Role of the Terminal Complement Pathway in Experimental Membranous Nephropathy in the Rabbit

GERALD C. GROGGEL, STEPHEN ADLER, HELMUT G. RENNKE, WILLIAM G. COUSER, and DAVID J. SALANT, *Evans Memorial Department of Clinical Research and Department of Medicine, University Hospital, Boston University Medical Center, Boston, Massachusetts 02118; Department of Pathology, Brigham and Womens Hospital, Boston, Massachusetts 02115*

ABSTRACT Our recent observations of a complement-mediated, cell-independent mechanism of altered glomerular permeability in rat membranous nephropathy suggested a possible role for the terminal complement pathway in the mediation of proteinuria in certain forms of glomerular disease. To directly determine whether the membranolytic terminal complement components (C5b-C9) are involved in glomerular injury, we studied the development of proteinuria in normal and C6-deficient (C6D) rabbits, in both of which a membranous nephropathy-like lesion develops early in the course of immunization with cationized bovine serum albumin (cBSA) (pI 8.9–9.2). C6 hemolytic activity of C6D was 0.01% that of control rabbits. After 1 wk of daily intravenous injections of cBSA, proteinuria developed in 71% of controls (median 154, range 1–3,010 mg/24 h, n = 24), whereas none of C6D were proteinuric (median 6, range 2–12 mg/24 h, n = 12, $P < 0.01$). After 1 wk of cBSA, both groups had qualitatively identical glomerular deposits of BSA, rabbit IgG, and C3 on immunofluorescence microscopy, predominantly subepithelial electron-dense deposits on electron microscopy, and minimal glomerular inflammatory cell infiltration of glomeruli. Glomeruli were isolated from individual animals after 1 wk of cBSA and deposits of rabbit IgG antibody were quantitated by a standardized in vitro assay using anti-rabbit IgG-^{125}I. Rabbit IgG deposits were found to be similar in control (29.8±13.2, range 12.7–48.6 μg anti-IgG/2,000 glomeruli, n = 6) and C6D rabbits (32.6±13.8, range 16.8–48.8 μg anti-IgG/2,000 glomeruli, n = 5, $P > 0.05$). After 2 wk, coincident with a prominent influx of mononuclear cells and neutrophils, proteinuria developed in C6D rabbits.

These results document, for the first time, a requirement for a terminal complement component in the development of immunologic glomerular injury. Since the only known action of C6 is in the assembly of the membrane attack complex, these observations suggest that the membranolytic properties of complement may contribute to glomerular damage.

INTRODUCTION

It has long been known that serum complement mediates tissue injury in the Arthus reaction (1) and heterologous phase of certain types of anti-glomerular basement membrane (GBM)[1] nephritis (2) through its leukocyte chemoattractant and immune adherent properties (3). More recently, in two models of experimental membranous nephropathy, we observed a form of complement-mediated glomerular injury that occurred independently of any inflammatory infiltrate and in the face of selective neutropenia and pancytopenia (4, 5). These observations, together with others demonstrating a cell-independent ameliorative effect of complement depletion on hyperacute cardiac allo-

Portions of this work were presented at the 14th Annual Meeting of the American Society of Nephrology, Chicago, IL, December 1982 and published in abstract form in 1983, *Kidney Int.*, 23:182.

Dr. Salant is the recipient of Clinical Investigator Award AM 00742 and Dr. Adler was the John P. Merrill Fellow of the National Kidney Foundation (1981). Drs. Groggel and Adler were recipients of the National Research Training Grant AM 07053. The present address of Drs. Adler and Couser is Department of Medicine, University of Washington, Seattle, WA 98195. Address reprint requests to Dr. Salant, Renal Section, Boston University Medical Center.

Received for publication 15 April 1983 and in revised form 18 July 1983.

[1] *Abbreviations used in this paper:* ABC-33, antigen-binding capacity 33; cBSA, cationized bovine serum albumin; C6D, C6 deficient; DDW, distilled and deionized water; Fx1A, proximal tubular epithelial cell brush border antigen; GBM, glomerular basement membrane; MAC, membrane attack complex of complement.

graft rejection (6, 7), suggested a hitherto undescribed role for complement in the mediation of tissue injury.

In addition to its inflammatory properties, for which C3b and C5a (3) are responsible, complement has a cytolytic action that is mediated by a cell membrane-bound complex of the terminal complement components, C5b-9, also known as the membrane attack complex (MAC) (8). Immunofluorescence studies of tissue from patients with systemic lupus erythematosus (9, 10), idiopathic membranous nephropathy and anti-GBM nephritis (11) have indicated the presence of the neoantigen of the MAC. Poly C9, a polymer of C9 that forms part of the MAC (12), has also been found in glomerular lesions in patients with a variety of renal diseases (13). While such findings support the hypothesis that the MAC is responsible for tissue injury, they by no means prove it.

Our previous studies on the cell-independent role of complement in glomerular injury were performed in the rat, which is the only species susceptible to the passive Heymann nephritis model of membranous nephropathy, but which lacks strains deficient in any of the terminal components of complement. To directly examine the role of terminal complement components in glomerular injury, we used the rabbit, a species in which a C6-deficient (C6D) strain exists, and in which a membranous nephropathy-like lesion develops early in the course of induction of chronic serum sickness with cationized bovine serum albumin (cBSA) (14). These studies compare the early, noninflammatory phase of glomerular injury in normal and C6D rabbits and document a requirement for C6 in the development of proteinuria in this model. Since the only known action of C6 is in the assembly of the MAC (8), these observations strongly suggest a role for the MAC in glomerular injury.

METHODS

Preparation and characterization of the antigen. Crystalline fraction V bovine serum albumin (Miles Laboratories, Inc., Elkhart, IN) was used to prepare cBSA. Cationization was accomplished by carbodiimide activation of carboxyl groups and substitution of ethylenediamine according to a modification of the method of Hoare and Koshland (15). In brief, the solution of ethylenediamine (Sigma Chemical Co., St. Louis, MO) was prepared by adding 67 ml of anhydrous ethylenediamine to 50 ml of distilled and deionized water (DDW) in a 1-liter flask. The pH was adjusted to 4.75 with 350 ml of 6 N HCl. The solution was cooled to 25°C and 25 ml of a 20% solution of BSA in DDW (wt/vol) was added. While maintaining pH and constant temperature, 1.8 g of 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (Sigma Chemical Co., St. Louis, MO) was added with constant stirring, and the reaction was continued for 2 h. The reaction was stopped by adding 30 ml of 4 M acetate buffer, pH 4.75. The product was extensively dialyzed against DDW, lyophilized, and stored at −70°C.

The isoelectric point (pI) of the cBSA was measured in a thin-layer polyacrylamide gel (5%), pH range 3.5–9.5 (Bio-Rad Laboratories, Richmond, CA) using a Bio-Rad horizontal electrophoresis cell (Bio-Rad Laboratories). The pH gradient was determined directly from the gel with a surface glass pH electrode and Corning pH meter (Corning Scientific Instruments, Chicago, IL). The molecular size of the cBSA was determined by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a Bio-Rad vertical electrophoresis cell (Bio-Rad Laboratories).

Experimental design and induction of chronic serum sickness. New Zealand white rabbits (1.5–2.0 kg, Pine Acres Rabbitry, Brattelboro, VT) and C6D rabbits (raised from a breeding colony obtained from the National Institutes of Health, Bethesda, MD) were used. Each animal received a primary intravenous immunization with 1 mg of cBSA and 1 μg of endotoxin (Difco Laboratories, Detroit, MI) dissolved in phosphate-buffered saline, pH 7.2 (PBS). 1 wk later, daily (5 d/wk) injections of cBSA, 25 mg i.v., were begun. The cBSA was dissolved in PBS (12.5 mg/ml) and centrifuged for 30 min at 27,000 *g* before injection. The animals were housed in individual cages and fed a standard diet. Blood was obtained before the first injection and weekly thereafter for C6 activity, C3 levels, serum creatinine and anti-BSA measurements. 24-h urine collections were obtained on the first day of each week, starting 6 h after the daily cBSA injection. Animals were killed after 1 or 2 wk of daily injections.

Assays. Urine protein was measured by a sulfosalicylic acid method on 24-h urine collections using a whole serum standard (Lab-Trol, Dade Diagnostics, Inc., Aguada, Puerto Rico). Serum creatinine was measured by the Jaffe method (Worthington Diagnostics, Freehold, NJ). Specific C6 hemolytic activity was measured with EAC1gp4hu and an excess of human C2, C3, C5, C7, and guinea pig C8 and C9 by a modification of the method of Kabat and Mayer (16) exactly as described by the suppliers of the reagents (Cordis Laboratories, Miami, FL). Results are expressed in CH50 units/ml of serum. Serum C3 was measured by single radial immunodiffusion (17). Serum antibody levels to cBSA were measured by the antigen-binding-capacity 33 (ABC-33) technique of Minden and Farr (18). cBSA was labeled with ^{125}I (New England Nuclear, Boston, MA) by a modification of the method of McConahey and Dixon (19) to a specific activity of 1.4×10^6 cpm/μg of protein. Serum samples, diluted 1:100, 1:500, and 1:2,500, were incubated with an equal volume (0.5 ml) of a solution containing 0.2 μg cBSA-^{125}I in PBS. Results are expressed as micrograms of cBSA bound per milliliter undiluted serum.

Quantitation of glomerular deposits of rabbit anti-BSA IgG. To determine the amount of rabbit IgG immunologically bound in the glomeruli of individual animals, the following procedure was used. The IgG fraction of sheep anti-rabbit IgG (heavy and light chains; Cappel Laboratories, Cochranville, PA) was radiolabeled with ^{125}I (19, 20). Specific activity was 9×10^4 cpm/μg protein, of which 95% was precipitable with 10% trichloroacetic acid. The labeled IgG was stored at −70°C and was diluted with unlabeled IgG before use. Glomeruli were isolated by differential sieving, as previously described (20), from the nonperfused kidneys of individual control (n = 6) and C6D (n = 5) rabbits after 1 wk of injections of cBSA, and a normal uninjected rabbit. After extensively washing the glomeruli with cold PBS, four test tubes of 2,000 glomeruli from each animal were incubated at 37°C for 48 h with 500, 1,000, 1,500, and 2,000 μg of anti-rabbit IgG-^{125}I in a reaction volume of 1 ml of Hanks' balanced salt solution containing 2% human serum albumin, penicillin (25 IU/ml), and streptomycin (25 μg/ml). After

incubation, the tubes were centrifuged at 160 g for 5 min, the supernatant was removed, and the glomeruli were washed three times and counted for ^{125}I. Pilot studies had shown that binding equilibrium was reached after 48 h at 37°C, incubation at lower temperatures required longer time for equilibrium to be reached, trichloroacetic acid precipitability of labeled antibody remained >90% and that no contamination with microorganisms occurred up to 48 h.

Specific binding of anti-rabbit IgG-^{125}I to glomeruli from experimental animals was determined by subtracting counts bound nonspecifically to normal rabbit glomeruli at each dose. For each experimental animal, specific binding of anti-rabbit IgG per 2,000 glomeruli in each of the four tubes was plotted against the total counts added to that tube. In six of 11 experimental rabbits (four control and two C6D) antigen saturation was demonstrated by a plateau of binding in response to increasing antibody concentration. Maximum binding was equated with the average of the tubes showing saturation. In assays of five experimental animals (two control and three C6D), antigen saturation was not achieved and Scatchard plots were therefore done to determine maximum binding (21). Counts were converted to micrograms of sheep IgG from the specific activity of the radiolabeled anti-rabbit IgG. Results are therefore expressed as micrograms of sheep anti-rabbit IgG bound per 2,000 rabbit glomeruli.

To validate this method, identical conditions were used to assess the correlation between the binding of anti-rabbit IgG in vitro to rat glomeruli which contained subepithelial deposits of rabbit IgG, and the actual amount of rabbit IgG that had been immunologically planted in these glomeruli in vivo in the form of antibody to rat proximal tubular epithelial cell brush border antigen (Fx1A). The IgG fraction of rabbit anti-rat Fx1A was prepared and radiolabeled with ^{131}I exactly as described before (20) and was shown to produce granular capillary wall deposits of rabbit IgG by immunofluorescence and subepithelial electron-dense deposits typical of passive Heymann nephritis (22). Five Charles River CD rats (Charles River Breeding Laboratories, Wilmington, MA) were given ^{131}I-labeled rabbit anti-rat Fx1A IgG in incremental doses (5, 10, 15, 20, and 30 mg i.v.) to produce subepithelial deposits of known antibody content. After 72 h, glomeruli were isolated and aliquots of 2,000 glomeruli from each rat and a normal control rat were incubated at 37°C for 48 h, as described above, with 50, 100, 150, and 200 μg sheep anti-rabbit IgG-^{125}I. After incubation, glomeruli were washed, counted for ^{131}I and ^{125}I and, after correcting for background, spillover, and nonspecific binding, the amounts of rabbit IgG deposited in vivo and the in vitro binding of anti-rabbit IgG were calculated. Antigen saturation was demonstrated at all antibody doses in every rat. The correlation between the in vitro binding of sheep anti-rabbit IgG and the actual amount of rabbit IgG present was therefore determined from the regression line relating the sheep IgG-^{125}I and rabbit IgG-^{131}I content of 2,000 glomeruli in each tube as shown in Fig. 1. There was an excellent correlation between the in vitro binding of anti-IgG and the amount of IgG deposited in vivo. In addition, a high degree of penetration of glomeruli by anti-rabbit IgG in vitro is demonstrated by the binding ratio of 12:1 (slope of the line) and a very small amount of nonspecific binding is indicated by the fact that the y intercept passes close to the origin (Fig. 1).

Tissue processing and immunofluorescence procedures. Tissue for light microscopy was fixed in 10% neutral buffered formalin, sectioned at 4 μm, and stained with hematoxylin and eosin and periodic acid-Schiff reagent.

Direct immunofluorescence procedures were performed on renal tissues snap frozen in dry ice-isopentane, sectioned at 4 μm in a cryostat, and fixed in ether-alcohol as previously described (22). All biopsies were stained with the fluoresceinated IgG fractions of monospecific antisera to rabbit IgG, rabbit C3, and BSA (Cappel Laboratories, West Chester, PA). The rabbit anti-BSA did not cross-react with rabbit albumin in Ouchterlony analysis.

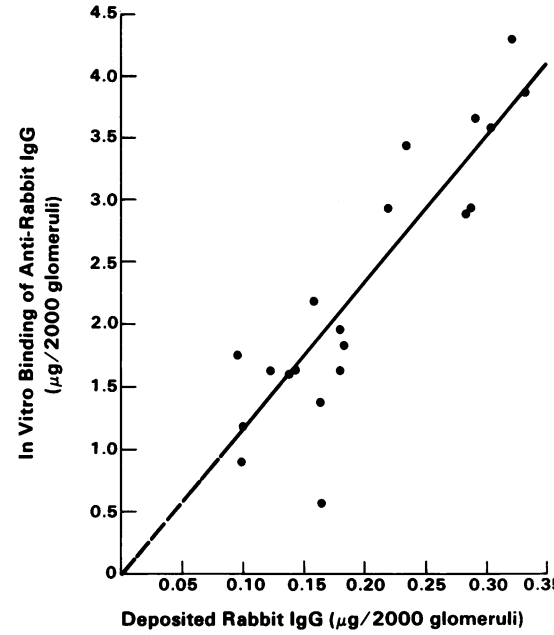

FIGURE 1 Relationship between the in vitro binding of anti-rabbit IgG and rabbit anti-Fx1A IgG deposited in vivo in glomeruli of rats. Glomeruli were isolated from five individual rats injected with incremental doses of rabbit anti-rat Fx1A-^{131}I and were incubated with sheep anti-rabbit IgG-^{125}I (50, 100, 150, and 200 μg). $y = 12.21\ x - 0.16$; $r = 0.896$; $P < 0.001$.

Tissue for electron microscopy was fixed by immersion in glutaraldehyde-paraformaldehyde mixture (2 g/100 ml paraformaldehyde, 2.5 g/100 ml glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4), rinsed in cacodylate buffer, postfixed in 1 g/100 ml of aqueous osmium tetroxide for 2 h, dehydrated in graded ethanols, cleared in propylene oxide, and embedded in epoxy resin. 1-μm thick sections and ultrathin sections (grey-silver interference color) were cut on an LKB-Ultrotome (LKB Instruments, Inc., Gaithersburg, MD). Thick sections were stained with toluidine blue and thin sections were stained on the grid with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope.

Statistical analysis. Results were evaluated by the Mann-Whitney U test for analysis of nonparametric data (23). Regression analysis and calculation of the correlation coefficient was done by standard methods (23). Parametric values are expressed as the mean±1 SD, and nonparametric values as the median and range.

RESULTS

The pI of cBSA was 8.8–9.3 with native BSA having a pI of 4.5–5.3. Sodium dodecyl sulfate-polyacrylamide

gel electrophoresis showed that the cationization process caused very little polymerization of the cBSA.

Specific C6 hemolytic activity in C6D rabbits was 6±0.8 CH50 units/ml, which represents 0.01% of C6 activity in control rabbits (60,000±14,140 CH50 units/ml). C3 levels in the C6D rabbits were 121±34% of pooled, control rabbit sera by radial immunodiffusion. Anti-BSA antibodies were measured in the serum of both groups by ABC-33 using 0.2 μg BSA as antigen. After 1 wk of daily cBSA, serum from control rabbits precipitated 6.5–99.0 μg BSA/ml (median 32.0, n = 14) and serum from C6D rabbits precipitated 2.0–46.9 μg BSA/ml (median 17.2, n = 13). Although the difference reached statistical significance (P = 0.05), there was considerable overlap between animals in the two groups. Serum creatinine did not change in any of the animals and after 1 wk of cBSA injections, it was 1.2±0.4 mg/dl in control and 1.1±0.4 mg/dl in C6D rabbits.

The results of 24-h urine protein excretion in control and C6D rabbits after 1 wk of daily cBSA are shown in Fig. 2. 24-h urine protein excretion exceeded the normal upper 99% confidence limit of 18 mg/24 h in 71% of controls (median 154, range 1–3,010 mg/24 h, n = 24), whereas none of the C6D rabbits were proteinuric (median 6, range 2–12 mg/24 h, n = 12, P < 0.01). In control rabbits there was a rough linear correlation between the serum anti-BSA levels, as measured by ABC-33, and the log urine protein excretion at 1 wk ($\log y = 0.0233\,x + 0.8311$, n = 14, r = 0.58, P < 0.05), which predicts that all animals with ABC-33 > 20 μg BSA/ml should have excreted urine protein >18 mg/24 h. Whereas this was true of 90% (nine of 10) of controls, none (zero of six) of the C6D rabbits with ABC-33 > 20 μg BSA/ml had abnormal protein excretion.

More direct evidence that the difference in proteinuria at 1 wk between controls and C6D rabbits was not the result of quantitative differences in glomerular antibody deposition at 1 wk is presented in Fig. 3. Glomeruli isolated from the kidneys of individual rabbits in each group, and incubated under defined conditions (see Methods), were found to bind equivalent amounts of radiolabeled anti-rabbit IgG (controls 29.8±13.2, range 12.7–48.6 μg/2,000 glomeruli, n = 6; C6D 32.6±13.8, range 16.8–48.8 μg/2,000 glomeruli, n = 5, P > 0.05), thereby indicating the presence of similar quantities of deposited rabbit IgG antibody.

Glomerular immune deposits in the two groups were also found to be qualitatively similar by immunofluorescent and electron microscopy. Fig. 4 demonstrates the similarity in glomerular capillary wall deposits of rabbit IgG and C3 in control (Fig. 4, *a* and *c*) and C6D rabbits (Fig. 4, *b* and *d*). IgG deposition at 1 wk was linear-granular in appearance and followed a peripheral capillary loop pattern (Fig. 4, *a* and *b*). With time, the granularity of IgG deposits became more apparent. At 1 wk, C3 was deposited in a granular, segmental pattern occupying peripheral capillary loops and mesangium (Fig. 4, *c* and *d*). With time, C3 deposits increased in intensity in both groups and involved peripheral capillary walls more diffusely. At 1 wk, BSA deposition was similar in both groups with a linear-granular appearance. Electron microscopy of glomeruli was identical in control (Fig. 5 *a*) and C6D rabbits (Fig. 5 *b*),

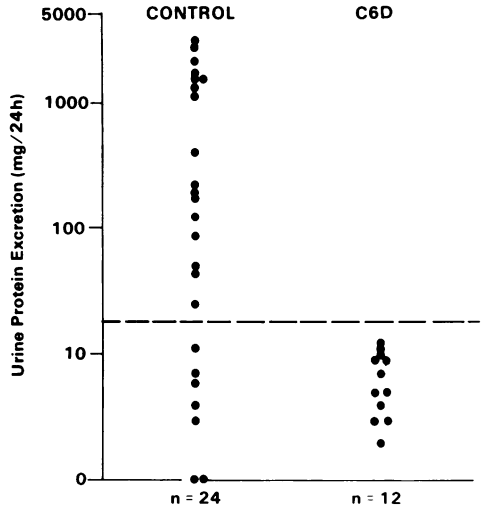

FIGURE 2 24-h urine protein excretion in control and C6D rabbits after 1 wk of daily cBSA. The dashed line represents the upper 99% confidence limit in normal rabbits (18 mg/24 h). The difference between the groups is significant (P < 0.01).

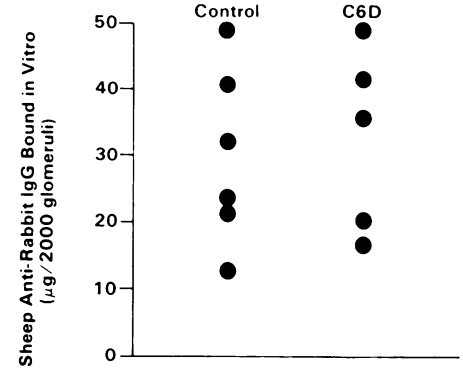

FIGURE 3 In vitro binding of anti-rabbit IgG to rabbit glomeruli containing rabbit anti-BSA antibody. Glomeruli from control and C6D rabbits were isolated after 1 wk of daily cBSA and were incubated with anti-rabbit IgG-^{125}I. No difference in binding was found between the groups (P > 0.05) indicating the presence of similar rabbit IgG deposits.

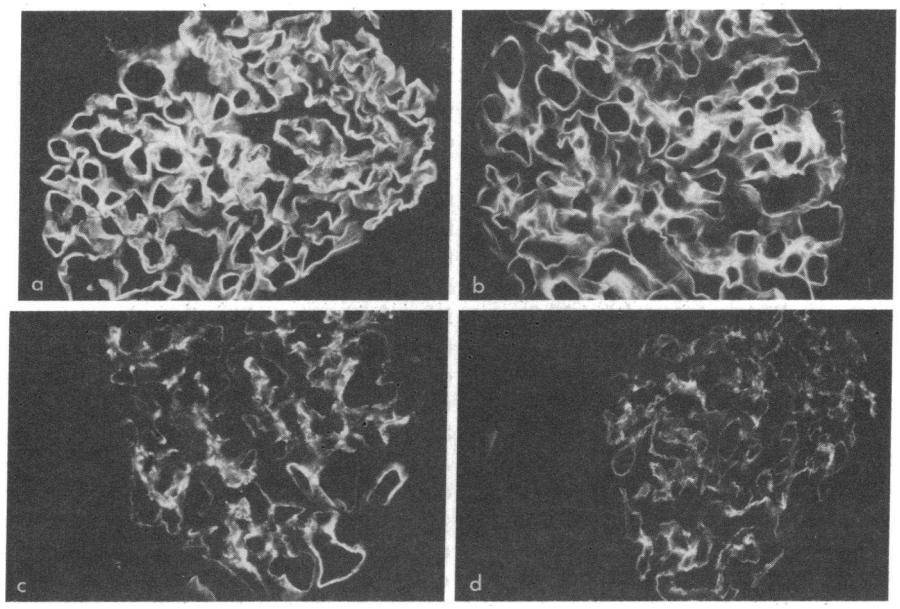

FIGURE 4 Immunofluorescent photomicrographs after 1 wk of daily cBSA in control (*a* and *c*) and C6D (*b* and *d*) rabbits. Similar deposits of IgG (*a* and *b*) are present in a peripheral capillary loop pattern. Rabbit C3 (*c* and *d*) is deposited in a granular segmental pattern on peripheral capillary loops and in the mesangium. × 250.

and showed small, predominantly subepithelial, and occasional subendothelial, electron-dense deposits. Glomerular morphology was well preserved and epithelial foot processes were intact in both groups (Fig. 5, *a* and *b*). Occasional mononuclear and polymorphonuclear leukocytes were noted in the glomerular capillary lumena of both groups at 1 wk. Light microscopy revealed a minimal increase in glomerular cellularity in both groups at 1 wk (Fig. 6, *a* and *b*). Glomerular morphology was otherwise normal.

Results after 2 wk of cBSA injection. After 2 wk of daily injections of cBSA, light and electron microscopy showed marked hypercellularity of the glomeruli of both groups due to the infiltration of polymorphonuclear leukocytes and mononuclear cells. Subepithelial deposits were much larger and more flocculent than at 1 wk and epithelial foot processes showed extensive effacement in both groups. Intense, granular, peripheral capillary deposits of rabbit IgG and C3 were detected by immunofluorescence. Coincident with the influx of inflammatory cells at 2 wk, C6D rabbits became proteinuric (median 421, range 156–878 mg/24 h, $n = 6$) so that they no longer differed significantly from the control group (median 359, range 13–4,451 mg/24 h; $P > 0.05$).

DISCUSSION

The aim of these studies was to examine the role of complement, in particular the terminal complement pathway, in the genesis of immune injury during the early, noninflammatory phase of serum sickness induced by cBSA in rabbits. During this early phase, immune deposits are predominantly subepithelial, glomerular morphology is normal, and inflammatory cell infiltration is minimal. The results of these studies show that proteinuria develops in the majority of normal rabbits during this phase of injury, whereas proteinuria is completely absent in C6D rabbits, despite qualitatively and quantitatively similar glomerular antibody and C3 deposits. Since the only established function of C6 is in the assembly of the MAC (8, 24), these findings strongly suggest that complement can produce glomerular injury in a manner analogous to its cytolytic effect (25).

These results are consistent with our earlier observations on a cell-independent form of complement-mediated, immunologic glomerular injury in rat membranous nephropathy (4, 5) but, in addition, indicate the probable mechanism of such injury. It has long been recognized that the development of proteinuria in rats immunized with a fraction of Fx1A to produce Heymann nephritis, corresponds with the presence of complement deposits in glomeruli (Noble, B., personal communication; and reference 26). By depleting rats of complement with cobra venom factor, we demonstrated the essential role for complement in the genesis of proteinuria and showed that only the complement-fixing, $\gamma 1$ subclass of sheep anti-Fx1A IgG was capable of altering glomerular permeability (4). Thus, rats de-

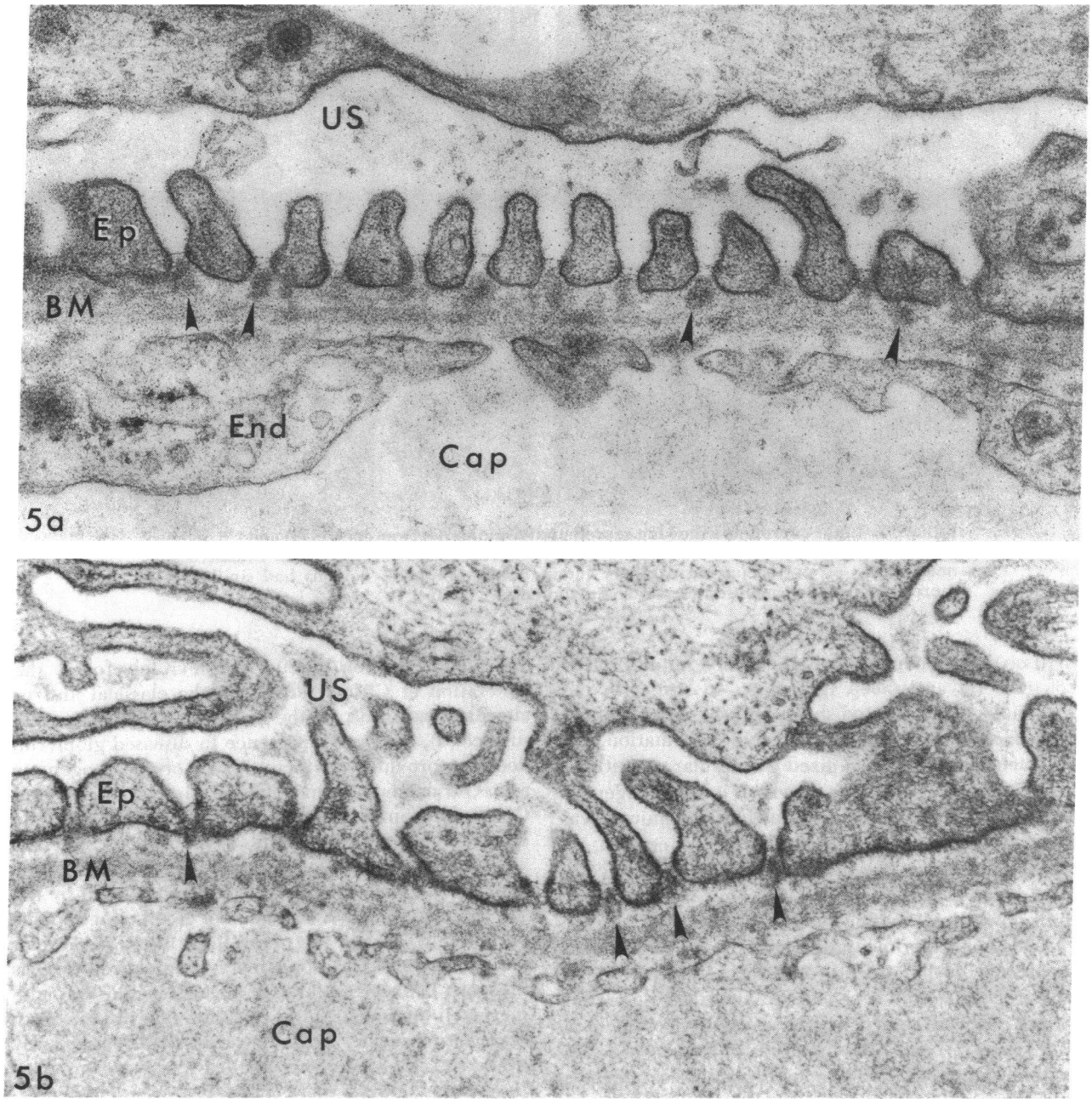

FIGURE 5 Electron micrographs of the glomerular capillary wall of control (*a*) and C6 deficient (*b*) rabbits after 1 wk of daily cBSA. The subepithelial layer of the basement membrane (*BM*) contains numerous, small electron densities (arrowheads), which are often located proximal to the filtration slit diaphragm. Epithelial cells (*Ep*) show normal interdigitating foot processes in both groups of animals. *US*, urinary space; *End*, endothelial cell; *Cap*, capillary lumen. × 45,900.

pleted of complement or given the noncomplement-fixing, $\gamma 2$ subclass of sheep anti-Fx1A IgG developed glomerular immune deposits that were similar in amount and distribution to rats given $\gamma 1$ antibody but did not become proteinuric (4). Identical observations were recently made in the autologous phase of injury

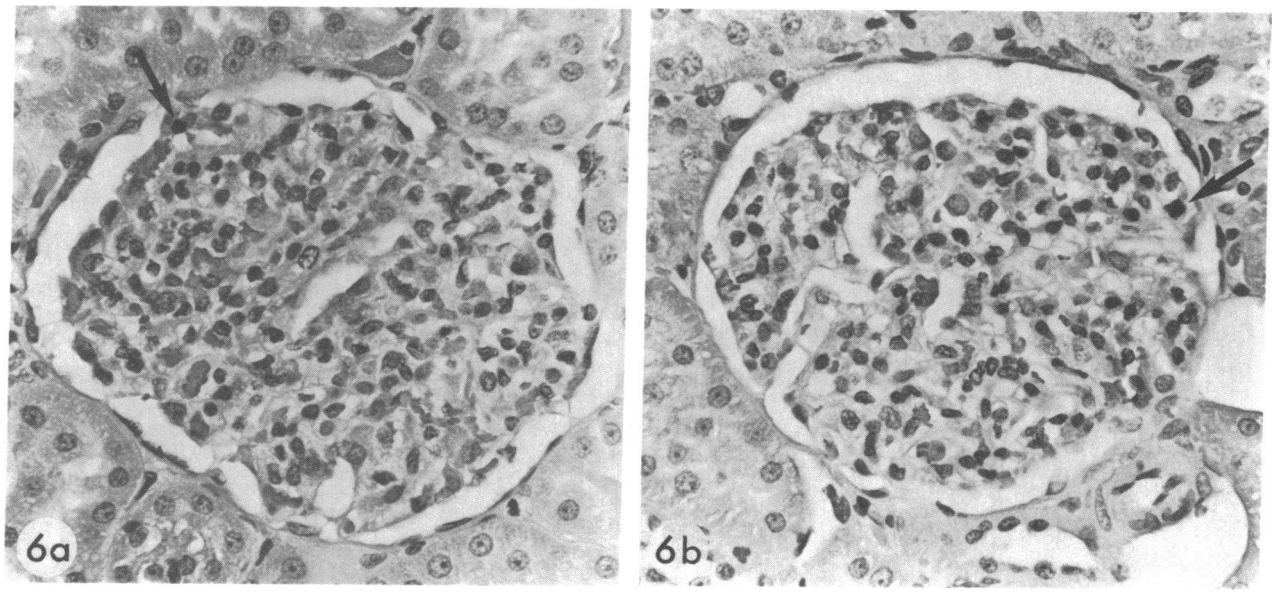

FIGURE 6 Light micrograph showing representative glomeruli from control (*a*) and C6 deficient (*b*) rabbits after 1 wk of daily cBSA. Glomerular tufts are minimally hypercellular with occasional neutrophils (arrow) occupying capillary lumina. Both groups of animals had similar, mild morphological abnormalities. Hematoxylin and eosin; × 370.

of passive Heymann nephritis in which autologous or isologous rat anti-sheep IgG reacts in situ with the immunologically planted sheep IgG (5). In both these rat models of in situ immune complex formation, one in which the antigen is a fixed glomerular constituent (22, 27, 28) and the other in which it is of exogenous origin (5, 29), the complement-induced injury was found to be independent of inflammatory cells (4, 5). A possible role for the membranolytic action of complement in glomerular injury was thus suggested but, until now, direct evidence was lacking.

Analogous experimental observations have been made regarding the hyperacute rejection of cardiac grafts. Hyperacute rejection of cardiac allografts in presensitized rats was found to be ameliorated by complement depletion (7) and uninfluenced by neutrophil and platelet depletion (6, 30), suggesting a direct effect of complement. Furthermore, hyperacute rejection of heart xenografts was delayed in C6D rabbits (31), indicating a possible cytolytic effect of complement in this form of immunologic vascular injury.

The current studies also add credence to recent observations that demonstrated, by immunofluorescence, the presence of the MAC neoantigen in glomerular deposits of patients with systemic lupus erythematosus (9), idiopathic membranous nephropathy, and anti-GBM nephritis (11), and of polyC9 is a variety of glomerular lesions (13). Since assembly of the MAC (32) and polymerization of C9 (12) inevitably follow the generation of C5 convertase by the classical and/or alternate pathways in the presence of an activating surface (8), their mere presence in diseased glomeruli does not provide convincing proof of a pathogenetic role. Our current findings present direct evidence that C6, and by inference the MAC, is essential for the development of certain forms of glomerular injury.

That these results were not due to impaired immune competence of C6D rabbits was demonstrated by the similar quantity and distribution of glomerular deposits of rabbit IgG in the two groups. In addition, glomerular deposits of rabbit C3 appeared as abundant in C6D as in control rabbits. While it is conceivable that glomerular immune deposits of some C6D rabbits with lower serum anti-BSA concentrations (and consequently higher free BSA levels) contained relatively more antigen than controls, there is no logical reason why this should have prevented them from becoming proteinuric. It is also unlikely that any disorder of leukocyte chemotaxis accounted for the lack of proteinuria in C6D rabbits since they have been shown to have normal chemotactic activity (24). A contribution of the terminal complement sequence to coagulation is inferred from studies in which delayed in vitro clotting of C6D rabbit blood was observed in response to activators of the alternate complement pathway (33, 34). While theoretically this clotting "defect" could have protected our C6D rabbits from glomerular injury (35), it seems

unlikely since there was no evidence of intraglomerular coagulation in our control group.

The cytolytic action of complement results from the insertion of the MAC, during its assembly, into the phospholipid bilayer of cell membranes. This leads to loss of the integrity of cell membranes due either to the formation of doughnut-shaped transmembrane channels (25) or to the loss and/or rearrangement of membrane phospholipids (36, 37). Since the GBM does not contain phospholipid bilayers, it seems likely that the site of action of the MAC in glomerular injury is the plasma membrane of either the endothelial or epithelial cells or the slit diaphragms that bridge the space between adjacent epithelial cell foot processes. Altered capillary wall permeability might then arise as a result of impaired synthesis or repair of the GBM by endothelial or epithelial cells, or as a result of disruption of the slit diaphragm which, itself, may provide a final barrier to macromolecular filtration (38). In this regard, it is of interest that recent studies have shown that the glomerular antigen responsible for subepithelial immune deposits in the Heymann models of rat membranous nephropathy is expressed on the surface of glomerular epithelial cell membranes (28, 39). It was in the passive Heymann model (4) and its autologous phase (5) that we first detected a direct action of complement in glomerular injury.

One can only speculate on the actual mechanism of complement-mediated glomerular injury in the rabbit model used in these studies. Current evidence favors the hypothesis that glomerular immune deposits, in this model, form in situ after the initial localization of cBSA which interacts electrochemically with anionic sites in the glomerular capillary wall, and the subsequent binding of anti-BSA antibody and fixation of complement (14, 40, 41). Antibody-induced complement fixation in the glomerular capillary wall would rapidly lead to assembly of the MAC which, because of the close proximity of the immune deposits to epithelial (and perhaps endothelial) cells, could become inserted into their plasma membranes and lead to cell damage. Closer scrutiny is required to determine whether the characteristic, doughnut-shaped MAC (25) can be visualized in the vicinity of glomerular immune deposits, or whether epithelial cells from such glomeruli exhibit alterations in the phospholipids of their plasma membranes (36, 37).

The absence of inflammation, despite fixation of complement, in the early phase of injury in these studies might reflect the predominantly subepithelial location of immune deposits which renders them inaccessible to circulating neutrophils, as previously discussed (4, 5). The subsequent appearance of proteinuria in C6D rabbits and its worsening in controls coincided with an intense inflammatory cell infiltrate and suggests the advent of a second, cell-dependent, form of injury. Whether or not the latter phase is dependent on the leukotactic (C5a) and immune adherent (C3b) properties of complement, as in the heterologous phase of anti-GBM nephritis (2, 3), remains to be resolved. In a similar model, in which in situ glomerular immune deposit formation was induced in rats with cationized human IgG, evidence was obtained for both cell-dependent and cell-independent forms of complement-mediated injury (42). It therefore seems likely that, in any form of antibody-induced tissue injury where immune deposits give rise to complement fixation, both cell-dependent and cytolytic forms of complement-mediated injury are potentially activated. The major determinant of which form predominates may be the site of immune deposition.

Thus, these results demonstrate, for the first time, that terminal complement components may mediate immunologic glomerular injury. They also provide a probable explanation of the mechanism of complement-dependent, cell-independent proteinuria. These findings, and those in rat membranous nephropathy (4, 5), suggest a possible mechanism for the development of proteinuria in idiopathic membranous nephropathy in man in which a bland-appearing glomerular lesion is accompanied by a high prevalence of glomerular deposits of complement (11, 43).

ACKNOWLEDGMENTS

The authors are grateful to Christine Darby and Neva Capparell for their expert technical assistance and Valerie Worrell for typing the manuscript.

Support for this work was provided by National Institutes of Health research grants AM 17722 and AM 30932.

REFERENCES

1. Cochrane, C. G., and A. Janoff. 1974. The Arthus reaction: a model of neutrophil and complement-mediated injury. *In* The Inflammatory Process. B. W. Zweifach, L. Grant, and R. T. McCluskey, editors. Academic Press, Inc., New York. Second ed. 85–162.
2. Couser, W. G., and D. J. Salant. 1982. Immunopathogenesis of glomerular capillary wall injury in nephrotic states. *In* Contemporary Issues in Nephrology. B. D. Myers and B. M. Brenner, editors. Churchill Livingstone, Inc., New York. 9:47–83.
3. Schreiber, R. D., and H. J. Müller-Eberhard. 1979. Complement and renal disease. *In* Contemporary Issues in Nephrology. C. B. Wilson, B. M. Brenner, and J. H. Stein, editors. Churchill Livingstone, Inc., New York. 3:67–105.
4. Salant, D. J., S. Belok, M. P. Madaio, and W. G. Couser. 1980. A new role for complement in experimental membranous nephropathy in rats. *J. Clin. Invest.* 66:1339–1350.
5. Adler, S., D. J. Salant, J. E. Dittmer, H. G. Rennke, M. P. Madaio, and W. G. Couser, 1983. Mediation of

proteinuria in membranous nephropathy due to a planted glomerular antigen. *Kidney Int.* 23:807–815.

6. Forbes, R. D. C., R. D. Guttman, D. V. M. Kuramochi, J. Klassen, and J. Knack. 1976. Nonessential role of neutrophils as mediators of hyperacute cardiac allograft rejection in the rat. *Lab. Invest.* 34:229–234.
7. Forbes, R. D. C., M. Pinto-Blonde, and R. D. Guttmann. 1978. The effect of anticomplementary cobra venom factor on hyperacute rat cardiac allograft rejection. *Lab. Invest.* 39:463–470.
8. Brown, E. J., and M. M. Frank. 1981. Complement activation. *Immunol. Today (Amst.).* 2:129–134.
9. Biesecker, G., S. Katz, and D. Koffler. 1981. Renal localization of the membrane attack complex in systemic lupus erythematosus nephritis. *J. Exp. Med.* 154:1779–1794.
10. Biesecker, G., L. Lavin, M. Ziskind, and D. Koffler. 1982. Cutaneous localization of the membrane attack complex in discoid and systemic lupus erythematosus. *N. Engl. J. Med.* 306:264–270.
11. Rauterberg, E. W., T. Gehrig, and P. L. Kohl. 1981. The attack complex of complement in epimembranous and anti-basement membrane antibody glomerulonephritis. *Kidney Int.* 20:160.
12. Podack, E. R., J. Tschopp, and H. J. Müller-Eberhard. 1982. Molecular organization of C9 within the membrane attack complex of complement. *J. Exp. Med.* 156:268–282.
13. Falk, R. J., Y. Kim, C. H. Tsai, J. I. Scheinman, H. Gewurz, A. Dalmaso, and A. F. Michael. 1983. Renal deposition of poly C9: neoantigen of the membrane attack complex. *Kidney Int.* 23:194.
14. Border, W. A., H. J. Ward, E. S. Kamil, and A. H. Cohen. 1982. Induction of membranous nephropathy in rabbits by administration of an exogenous cationic antigen. Demonstration of a pathogenic role for electrical charge. *J. Clin. Invest.* 69:451–461.
15. Hoare, D. G., and D. F. Koshland, Jr. 1967. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. *J. Biol. Chem.* 242:2447–2453.
16. Kabat, E. A., and M. M. Mayer. 1961. Complement and complement fixation. *In* Experimental Immunochemistry. Charles C Thomas, Springfield, IL. Second ed. 133–240.
17. Mancini, O. A., A. O. Carbonara, and H. R. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* 2:235–245.
18. Minden, P., and R. S. Farr. 1978. Ammonium sulfate method to measure antigen-binding capacity. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publication, Oxford. Third ed. 1:13.1–13.22.
19. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* 29:185–189.
20. Salant, D. J., C. Darby, and W. G. Couser. 1980. Experimental membranous glomerulonephritis in rats. Quantitative studies of glomerular immune deposit formation in isolated glomeruli and whole animals. *J. Clin. Invest.* 66:71–81.
21. Scatchard, G. 1946. The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51:660–672.
22. Couser, W. G., D. R. Steinmuller, M. M. Stilmant, D. J. Salant, and L. M. Lowenstein. 1978. Experimental glomerulonephritis in the isolated perfused rat kidney. *J. Clin. Invest.* 62:1275–1287.
23. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames. Sixth ed. 120–134, 135–171.
24. Snyderman, R., J. Phillips, and S. E. Mergenhagen. 1970. Polymorphonuclear leukocyte chemotactic activity in rabbit serum and guinea pig serum treated with immune complexes: evidence for C5a as the major chemotactic factor. *Infect. Immun.* 1:521–525.
25. Mayer, M. M., D. W. Michaels, L. E. Ramm, M. B. Whitlow, J. B. Willoughby, and M. L. Shin. 1981. Membrane damage by complement. *Crit. Rev. Immunol.* 2:133–165.
26. Edgington, T. S., R. J. Glassock, and F. J. Dixon. 1968. Autologous immune complex nephritis induced with renal tubular antigen. I. Identification and isolation of the pathogenetic antigen. *J. Exp. Med.* 127:555–572.
27. Van Damme, B. J. C., G. J. Fleuren, W. E. Bakker, R. L. Vernier, and P. J. Hoedemaeker. 1978. 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.
28. Kerjaschki, D., and M. G. Farquhar. 1982. The pathogenetic antigen of Heymann nephritis is a membrane glycoprotein of the renal proximal tubule brush border. *Proc. Natl. Acad. Sci. USA.* 79:5557–5561.
29. Van Es, L. A., A. P. R. Blok, L. Schoenfeld, and R. J. Glassock. 1977. Chronic nephritis induced by antibodies reacting with glomerular-bound immune complexes. *Kidney Int.* 11:106–115.
30. Forbes, R. D. C., and R. D. Guttman. 1982. Evidence for complement-induced endothelial injury in vivo. A comparative ultrastructural tracer study in a controlled model of hyperacute rat cardiac allograft rejection. *Am. J. Pathol.* 106:378–387.
31. Chartrand, C., S. O'Regan, P. Robitaille, and M. Pinto-Blonde. 1979. Delayed rejection of cardiac xenografts in C-6 deficient rabbits. *Immunology.* 38:245–248.
32. Kolb, W. P., and H. J. Müller-Eberhard. 1975. The membrane attack mechanism of complement. Isolation and subunit composition of the C5b-9 complex. *J. Exp. Med.* 141:724–735.
33. Zimmerman, T. S., C. M. Arroyave, and H. J. Müller-Eberhard. 1971. A blood coagulation abnormality in rabbits deficient in the sixth component of complement (C6) and its correction by purified C6. *J. Exp. Med.* 134:1591–1600.
34. Zimmerman, T. S., and H. J. Müller-Eberhard. 1971. Blood coagulation initiation by a complement-mediated pathway. *J. Exp. Med.* 134:1601–1607.
35. Cochrane, C. G. 1979. Mediation systems in neutrophil-independent immunologic injury of the glomerulus. *In* Contemporary Issues in Nephrology. C. B. Wilson, B. M. Brenner, and J. H. Stein, editors. Churchill Livingstone, Inc., New York. 3:106–121.
36. Inoue, K., T. Kinoshita, M. Okada, and Y. Akiyama. 1977. Release of phospholipids from complement-mediated lesions on the surface structure of Escherichia coli. *J. Immunol.* 119:65–72.
37. Podack, E. R., G. Biesecker, and H. J. Müller-Eberhard. 1979. Membrane attack complex of complement: generation of high-affinity phospholipid binding by fusion

of five hydrophilic plasma proteins. *Proc. Natl. Acad. Sci. USA.* 76:897–901.
38. Rennke, H. G., J. L. Olson, and M. A. Venkatachalam. 1981. Glomerular filtration of macromolecules. Normal mechanisms and the pathogenesis of proteinuria. *In* Contributions to Nephrology. G. M. Berlyne, S. Giovanetti, and S. Thomas, editors. S. Karger AG, Basel. 24:30–41.
39. Kerjaschki, D., and M. G. Farquhar. 1983. Immunocytochemical localization of the Heymann nephritis antigen (GP 330) in glomerular epithelial cells of normal Lewis rats. *J. Exp. Med.* 157:667–686.
40. Adler, S. G., H. Wang, H. J. Ward, A. H. Cohen, and W. A. Border. 1983. Electrical charge. Its role in the pathogenesis and prevention of experimental membranous nephropathy in the rabbit. *J. Clin. Invest.* 71:487–499.
41. Wang, H., and W. A. Border. 1983. Influence of antigen charge on isoelectric point and biologic properties of preformed immune complexes. *Kidney Int.* 23:191.
42. Thaiss, F., S. Batsford, M. Mihatsch, D. Bitter-Suermann, and A. Vogt. 1983. Mediation systems in in situ immune complexes glomerulonephritis. *Kidney Int.* 23:199.
43. Berger, J., L. H. Noel, and H. Yanerva, 1974. Complement deposition in the kidney. *In* Advances in Nephrology. J. Hamburger, J. Crosnier, and M. H. Maxwell, editors. Year Book Medical Publishers, Inc., Chicago. 4:37–48.