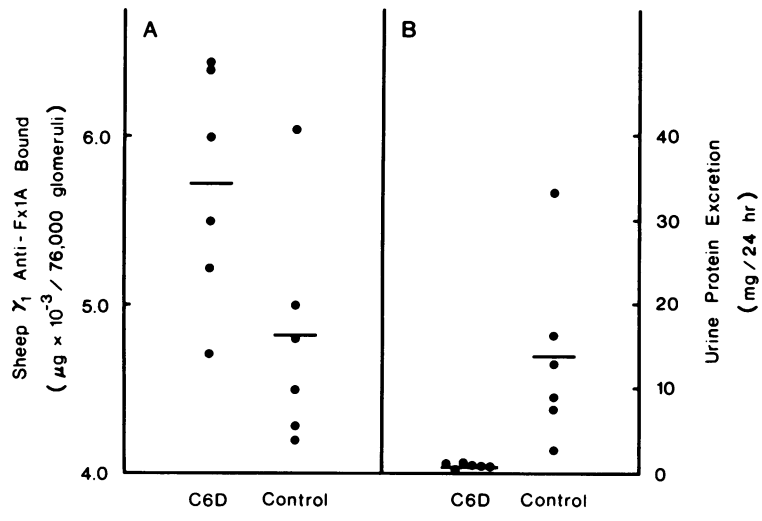

Figure 3. *The effect of C6 depletion on binding of anti-Fx1A IgG and on urinary protein excretion. ^{125}I-anti-Fx1A IgG deposition was higher in C6D rats but the difference was not statistically significant* (A). *However, depleting rats of C6 throughout the course of development of PHN completely abolished proteinuria* (B).

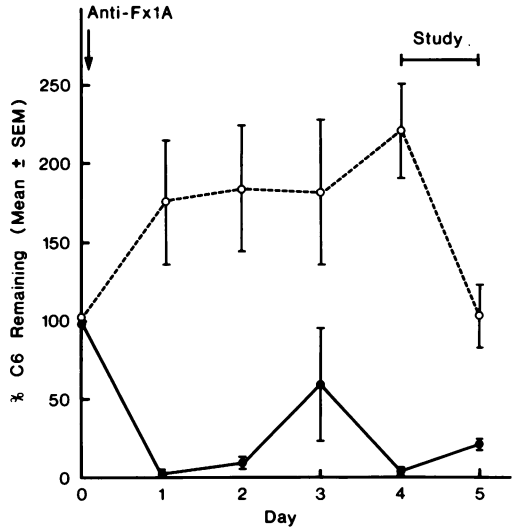

Figure 4. *Plasma C6 hemolytic activity was measured for 5 days after initiation of C6 depletion with F(ab')$_2$ anti-C6. PHN was initiated 2 hours after beginning C6 depletion and urine protein excretion was measured at day 4 to 5. ○, controls; ●, C6D.*

group (Figure 4). Of 12 C6 values obtained both 2 hours and 8 hours after administration of anti-C6 F(ab')$_2$, 10 were less than 10% of baseline values. In contrast, animals receiving nonantibody F(ab')$_2$ fragments had C6 levels that were always in excess of 100% of baseline values.

C3 levels in the animals depleted of C6 with anti-C6 F(ab')$_2$ were 106% ± 13% of baseline at the time of anti-Fx1A administration *vs.* 106% ± 23% in the control animals receiving nonantibody F(ab')$_2$; at no time were levels less than 80% of baseline (Table 1).

The effect of C6 deficiency on subsequent development of proteinuria in rats given anti-C6 F(ab')$_2$ was tested. Although the numbers of animals were small, the proteinuria in the C6D group (10.7 ± 5.7 mg/24 hours; N = 3) was significantly lower than in control animals (31.2 ± 1.9 mg/24 hours; N = 4; $P < 0.02$) with no overlap between the two groups.

Morphology

Biopsies obtained on day 4 showed no significant histologic changes in control rats or in those depleted of C6 with anti-rat C6 IgG or F(ab')$_2$. By immunofluorescence, all animals in the C6D and control groups had 3 to 4$^+$ diffuse granular capillary wall deposition of sheep IgG at 4 days, a finding characteristic of the PHN model.[9] Stains for rat IgG were negative; some increase in mesangial deposition of goat IgG was seen in the C6D animals compared with controls. Rat C3 was present in a similar granular pattern with similar intensity in control and C6D animals (Figure 5). Control animals had 1 to 2$^+$ granular capillary wall staining for rat C6. C6 staining was reduced to trace or was absent in the C6D group. Similarly, control animals had 1 to 3$^+$ deposition of rat C5b-9 neoantigens, whereas C6D animals had only trace amounts of C5b-9 detectable in glomerular immune deposits (Figure 6).

By electron microscopy, control rats showed electron dense deposits localized in the subepithelial space and slit pore regions with extensive effacement of epithelial cell foot processes (Figure 7). C6D rats had equivalent amounts of subepithelial immune deposits but no significant change in epithelial cell morphology (Figure 7).

Discussion

Until recently, the role of complement in the mediation of antibody-induced injury to the glomerular capillary wall was believed to be primarily an indirect one with complement-derived opsonins, chemotactic factors, or both leading to localization of inflammatory cells that produced tissue injury through release of proteases, reactive oxygen species, or both.[1,2]

However, we have postulated that the membrane attack complex, C5b-9, directly mediates glomerular injury associated with subepithelial immune deposit formation.[9,30,31] In support of this hypothesis, studies in a rabbit model of chronic serum sickness induced by immunization with cationized BSA showed that C6D rabbits developed significantly less proteinuria than controls despite equivalent glomerular deposits of both antigen and antibody.[13] Later studies by Cybulsky et al[19,20] also indicated a role for C5b-9 in membranous nephropathy in rats. These authors used an isolated perfused kidney model in which rat kidneys bearing nephritogenic amounts of subepithelial anti-Fx1A were perfused with dilute normal human plasma.[20] A significant increase in protein excretion was seen, which did not occur when the perfusate was C8-deficient human or C6-deficient rabbit plasma. A similar increase in proteinuria occurred when the isolated kidney contained the nonnephritogenic, noncomplement-fixing γ2 fraction of anti-Fx1A and when the perfusate contained heat-inactivated guinea pig antisera to sheep IgG and a complement source.[19] However, the isolated kidney is a difficult model to study because of markedly elevated protein excretion in normal kidneys,[10] and because the time course of disease development in this system does not resemble that documented *in vivo*.[25]

Others have documented the role of C5 in the mediation of glomerular immune complex localization and neutrophil influx, necrosis, and crescent formation in C5-deficient mice compared with controls.[32] However, these studies do not distinguish between effects of the chemo-

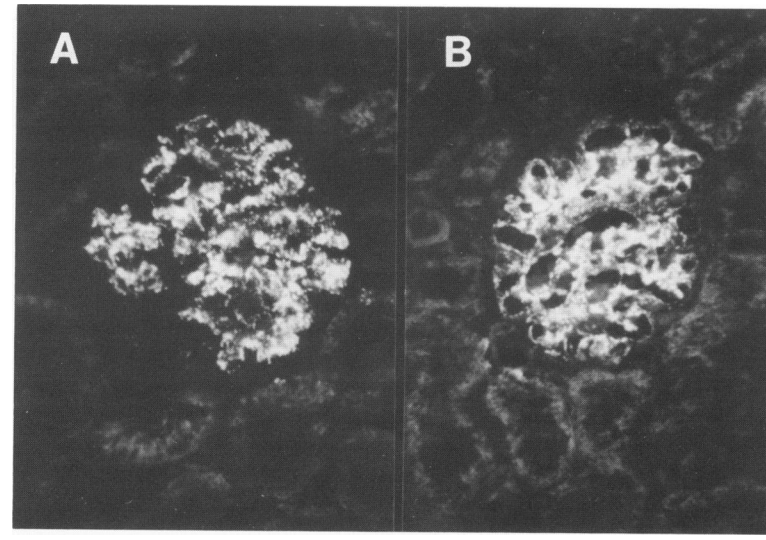

Figure 5. *Immunofluorescent photomicrographs of deposits resulting from injection of anti-Fx1A IgG.* **A**: *Control glomeruli with C3 deposits in the capillary walls.* **B**: *C6D glomeruli have no apparent reduction in staining for C3.*

tactic peptide C5a and the terminal C5b-9 membrane attack complex.

Thus, none of these studies directly test the hypothesis that, in PHN in the intact animal, antibody to a glomerular epithelial cell membrane antigen *in vivo* results in a loss of glomerular barrier function through a C5b-9 dependent mechanism. Because no complement-deficient rat strains are available, we tested this hypothesis by inducing C6 depletion *in vivo* with polyclonal antibody specific for rat C6. The final preparation of goat anti-rat C6 IgG reacted with C6 by immunoblotting, neutralized C6 hemolytic activity *in vitro,* and induced a decrease in serum C6 levels to less than 5% of normal within 2 hours of injection. C6 depletion persisted for over 12 hours. To ensure the adequacy of C6 depletion, C6 hemolytic levels were measured daily at peak levels of C6 just before the next maintenance injection of anti-C6 antibody. Thus, peak C6 levels in depleted PHN rats were maintained at less than 4% of baseline values in 25 of 30 serial measurements over the 4 day study period. Values exceeding 4% of baseline (38% to 75%) were present transiently in 2 of 6 animals at isolated time points during the study (Figure 2). However, animals with transient C6 increases demonstrated little detectable glomerular C5b-9 deposition and did not develop proteinuria in excess of that observed in animals with C6 levels maintained consistently below 4%, a finding that suggests that the degree of depletion achieved was adequate to abrogate nephritogenic amounts of glomerular C5b-9 formation. C6 levels in control animals given normal goat IgG were always in excess of 100% of baseline.

The C6 depletion protocol used was not entirely selective for depletion of C5b-9 because animals depleted of C6 with IgG antibody also exhibited some depression of C3 levels as well. This may have resulted from C3 activation due to intravascular formation of C6-anti-C6 and other

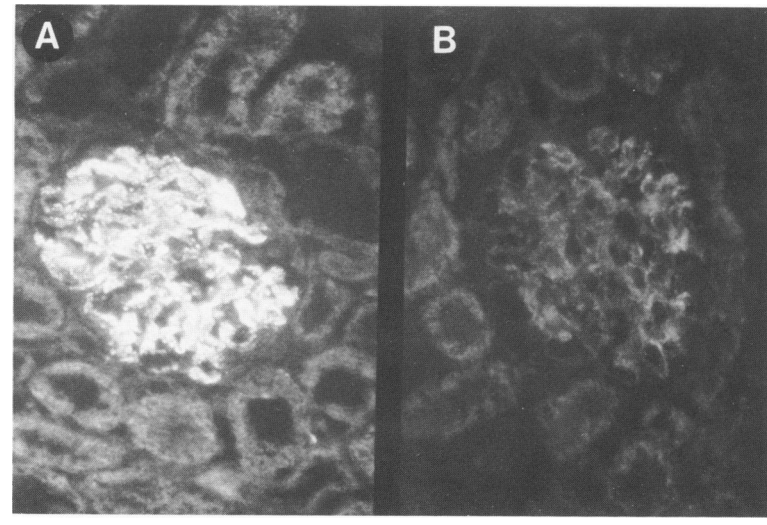

Figure 6. *Immunofluorescent photomicrographs of C5b-9 deposits resulting from injection of anti-Fx1A IgG.* **A**: *Control glomeruli with C5b-9 complexes in the capillary walls.* **B**: *C6D glomeruli have a marked reduction in staining for C5b-9.*

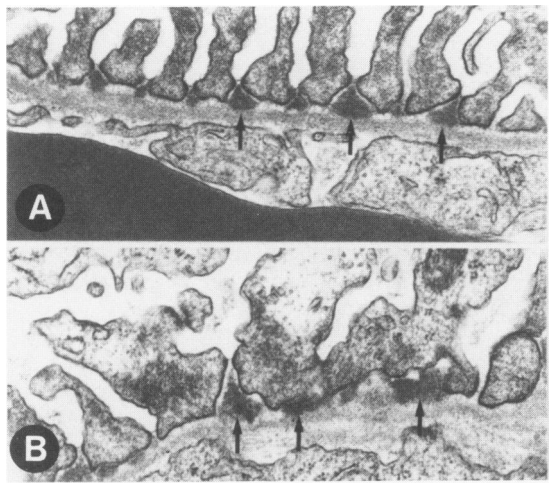

Figure 7. *Electron micrographs of the glomerular capillary wall from C6D* **(A)** *and control* **(B)** *rats. Equivalent subepithelial immune deposits are seen in both animals (arrows). Extensive epithelial cell foot process effacement is seen in the control but is not present in the C6D rat.*

types of immune complexes. C3 levels in C6D were depressed approximately 33% on day 1 but then rose and were not significantly different from C3 levels in control animals at any point during the study. Moreover, glomerular C3 deposition in subepithelial deposits was not reduced in C6D compared with control animals, although mesangial deposits of C3 and IgG were somewhat more pronounced in C6D rats. Although the possibility cannot be excluded that the 20% difference in C3 levels between control and C6D animals on day 1 may have contributed to the lack of proteinuria in the C6D group, all animals, including those with C3 levels within 1 standard deviation of controls, failed to develop proteinuria. Moreover, previous studies of C3 depletion with CVF suggested that more than 50% depletion of C3 was required to significantly reduce proteinuria in the PHN model (unpublished observations, W. G. Couser, 1985).

To further document that C3 depletion did not contribute significantly to our findings, the studies were repeated using the $F(ab')_2$ portion of anti-C6 IgG to induce C6 depletion. Antibody lacking the Fc piece was approximately equivalent in efficacy to whole IgG in depleting serum C6 hemolytic activity and in abolishing glomerular C5b-9 deposition and proteinuria. However, C3 levels remained in excess of 80% of baseline. In addition to C3, we also measured levels of C7, C8, and C9 in representive C6D rats throughout the IgG depletion experiment. Quantitation of C5 titers by hemoytic assay proved impossible as C5 levels are relatively low and the anti-C6 present in the rat plasma samples neutralized the excess Diamedix C6 in the assay, even when C6 was increased further. However, this did not prove to be a problem in measuring C7, C8, or C9 because of their high serum concentrations. In all cases, C7, C8, and C9 titers throughout the experiment were equal to or greater than the control serum titers collected before initiation of the experiment. Because early complement components were not examined, we cannot exclude the possibility that depletion of C1, C4, or C2 may have contributed to the reduced proteinuria observed in C6D rats. However, this seems unlikely because plasma C3 levels remained high and C3 deposits in the renal biopsies of the C6D rats were comparable to those in controls receiving normal goat IgG. We therefore conclude that the marked reduction in proteinuria observed in C6D rats was due primarily to the inability of the C6D animals to form the terminal C5b-9 complex.

The effect of C6 depletion with either IgG or $F(ab')_2$ antibody to rat C6 was to essentially abolish glomerular deposition of C6 and C5b-9 and to prevent the development of significant proteinuria or epithelial cell foot process effacement despite equivalent deposits of antibody in the subepithelial space as shown by immunofluorescence, electron microscopy, and quantitative measurements of glomerular deposition of radiolabeled antibody. The $F(ab')_2$ results, therefore, confirm our hypothesis that glomerular injury in this model is mediated by the C5b-9 portion of the complement system. Our findings are in accord with previous immunopathologic studies demonstrating a correlation between glomerular C5b-9 deposits and complement-dependent proteinuria in experimental glomerular disease.[15–17] The presence of C5b-9 deposits in a similar pattern has been reported in several human glomerular diseases, including membranous nephropathy, suggesting that this mechanism may operate in man as well.[33–35]

The mechanism by which C5b-9 induces injury to the glomerular filtration barrier has not been defined. The pathobiology of the complement membrane attack complex has been recently reviewed[36] and possible mechanisms by which C5b-9 may injure glomeruli are discussed more extensively elsewhere.[31] A complement-mediated reduction in glomerular filtration rate (GFR) associated with an increase in filtration fraction could result in some increased protein filtration on a hemodynamic basis.[2,37] Antibody-induced reduction in GFR has been shown to be partially complement dependent in nephrotoxic nephritis in the rat.[38] Glomerular hemodynamics during development of PHN have been studied by Gabbai et al,[39] who also demonstrate a reduction in GFR and increase in filtration fraction in PHN at 5 days accompanied by a significant increase in intraglomerular pressure.[39,40] These changes were partially prevented by complement depletion and suggest that complement-mediated intrarenal hemodynamic effects may have contributed to proteinuria, particularly to the rise in intraglomerular pressure that is clearly related to the magnitude of deficit in the filtration barrier in PHN.[41] Thus, complement activation may induce

hemodynamic changes that can exacerbate proteinuria, but there is little evidence of a role for C5b-9 in this process as opposed to other vasoactive complement peptides such as C5a.[42] Thus, C5b-9 probably has a more important direct effect on the functional and structural integrity of the filtration barrier. Moreover, it is unlikely that hemodynamic factors alone are sufficient to account for the marked differences in protein excretion observed between complement-depleted and control animals with PHN.[10,15]

Immunoultrastructural studies in human and experimental glomerular disease suggest that C5b-9 deposits are usually found in association with cell membranes or membrane fragments.[35,43] We have studied by immunoelectron microscopy the deposition of C5b-9 in the kidneys of PHN rats.[21] By day 3, C5b-9 could be localized not only in the subepithelial immune deposits but also on the cell membranes of foot processes in clathrin-coated pits, and in membrane vesicles within the cytoplasm, as well as in high concentration within large multivesicular cytoplasmic bodies. C5b-9 was also seen within disrupting membrane vesicles on the urinary side of the glomerular epithelial cell. Freeze fracture electron microscopy showed 200 to 250 A intramembrane particles believed to be membrane-inserted C5b-9 complexes in the "soles" of the foot processes. Thus, proteinuria in PHN is most likely a consequence of C5b-9 attack on the glomerular epithelial cell initiated by membrane insertion of the complex.

Recently it has become apparent that membrane inserted C5b-9 may induce nonlytic perturbations of nucleated cell membranes that are accompanied by marked changes in cell metabolism. C5b-9 has been shown to be a potent stimulator of prostaglandin release by platelets, PMN's, macrophages, and tumor cells. C5b-9 can also activate glomerular mesangial cells to release several potential inflammatory mediators including prostaglandins, an interleukin I-like cytokine,[44,45] and reactive oxygen species.[46] C5b-9 can activate rat glomerular epithelial cells to produce increased prostaglandins and thromboxanes[47] and to increase type IV collagen production.[48] Although the mechanism(s) by which C5b-9-induced alterations in cell metabolism may lead to loss of the glomerular barrier to protein filtration are unknown at the present time, this issue should provide a fruitful avenue for future research.

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