

LOCALIZATION OF THE MEMBRANE ATTACK COMPLEX (MAC)
IN EXPERIMENTAL IMMUNE COMPLEX
GLOMERULONEPHRITIS*

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The membrane attack complex (MAC)¹ and immune complexes have been localized by immunofluorescence within renal (1) and skin lesions (2) of patients with systemic lupus erythematosus (SLE). Concordance between the MAC and immune complex deposition was observed in affected glomeruli, one-third of tubulointerstitial lesions, and in all active cutaneous lesions studied. The MAC was restricted to areas of tissue injury but was not observed in conjunction with C3-containing immune complexes found at the dermal-epidermal junction of normal skin obtained from patients with SLE. These observations raise several questions: (a) Are immune complexes and/or nonimmune factors responsible for the generation of the MAC found in areas of tissue injury? (b) Does the MAC disrupt the integrity of membranes by mechanisms similar to those observed for in vitro lysis of erythrocytes (3), tumor cells (4), and bacteria (5)? (c) Do basement membranes, cell membranes, and other structures such as collagen serve as substrates for insertion of the MAC? (d) What factors inhibit the generation of MAC by putative complement fixing immune complexes such as junctional deposits found in normal skin of patients with SLE?

In order to gain insight into these questions, an experimental immune complex glomerulonephritis induced by bovine serum albumin (BSA) immunization of rats was studied (6, 7). The immunopathologic changes occurring in renal tissues manifesting proliferative glomerulonephritis were examined by light, fluorescence, and electron microscopy. The distribution of the MAC, C3, and immune complexes within glomerular and tubulointerstitial lesions was assessed in order to evaluate the role of the MAC as a mediator of immune-complex-initiated tissue injury.

Materials and Methods

Experimental Protocol. Female Lewis rats weighing ~125 g (Microbiological Associates, Wilkesville, MD) were immunized according to a previously published protocol (7). 3 mg of BSA

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¹*Abbreviations used in this paper:* BC, Bowman's capsule; BSA, bovine serum albumin; CL, capillary lumen; E, endothelium; FLR, fluorescein-labeled rabbit; GBM, glomerular basement membrane; HRPO, horseradish peroxidase; I, interstitium; i.v., intravenous; M, mesangium; MAC, membrane attack complex; P, podocyte; PAP, peroxidase anti-peroxidase; PBS, phosphate-buffered saline; SLE, systemic lupus erythematosus; TBM, tubular basement membrane; TC, tubular cell; US, urinary space.

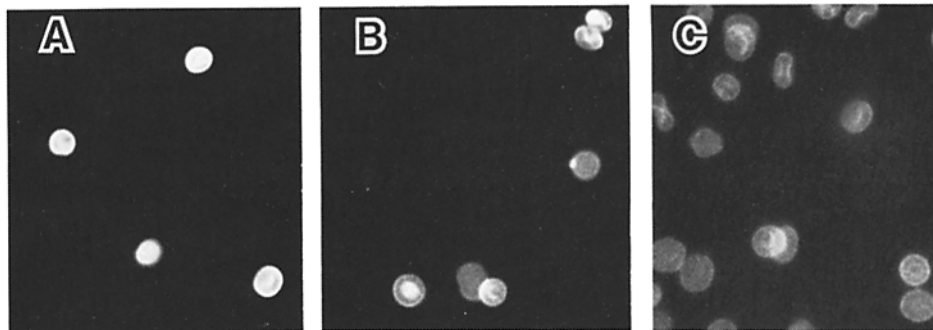


FIG. 1. Glutaraldehyde-fixed unlysed rabbit erythrocytes, incubated with fresh rat or human serum. Cells coated with rat complement components: (A) stained with fluorescein-labeled rabbit (FLR) anti-rat C3; (B) stained with FLR anti-human neoantigen(s); cells coated with human complement components; (C) stained with FLR anti-human neoantigen(s) ($\times 600$). The erythrocytes stain most intensely for C3 due to the greater amount of C3 deposited during complement activation. Cells incubated with rat and human serum containing ethylene diamine tetra-acetic acid to inhibit complement activation showed no staining.

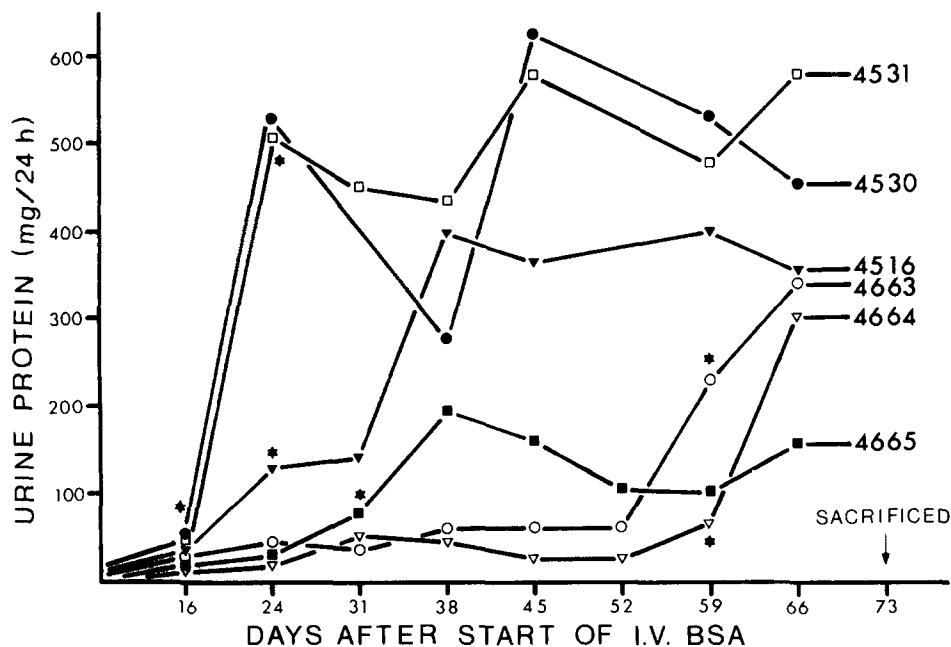


FIG. 2. Diagram of temporal relationship between daily i.v. BSA administration and 24-h urinary protein excretion. Serum anti-BSA antibody titers were initially 1:4. The asterisks denote the time at which precipitating antibody was no longer detectable. The onset of proteinuria coincided with the disappearance of precipitating antibodies.

(Miles Laboratories, Kankakee, IL) emulsified in incomplete Freund's adjuvant was injected subcutaneously on days 0 and 14. 2 wk hence (day 28) the animals received intravenous (i.v.) injections—maximal dose 2 mg of BSA daily. At the start of i.v. injections an anti-BSA titer of 1:4 was detected in all sera by agar gel immunodiffusion tests. 24-h urinary protein was measured on samples collected from rats placed in metabolism cages (8). The rats were killed 73 d after the start of i.v. injections. Three nonimmunized female Lew rats weighing ~ 125 g served as controls.

Preparation of Antigens. Rabbit and rat proteins were prepared by the following methods:

TABLE I
Immunofluorescence Localization of Rat IgG, Rat Complement Proteins, and BSA

Tissue localization* of:	Glomeruli [‡]	Peritubular basement membrane region
IgG	++ to +++ [§]	0
C3	++	+ to ++
BSA antigen	++ to +++	0
BSA antibody	+	0
MAC [¶]	++	+

* Results tabulated for six rats with moderate to severe glomerulonephritis and three nonimmunized control rats.

[‡] Glomeruli of nonimmunized control rats were negative for all antigens.

[§] 0, neg, +, ++, and ++++, weak, moderate, and strong fluorescence staining.

^{||} Isolated groups of tubules of non-immunized control rats showed peritubular staining.

[¶] No significant difference was observed for the distribution and staining intensity for C9 and neoantigen(s)—markers for the MAC.

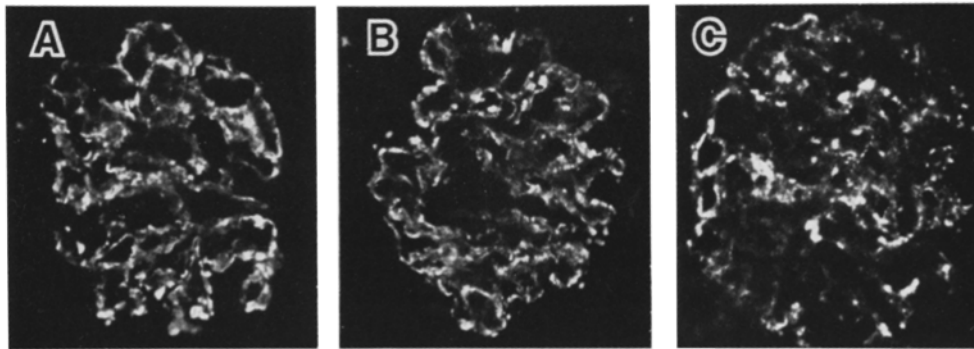


FIG. 3. Kidney (rat #4516) stained with: (A) FLR anti-rat IgG, diffuse finely and coarsely granular deposits along capillary walls ($\times 250$); (B) FLR anti-rat C3, diffuse deposits similar to IgG ($\times 250$); (C) FLR anti-human neoantigen(s), diffuse granular deposits, bright fluorescence with a more restricted distribution along glomerular capillary walls ($\times 250$).

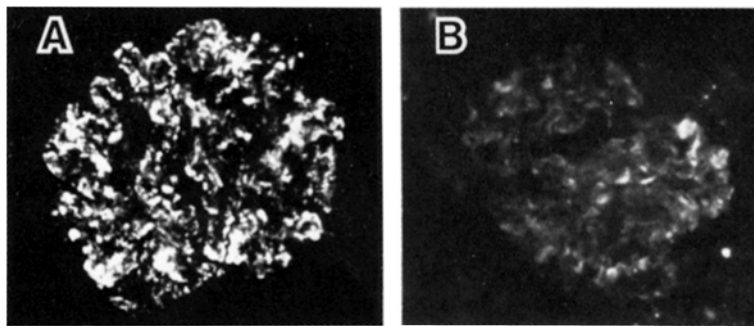


FIG. 4. Kidney (rat #4530) stained with: (A) FLR anti-BSA, granular deposits diffusely scattered throughout glomerulus outlining capillary walls ($\times 250$); (B) Fluorescein-labeled BSA, few weakly staining deposits detectable along glomerular capillary walls ($\times 250$).

rabbit and rat IgG (9); rat C3 (10); and rat C9 (11). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of these proteins showed characteristic banding patterns. The functional integrity of rat C3 and C9 was assessed. Hemolytic activity of C3 was assayed using activated sheep erythrocytes bearing human complement components C1, C4, and C2, which were incubated with aliquots of rat C3 and guinea pig serum depleted of C3 to which rat C3 was

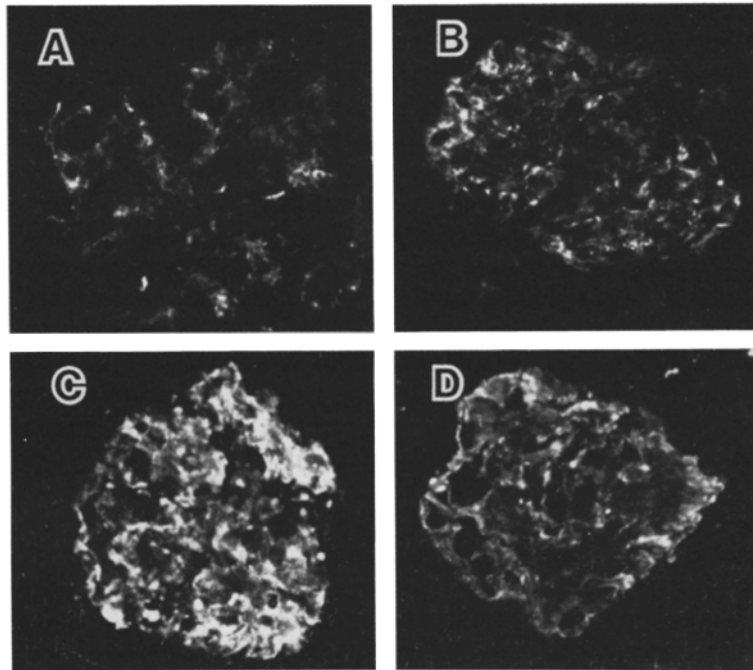


FIG. 5. Kidney (rat #4663), unfixed cryostat sections before and after incubation with acid buffer: (A) FLR anti-rat IgG, glomerulus shows a few residual faintly fluorescent granules along capillary walls ($\times 250$). (B) FLR anti-rat C9, granular deposits of rat C9 unaffected by acid buffer treatment for 2 h ($\times 250$). Note: FLR anti-rat C9 and FLR anti-human neoantigen(s) showed parallel distribution in tissue sections and both were considered to be markers for rat MAC. (C) FLR anti-rat IgG, granular deposits of IgG prior to elution with acid buffer ($\times 250$). (D) FLR anti-rat C9, granular deposits of MAC prior to elution with acid buffer ($\times 250$).

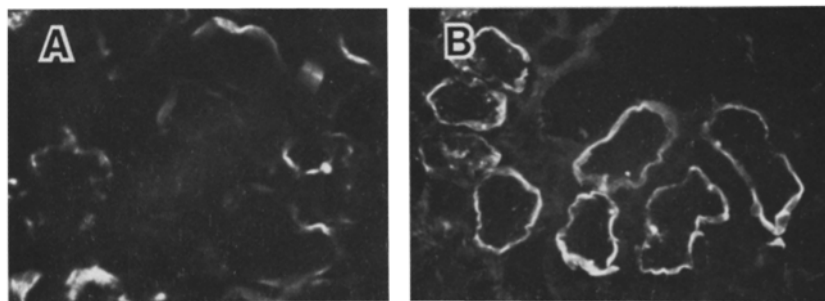


FIG. 6. (A) Kidney (rat #4663); peritubular interstitial deposits of C3, patchy localization within irregularly thickened TBM ($\times 400$). (B) Nonimmunized control rat kidney; cluster of tubules showing homogeneous linear fluorescence for rat C3 in peritubular region ($\times 250$). The discontinuous pattern observed in A was uncommon in nonimmunized animals.

added (12). Rat C9 was assayed with erythrocytes coated with the C5b-8 complex (11). Human MAC was isolated and characterized as previously described (13).

Preparation of Antisera. Rabbits were immunized with rat IgG, rat C3, human MAC, crystalline BSA, and horseradish peroxidase (HRPO) (Sigma Chemical Co., St. Louis, MO). Goats were immunized with rabbit IgG. The specificity of each antiserum was determined using immunoelectrophoresis except for rabbit anti-MAC. Monospecific antiserum to human MAC neoantigen(s) was prepared and characterized as previously described (1, 14). Antiserum titers were assayed by double agar gel immunodiffusion in 1% agarose. Purified antigen (0.2

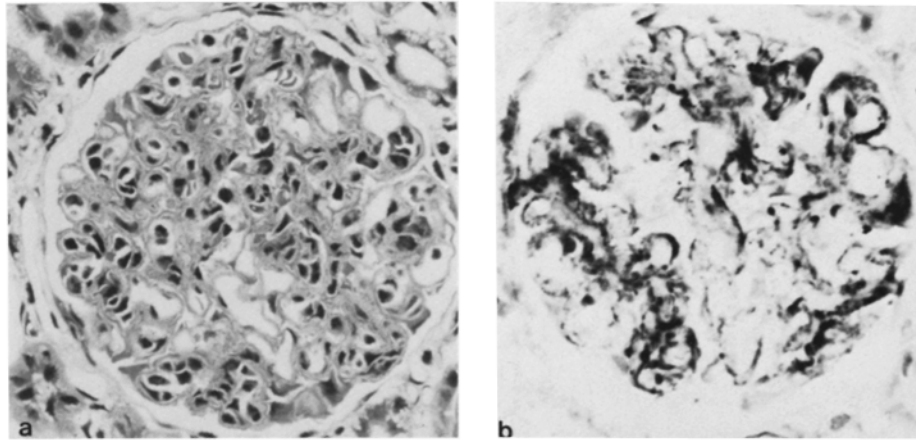


FIG. 7. Kidney (rat #4664): (A) Plastic embedded section stained with hematoxylin and eosin demonstrating moderate proliferative glomerulonephritis; (B) Cryostat section stained with rabbit anti-rat C3 (PAP method). Granular deposits of C3 in peripheral capillary loops and mesangium show similar distribution and reactivity as that obtained by immunofluorescence ($\times 200$).

TABLE II
Ultrastructural Localization of Immune Complex, C3, and the MAC

Glomerular localization of:	Cell membranes*	Lamina rara interna [‡]	Lamina densa	Lamina rara externa [§]	Podocyte membranes
BSA	0	+ ¹	+	+	0
IgG	0	++	++	+++	0
C3	0	++	++	+++	+
C9	0	++	+	+++	++
Neoantigen(s)	0	+	0	+++	+++

* Endothelial, mesangial, and visceral epithelium (excluding podocyte membranes).

[‡] Subendothelial region of GBM.

[§] Subepithelial region of GBM.

¹ Comparative grading (qualitative) of peroxidase reactivity with respect to staining intensity and distribution; dense deposits considered +++, used as standard; ++, moderate staining with more limited distribution; +, mild, weak staining reaction with limited distribution; 0, not demonstrable.

mg/ml) was placed in the center well and antiserum titers were expressed as the highest dilution that produced a positive precipitin reaction: anti-rat IgG, 1:16; anti-rabbit IgG, 1:16; anti-rat C3, 1:8; anti-rat C9, 1:16; anti-BSA, 1:32; anti-HRPO, 1:16; and anti-human MAC neoantigen(s), 1:4.

Each antiserum and BSA antigen was conjugated with 0.02 mg of fluorescein isothiocyanate (Becton, Dickinson and Co., Cockeysville, MD) per mg of protein (15). Fluorescein-labeled gamma globulins were rechromatographed on DEAE-cellulose. Antisera with the following fluorescein/protein ratios were used for immunofluorescence staining: anti-rat IgG, 2.1; anti-rat, C3, 2.3; anti-rat C9, 2.6; anti-BSA, 3.5; and anti-neoantigen(s), 2.5.

In order to test the *in vitro* reactivity and specificity of the above fluorescein-labeled reagents glutaraldehyde-fixed rabbit erythrocytes coated with complement were used. Fresh rabbit erythrocytes suspended in normal saline containing 0.02 M phosphate, pH 7.2 (PBS) were fixed with 0.05% glutaraldehyde for 1 h at room temperature. The cells were washed five times with veronal-buffered saline containing CaCl₂ and MgCl₂, followed by reaction with either fresh rat or human serum for 1 h at 37°C, then washed and resuspended in PBS. The erythrocytes were incubated with fluorescein-labeled antibody for 1 h, washed, and 10- μ l aliquots were placed on a glass slide and examined. The cross-reactivity of human MAC with rat MAC was demon-

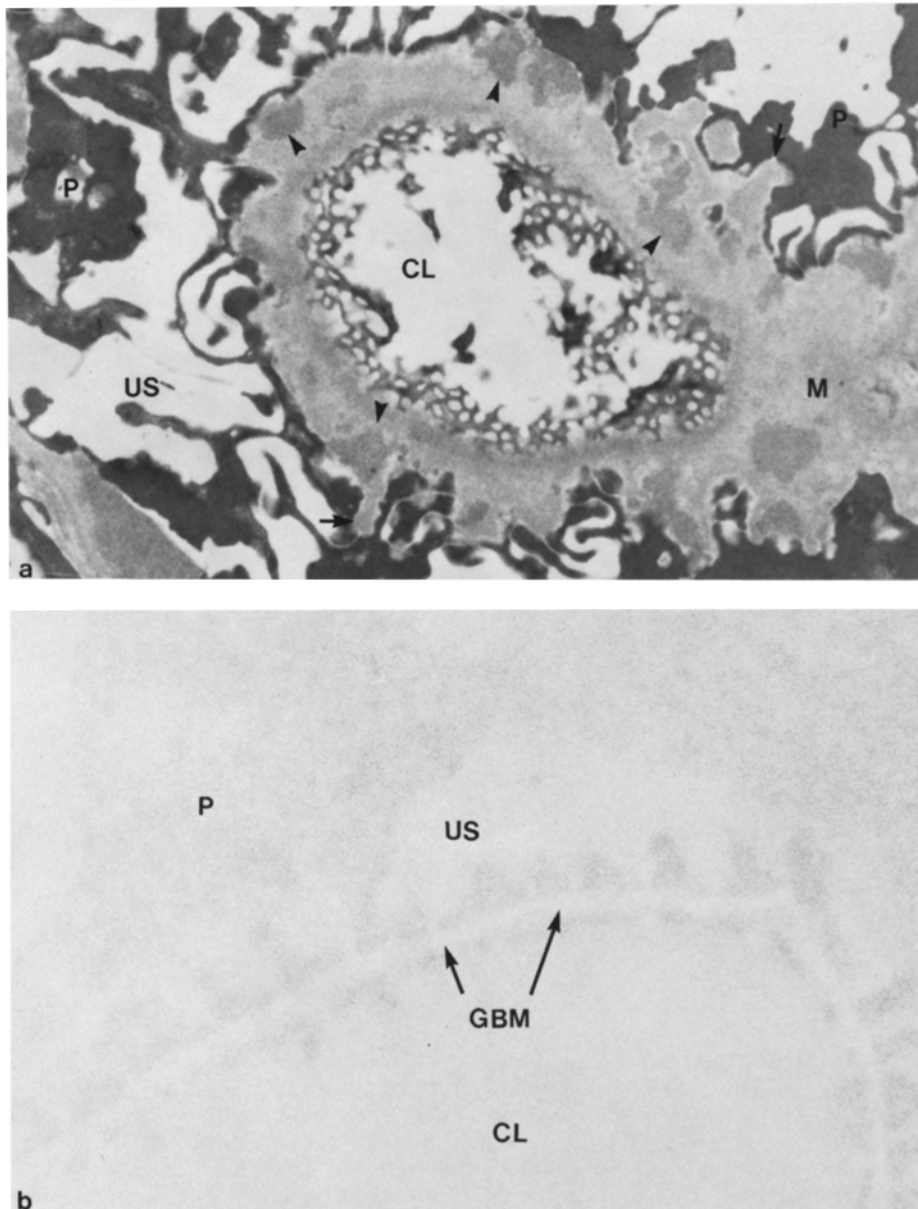


FIG. 8. Kidney (rat #4665): (A) Routine electron microscopy of peripheral capillary loop showing endothelial fenestrae, subepithelial (*arrowheads*) and mesangial electron dense deposits. Prominent GBM spikes are present (*arrows*) ($\times 10,600$). (B) Electron microscopic immunohistochemistry, sections of nonimmunized normal rat kidney reacted with rabbit anti-rat C3 (PAP method used for demonstration of tissue bound antigens). Glomerular structures are negative. The low contrast is due to absence of counterstain ($\times 20,000$). CL, capillary lumen; M, mesangium; P, podocyte; US, urinary space.

strated using complement-coated unlysed glutaraldehyde-fixed erythrocytes (Fig. 1). Rabbit erythrocytes coated with rat and human complement components C3 and C5b-9 were incubated with fluorescein-labeled anti-rat C3, anti-rat C9, and anti-human neoantigen(s). Anti-rat C3 and C9 gave bright fluorescence of the rabbit erythrocytes, which was specifically inhibited by

purified antigen. The anti-human neoantigen(s) serum reacted with both rat and human MAC bound to rabbit erythrocytes and was inhibited by isolated human MAC.

Purified Fab' fragments of anti-HRPO were prepared by papain digestion and chromatography on carboxymethyl-cellulose. The peroxidase antiperoxidase (PAP) method was used as previously described (16-20).

Immunofluorescence Techniques. Small blocks of tissue for immunofluorescence were quick-frozen in isopentane/liquid nitrogen at -178°C . Frozen sections ($5\ \mu\text{m}$) were cut using a Harris cryostat (Harris Mfg. Co., Inc., N. Billerica, MA). Sections were stained by the direct immunofluorescence method and examined by epi-illumination using a Leitz Orthoplan microscope. Photographs were taken using Ektachrome 400 professional film (Eastman Kodak Co., Rochester, NY). Specificity controls included the absence of glomerular staining of kidneys from normal rats and selective blocking of positive staining by prior incubation with purified antigen. In addition, anti-neoantigen(s) serum was incubated with inactivated whole serum and with zymosan-activated serum before staining. Acid elution of cryostat sections was performed by incubation in pH 2.4, 0.2 M glycine for 0.25, 0.5, 1, and 2 h before staining (21).

Immunoperoxidase Techniques. Rats under light ether anesthesia were perfused via the abdominal aorta with PBS for 1 min, followed by perfusion for 20 min with 4% paraformaldehyde in PBS according to previously described methods (17-20). Kidney blocks ($4 \times 4 \times 1\ \text{mm}$) were postfixed in 4% paraformaldehyde for 2 h at 4°C and washed in multiple changes of PBS containing 4% sucrose at 4°C overnight. Tissues were quick-frozen in isopentane/liquid nitrogen and $8\text{-}\mu\text{m}$ frozen sections were mounted on albumin-coated slides. After reduction of free aldehyde groups with sodium borohydride, the sections were incubated with 3% normal goat serum for 1 h, primary antibody overnight at 4°C , goat anti-rabbit IgG for 2 h and PAP (Fab') for 2 h, with appropriate washes in PBS. Sections were incubated in H_2O_2 -diaminobenzidine solution for 15 min (18), washed in PBS, reacted with 1% OsO_4 for 1 h and embedded in plastic. Ultrathin sections were cut with an LKB-V Ultramicrotome, mounted on uncoated grids, examined with a Zeiss EM 10 without any counterstain and photographed using Dupont EM film.

Evaluation of Histopathology. Tissue specimens were evaluated by light microscopy for increased cellularity of glomeruli, frequency of polymorphonuclear leukocytes, thickness of the glomerular basement membrane (GBM), and interstitial infiltration, according to a scheme described in detail elsewhere (8).

Results

Clinical and Morphologic Findings

The pattern of urinary protein excretion of six immunized rats used in this study is shown in Fig. 2. Each animal exhibited a titer of 1:4 for anti-BSA antibody before the onset of IV BSA administration. In three animals precipitating antibodies to BSA were no longer detectable 24 d after the start of i.v. BSA injections. Those animals developed 24-h urinary protein excretion ranging from 350 to 600 mg at 66 d and histologically severe glomerulonephritis was observed. In one rat precipitin titers became negative at day 31; that animal showed moderate glomerulonephritis with focal interstitial inflammation when killed on day 73. Precipitin titers became negative by day 59 in two rats. Those animals also exhibited moderate glomerulonephritis. The onset of proteinuria was correlated with disappearance from circulation of antibodies detectable by immunodiffusion tests. Severity of the histological lesion was related to the duration of proteinuria. Conventional light microscopic examination of sections stained with silver methenamine revealed irregular thickening of the GBM with fine granular subepithelial deposits. Electron microscopic study of kidney sections of three rats (No. 4663, 4664, 4665) showed extensive glomerular alterations that included proliferative changes, thickening of the basement membrane and dense deposits primarily in the region of the lamina rara externa. The tubulointerstitial region showed focal mononuclear cell infiltrates with groups of tubules surrounded

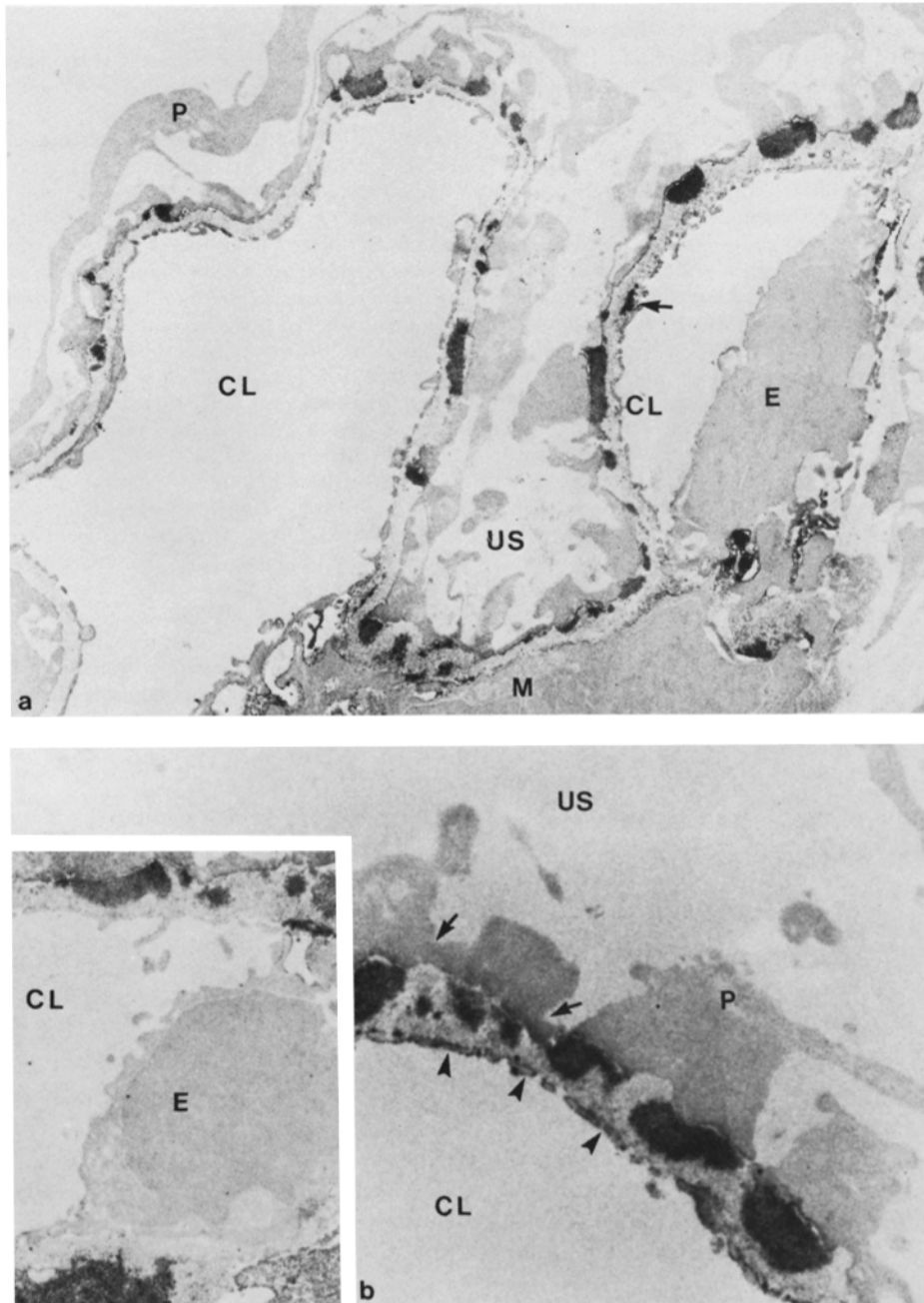
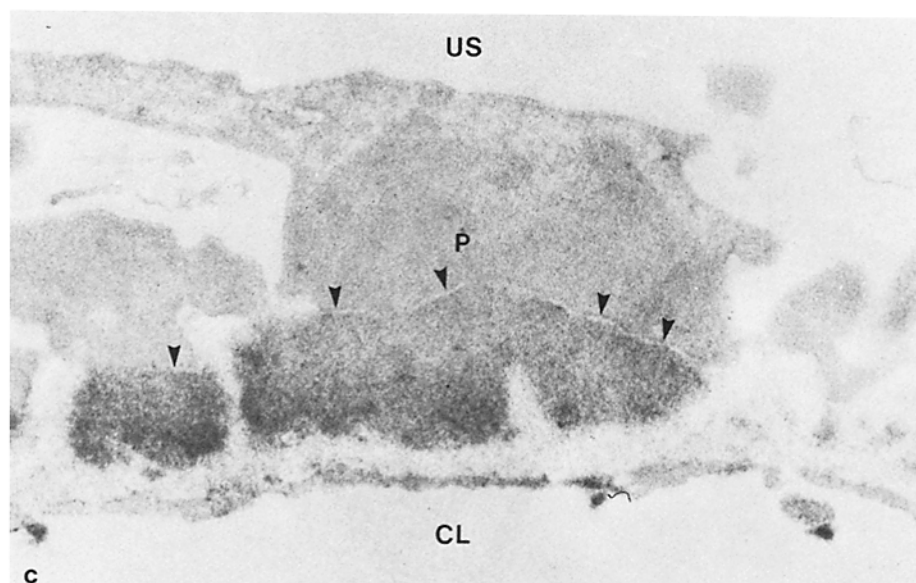


FIG. 9. Kidney (rat #4665): Electron microscopic immunohistochemistry, sections stained with rabbit anti-rat IgG. (A) Renal glomerulus containing diffuse peripheral and mesangial deposits of IgG. Dense deposits are prominent in subepithelial location, whereas small deposits (*arrow*) are found in subendothelial region. Endothelial cell and podocyte membranes are negative ($\times 10,000$). (B) Detail of IgG localization in a peripheral capillary loop. Dense deposits of IgG in subepithelial region with scattered small deposits in subendothelial location (*arrowheads*) and fine granules in lamina densa. Note the absence of foot process membrane localization of IgG (*arrows*) ($\times 18,000$). *Inset, lower left*: Endothelial cell membranes were negative for IgG ($\times 16,000$). (C) Higher magnification view of subepithelial immune complex deposits. IgG staining of the deposit is present whereas the contiguous podocyte membrane is negative (*arrowheads*) ($\times 30,000$). E, endothelium.



by mild to moderate irregularly thickened tubular basement membranes (TBM). Dense deposits were not observed in the tubulointerstitium.

Immunohistochemical Studies

IMMUNOFLUORESCENCE. Multiple sections of kidneys of six rats with clinical and histological evidence of glomerulonephritis and three nonimmunized control rats were examined (Table I). IgG and C3 were distributed in a uniform pattern in glomeruli as fine to coarse granular deposits or larger aggregates involving capillary walls of animals with moderate to severe proliferative glomerulonephritis (Fig. 3A and B). Markers for the MAC (C9 and neoantigen) were limited to sites of immune complex deposition (Fig. 3C) but exhibited a more restricted distribution than IgG, C3, or BSA antigen. Discrete mesangial deposits were scattered within glomeruli and showed a less intense staining pattern for IgG, C3, and the MAC.

BSA antigen was identified in a similar pattern as IgG and C3 utilizing fluorescein-labeled anti-BSA antibody (Fig. 4A). In contrast to the strong reactivity observed for antigen, fluorescein-labeled BSA revealed only weak staining reactions for free glomerular-bound anti-BSA antibody sites (Fig. 4B). Acid buffer elution of unfixed cryostat sections resulted in marked diminution of fluorescence staining for IgG (Fig. 5A), C3, BSA antigen, and BSA antibody. Sequential elution with acid buffer at 15 min, 30 min, 1 h, and 2 h did not uncover antibody sites within glomerular capillary walls. However, this procedure demonstrated that immune complexes were eluted after periods of 15–30 min of incubation with acid buffer, whereas the MAC was essentially unaffected by this treatment (Fig. 5B).

C3 localization was observed in the peritubular region of clusters of proximal convoluted tubules (Fig. 6A) associated with faint staining for the MAC, both occurring in a patchy distribution. IgG was not detectable in either the peritubular or interstitial regions. Interstitial infiltrates were relatively sparse and did not correlate with peritubular C3 and MAC. Neither BSA antigen nor antibody were detectable in the tubulointerstitial region using specific fluorescein-labeled reagents.

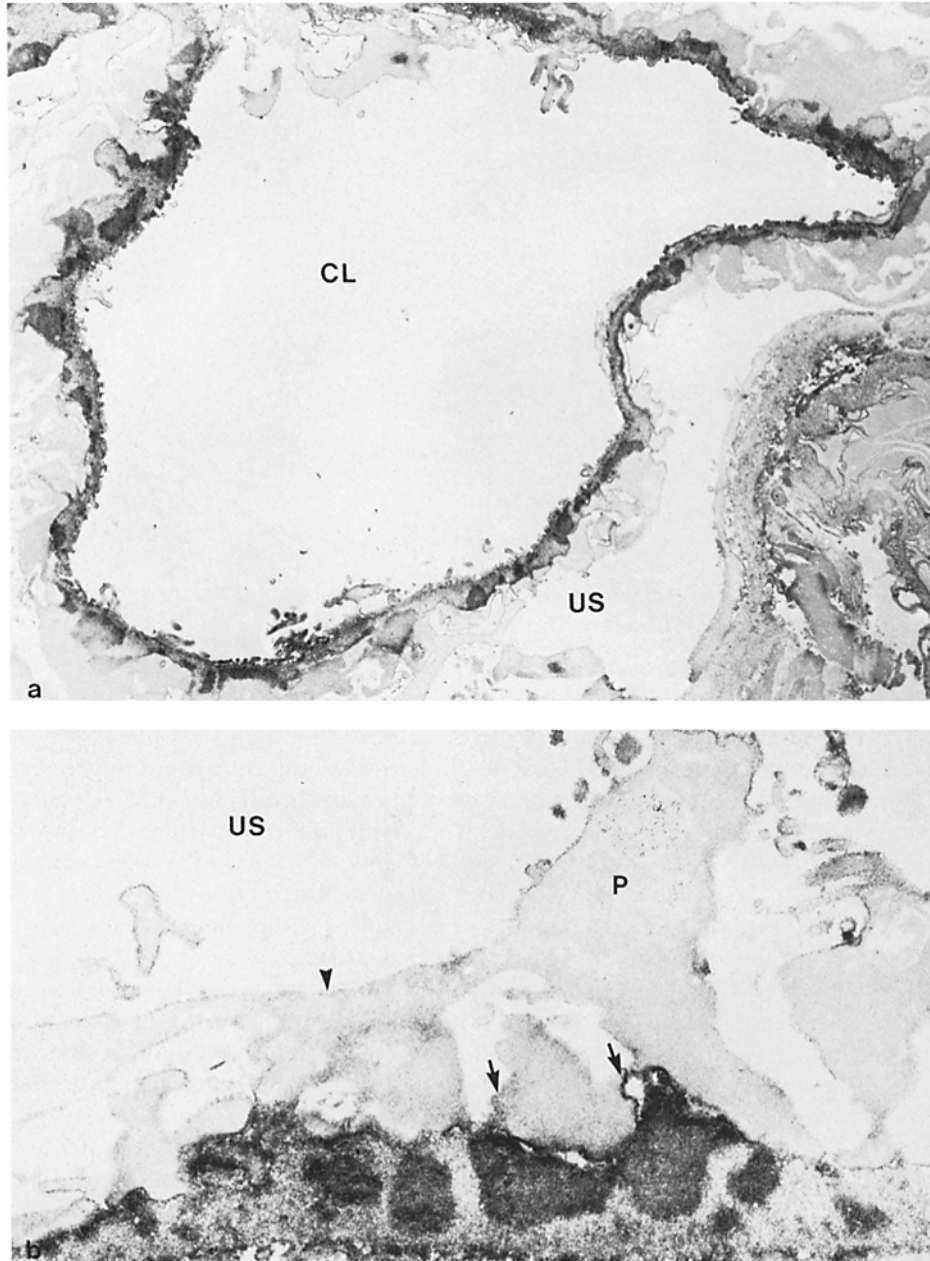


FIG. 10. Kidney (rat #4665): Electron microscopic immunohistochemistry, sections stained with rabbit anti-rat C3 showing: (A) Reaction with granular and dense deposits throughout the basement membrane ($\times 7,500$); (B) Detail of peripheral capillary loop. Large subepithelial, small subendothelial deposits and fine granular particles are present throughout the GBM. Note the distinct granular and linear deposits of C3 on foot process membranes contiguous to the GBM ($\times 25,000$).

Nonimmunized control rats exhibited isolated groups of tubules that showed peritubular linear fluorescence for C3 (Fig. 6 B) without demonstrable IgG or MAC. Renal glomeruli of these animals were negative for immune complexes, C3, and the

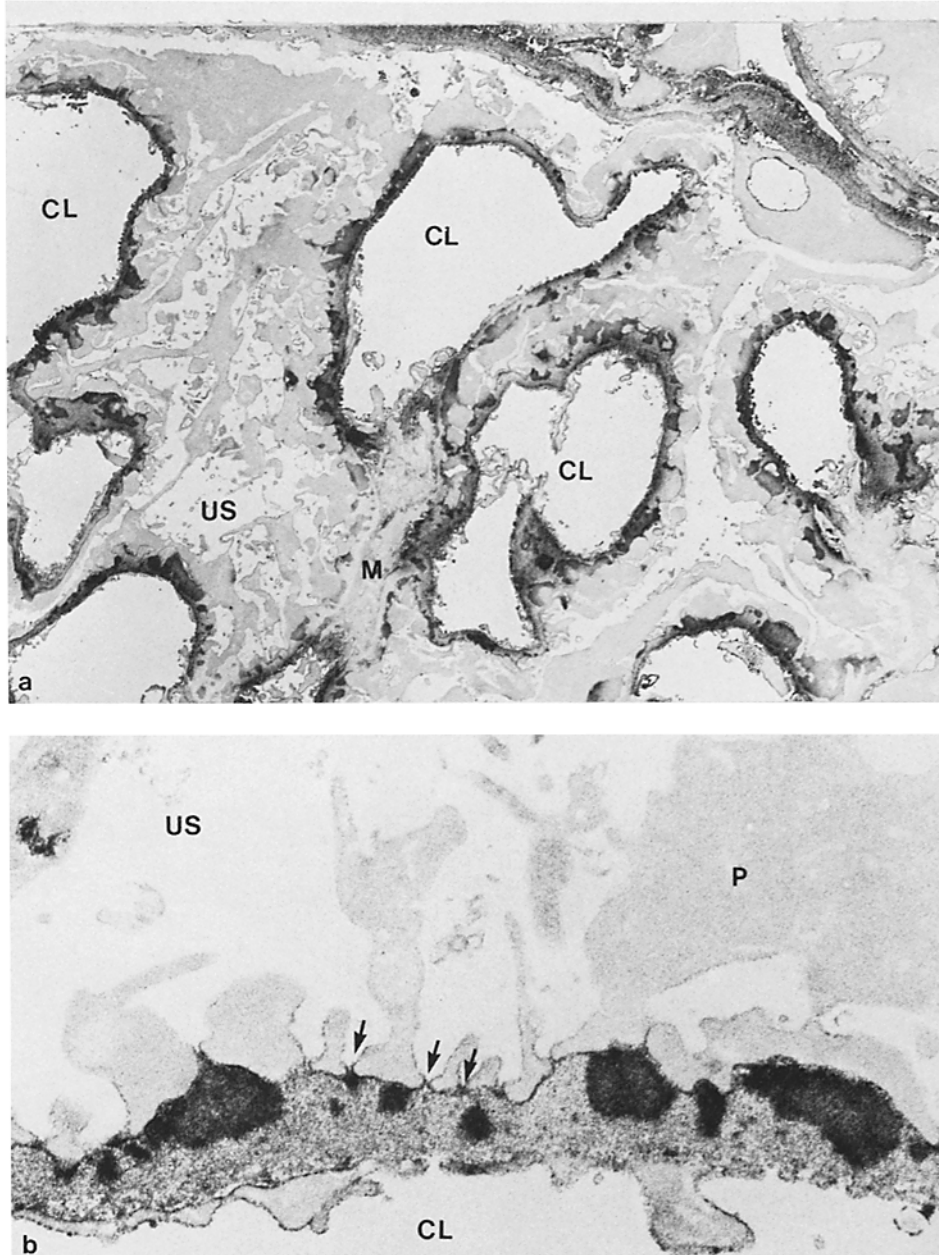


FIG. 11. Kidney (rat #4665): Electron microscopic immunohistochemistry, sections stained with rabbit anti-rat C9 showing: (A) Intense reactivity of MAC in several capillary loops. Mesangial matrix is essentially negative ($\times 4,000$); (B) Detail of peripheral capillary loop. Large subepithelial deposits, granular subendothelial and basement membrane deposits are less prominent than C3 deposits (compare with Fig. 10 B). Note the fine granular and linear deposits of C9 on foot process membranes adjacent to the GBM ($\times 24,600$).

MAC.

IMMUNOPEROXIDASE REACTIONS. Cryostat sections from the kidneys of three nephritic rats were stained by the PAP method and compared to sections incubated

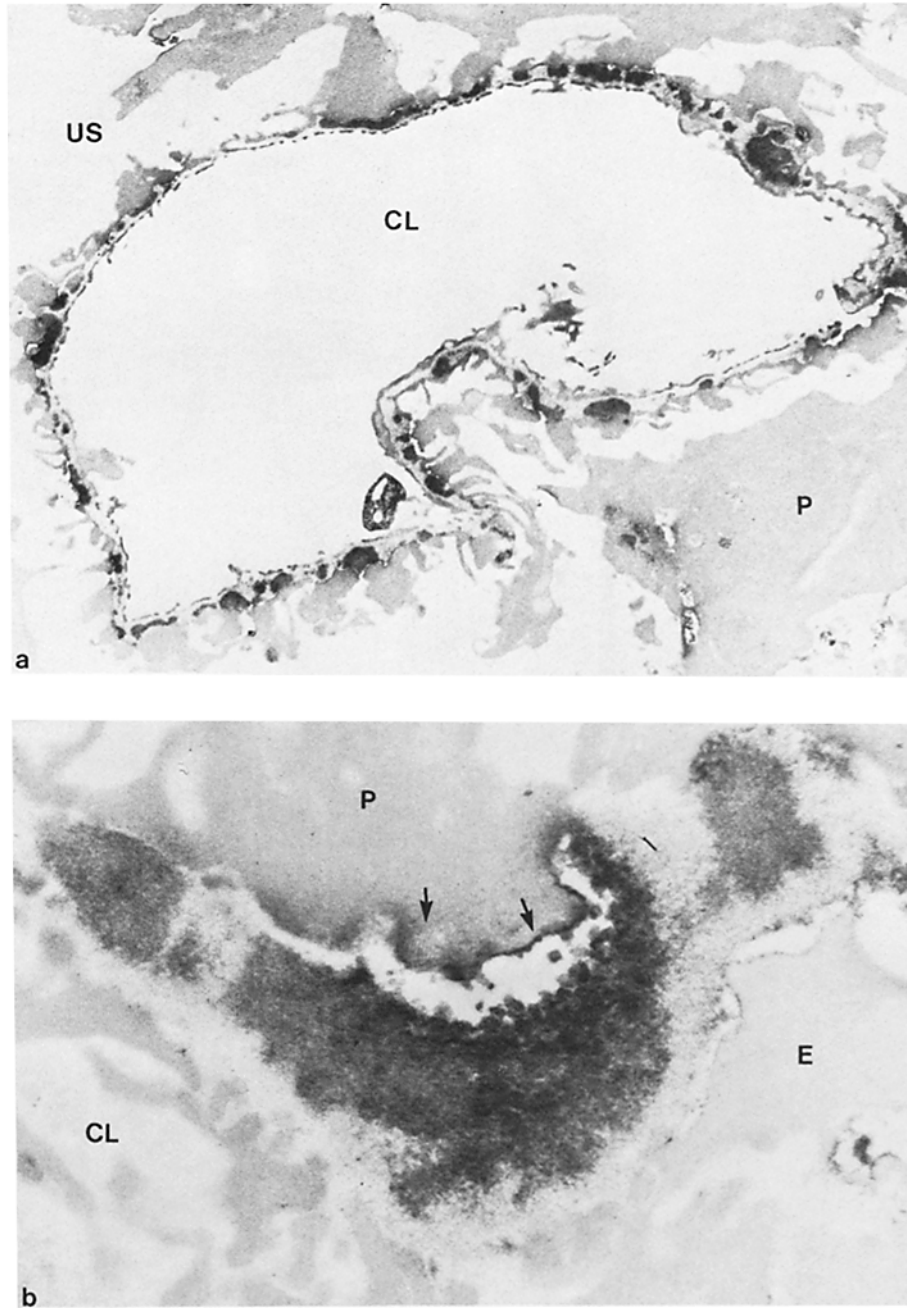


FIG. 12. Kidney (rat #4665): Electron microscopic immunohistochemistry, sections stained with rabbit anti-human neoantigen(s). (A) Intense, selective reaction in subepithelial region ($\times 8,200$). (B) Detail of subepithelial deposits and podocyte membrane foot processes. Strong reactivity of dense deposits with negative basement membrane regions between deposits. Podocyte membrane of visceral epithelial cell overlying deposit is detached and exhibited strong reactivity. C9 and C3 showed moderate staining whereas IgG and BSA antigen were absent from podocyte membranes ($\times 31,600$).

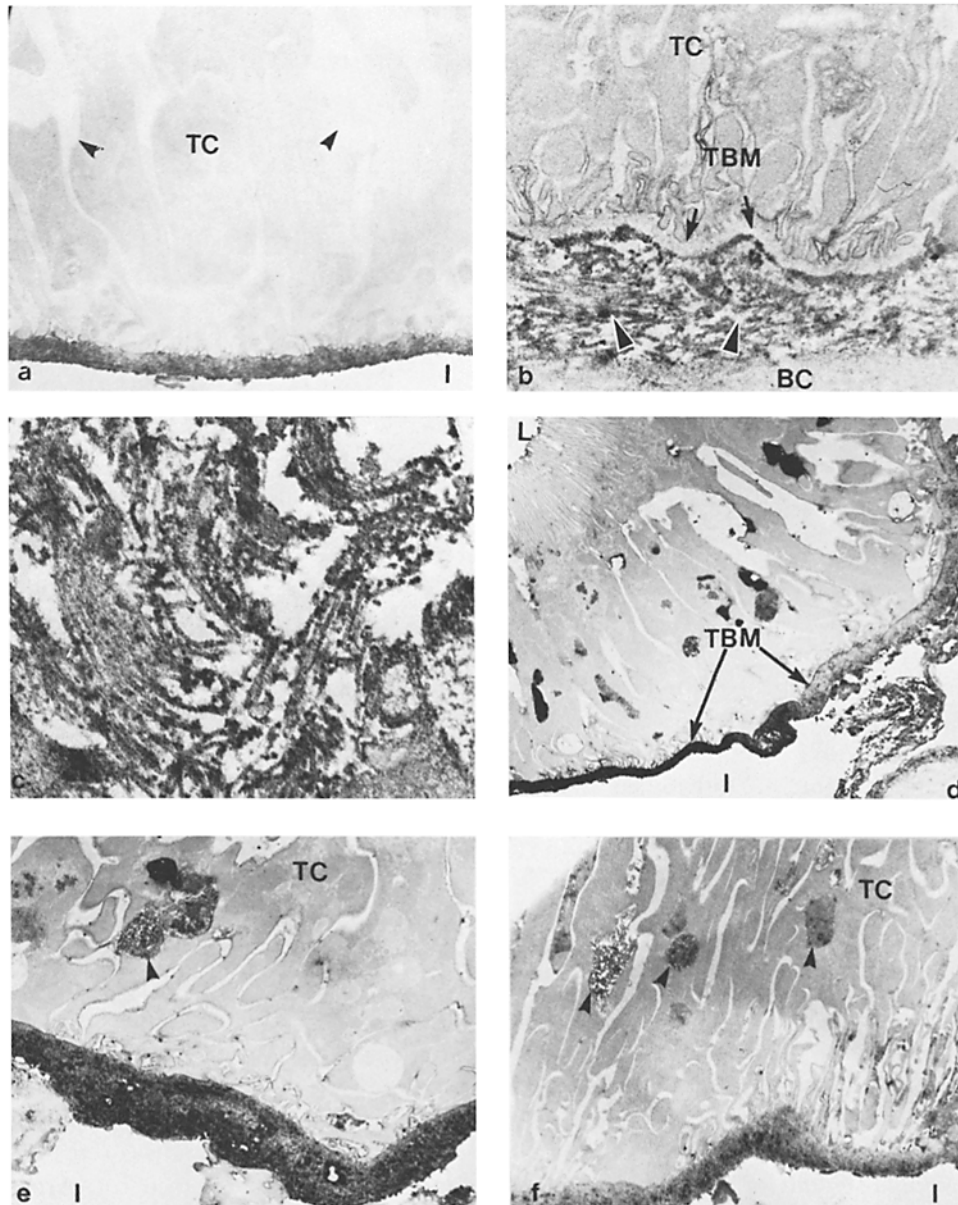


FIG. 13. Electron microscopic immunohistochemistry, tubulointerstitial localization. (A) Nonimmunized control rat kidney section stained with rabbit anti-rat C3. Proximal convoluted tubule shows moderate homogeneous staining reaction of basement membrane. The interstices between basal infoldings (*arrowheads*) are negative ($\times 7,600$). Note that sections B-F are obtained from nephritic rat kidneys. (B) Section (rat #4665) stained with rabbit anti-rat IgG. Interstitial collagen fibers (*arrowheads*) are surrounded by IgG and small aggregates are found close to the TBM (*arrows*). Tubular epithelial cells, basement membrane and adjacent Bowman's capsule are negative for IgG ($\times 12,500$). (C) Section (rat #4665) stained with rabbit anti-rat IgG. Interstitial collagen fibers showed surface IgG. In addition, small aggregates of IgG are present in the interstitial region ($\times 17,300$). (D) Section (rat #4665) stained with rabbit anti-rat C3. TBM of proximal convoluted tubule shows segment of intense homogeneous C3 deposit. Note the difference between intensely positive (*left*) and weakly reactive (*right*) areas of the TBM (*arrows*). Interstitial collagen fibers show scattered C3 deposits. The inner luminal surface of microvilli contains C3 deposits ($\times 3,900$). (E) Section (rat #4665) stained with rabbit anti-rat C9. TBM shows segmental deposit of C9 which has a more granular pattern (compare with D) ($\times 5,600$). The cell interstices contain C9 (*arrowhead*). (F) Section (rat #4665) stained with rabbit anti-human neoantigen(s). TBM shows discrete segmental staining which is less intense than that observed with C3 or C9 (D and E) ($\times 4,300$). The cell interstices contain neoantigen (*arrowheads*). BC, Bowman's capsule; I, interstitium; TC, tubular cell.

TABLE III
Ultrastructural Localization of Tubulointerstitial Protein

	TBM		Interstitial*
	Nephritic (3)	Normal (3)	Nephritic (3)
IgG	0 [‡]	0	++ [§]
C3	+++	++	+ [§]
C9	++	+	0
Neoantigen(s)	+	+	0
BSA antigen	0	0	0

* No proteins were identified in the interstitial region of nonimmunized control animals.

[‡] 0, negative; +, ++, and +++, weak, moderate, and strong peroxidase reactivity.

[§] Not demonstrated by immunofluorescence.

with fluorescein-labeled reagents. Both techniques detected a similar distribution of immune complexes, C3, and the MAC in renal lesions, and manifested a comparable degree of sensitivity (Fig. 7 *B*).

IMMUNOELECTRON MICROSCOPY. IgG, C3, BSA antigen, and MAC markers were localized in glomerular structures in three nephritic animals utilizing the PAP method (Table II). Concordant localization of IgG, C3, BSA antigen, and the MAC were observed within fine granular deposits located on the subendothelial surface of the lamina rara interna, distributed in a patchy fashion within the lamina densa and as prominent subepithelial dense deposits present in the region of the lamina rara externa (Figs. 8–12). In contrast to IgG and C3, C9 and particularly neoantigen(s) exhibited a more restricted distribution with intense peroxidase reaction products observed primarily in subepithelial dense deposits (Figs. 11 and 12). The membranes of visceral epithelial cells overlying these deposits showed foot process fusion and retraction associated with a distinct granular and irregular linear staining pattern for C3 and the MAC (Figs. 10–12). IgG was not observed along podocyte membranes (Fig. 9), the only glomerular site manifesting a uniform discordance between IgG and complement component deposits. Immune complexes, C3, and the MAC were not demonstrable on membranes of endothelial cells, mesangial cells, or visceral epithelium located distal from the GBM (Figs. 9–12). Within the mesangial matrix decreased staining reactions for both immune complexes and the MAC were observed, compared with glomerular capillary walls. Scattered amorphous deposits of immune complexes and MAC were also found in Bowman's capsule (Figs. 10 *A* and 11 *A*). Nonimmunized control rats did not demonstrate glomerular staining for any antigens localized in animals with proliferative glomerulonephritis (Fig. 8 *B*).

Proximal convoluted TBM of nephritic animals showed focal deposits of C3 with significantly less granular MAC present within the TBM (Fig. 13 and Table III). The immunoperoxidase reaction for C3 was visualized as segmental bands of homogeneous staining. IgG and C3 were found on the surface of collagen fibers and in small aggregates within the interstitium close to the TBM (Fig. 13 *B* and *C*), but IgG was not demonstrable within the TBM (Fig. 13 *B*).

Nonimmunized control rats exhibited homogeneous linear peritubular C3 deposits (Fig. 13 *A*) in fewer tubules, i.e., <10% as compared with 30% to 50% of tubules in

nephritic animals. Small focal deposits of the MAC were observed within the TBM (Figs. 13*E* and *F*) but IgG was absent from both the interstitium and the TBM.

Discussion

Diffuse glomerular deposits of immune complexes and the MAC were observed in the kidneys of rats with proliferative glomerulonephritis induced by chronic BSA administration. Concordant deposits of IgG, C3, BSA antigen, and the MAC were visualized by immunofluorescence predominantly along glomerular capillary walls, whereas the anti-BSA antibody component of immune complex deposits showed minimal reactivity with fluorescein-labeled BSA. Ultrastructural localization of immune complexes, C3, and the MAC by immunohistochemical techniques permitted a more precise delineation of the anatomic distribution of these proteins. Prominent dense deposits confined primarily to the subepithelial aspect of the GBM contained IgG, BSA antigen, C3, and MAC markers. The visceral epithelial cells showed fusion and retraction of foot processes in areas overlying dense deposits. Limited reactivity for C3 and intense staining for the MAC were observed along segments of fused podocyte membrane although membrane-associated IgG was not demonstrated. IgG, C3, and the MAC were also detected in the region of the lamina rara interna, scattered within granular deposits in the lamina densa and in the mesangial matrix. Neoantigen(s) exhibited a more restricted immunoperoxidase staining pattern than that observed for C9, which may be related to the occurrence of fewer neoantigenic epitopes on the MAC or to the presence of tissue-bound C9 that was not a component of the terminal complement complex. IgG, BSA, C3, and MAC markers were not identified along cell membranes of endothelial cells, mesangial cells, or the non-GBM-associated membranes of visceral epithelium.

These observations suggest that the MAC is generated locally by immune complex activation of the classical complement pathway, but the MAC is restricted to membrane sites that serve as a substrate for insertion, i.e., where immune deposits are embedded or partially covered by basement membrane or contiguous to cell membranes. Previous studies indicate that the MAC may diffuse and insert into membranes at sites distant from the activation of the classical complement pathway (22) indicating that MAC may bind independently of C3 to cell membranes. The foot process membrane of visceral epithelium is the only site within the glomerulus at which C3b receptors have been demonstrated (23–28), although immune complexes and C3 were observed throughout the basement membrane of immunized rats. The predominant localization of the MAC in the region of the lamina rara externa suggests that immune complexes migrate through a damaged GBM and accumulate in the subepithelial region with consequent activation of the classical complement pathway and deposition of C3 and the MAC on podocyte membranes. The failure to demonstrate IgG, C3, BSA, or the MAC on endothelial and other cell membranes is most likely related to the absence of Fc and C3b receptors on these membranes except for unique circumstances such as induction of receptors by viral infection (29).

Mechanisms responsible for the occurrence of proteinuria both in immune-complex-induced and other forms of glomerulonephritis have been the source of considerable speculation. The GBM and visceral epithelium are major components of the glomerular filtration barrier (30–32). Morphologic evidence of injury to the GBM and podocyte membrane associated with deposits of the MAC, a known membran-

olytic agent, suggests that this complement complex is a mediator of proteinuria. Patients with nonimmune renal disease resulting from the administration of puromycin (33–37) and adriamycin (38) manifest massive proteinuria and nephrotic syndrome. These drugs primarily affect cell metabolism but are associated with both cell and basement membrane injury, which may be mediated by the MAC or analogous agents. Although fusion of foot processes may occur secondary to diffusion of excess albumin through the basement membrane (39, 40), it appears that significant proteinuria occurs only when injury to both the GBM and podocyte membranes is manifest. The predominant localization of immune complexes and the MAC in the subepithelial GBM-podocyte membrane region is consistent with this thesis. In experimental immune complex and human glomerulonephritis abnormalities of the GBM, fusion of visceral epithelial foot processes (41) and “loss” of C3b receptors from foot process membranes of human kidney have been demonstrated (42–46). Based on observations derived from this study, the loss of the putative C3b receptor, but not of intact C3b containing immune complexes is related to binding of C3. The failure to demonstrate C3b receptors may also result from injury to the podocyte membrane with subsequent functional loss of these receptors.

The presence of glomerular-bound immune complexes containing excess BSA antigen is characteristic for this experimental model of rat glomerulonephritis. Lew rats manifest moderate to severe proliferative glomerulonephritis following two or more months of daily i.v. BSA administration (6–8). Deposition of circulating immune complexes in antigen excess and/or the addition of BSA antigen to in situ antigen-antibody deposits may perpetuate complement activation and thereby enhance both the severity and progression of glomerulonephritis. The ability of acid buffer to elute immune complexes and C3 from glomeruli of each animal studied suggests that the nephritis has not reached the advanced stage found in SLE, where dissociating agents may only partially release glomerular-bound complexes from kidneys manifesting severe nephritis (1). The ease with which immune complexes are eluted from the GBM may reflect potential reversibility of a particular glomerular lesion, although further experiments are required to substantiate this hypothesis. In contrast to immune complexes, the MAC is not significantly affected by acid buffer treatment in both experimental immune complex glomerulonephritis and human SLE renal disease, suggesting that the MAC is firmly embedded in basement and cell membranes. In vitro experiments have demonstrated that the MAC is resistant to proteolysis and dissociation from cell membranes (47–48). Therefore, the evaluation of serial renal biopsies that manifest decreasing amounts of immune complex reactants or a diagnostic biopsy that fails to disclose IgG or C3 may reflect more rapid catabolism or solubilization of glomerular-bound immune complexes. Further studies are warranted to determine the following: the stability of the MAC as a marker of immune or nonimmune induced tissue injury; the correlation of MAC deposition compared to immune complex localization for monitoring progression of glomerulonephritis; and the possibility of uncovering immune renal disease for which neither IgG nor C3 have been demonstrated (49).

Tubulointerstitial lesions have not been found to be consistently associated with animal models of experimental immune complex glomerulonephritis (50–52). Immunization schedules utilizing antigens such as BSA, which result in the formation of circulating immune complexes in antigen excess persisting for long periods of time,

have been most closely correlated with the occurrence of tubulointerstitial disease. Previous studies indicate that immune complexes diffuse into the renal parenchyma from peritubular capillaries rather than reabsorption from tubular fluids. Kidneys of five of six rats with moderate to severe glomerulonephritis manifested interstitial infiltrates and irregular peritubular C3 and scant MAC deposits in clusters of tubules, but IgG was not demonstrable in the tubulointerstitial region utilizing fluorescein-labeled reagents. Immunoelectron microscopy studies, however, indicated that IgG and C3 were detectable on the surface of interstitial collagen fibers contiguous to thickened TBM. Segments of the TBM showed homogeneous C3 deposits and smaller foci of the MAC, but IgG was not demonstrable within the TBM. These data are consistent with immune complex diffusion from peritubular capillaries, although tubular reabsorption of complexes cannot be excluded. Following activation of the classical complement pathway C3 and the MAC are deposited within the TBM thereby serving as a potential mediator of injury of the TBM. Several observations suggest that nonimmune agents, such as bacteria, may activate the alternative complement pathway and contribute to the occurrence of tubulointerstitial lesions: (a) the absence of extraglomerular BSA antigen or antibody in the kidneys of nephritic animals; and (b) the occurrence of scattered foci of peritubular C3 associated with scant deposits of the MAC without interstitial IgG or C3 in control animals.

The strong cross-reactivity between rat and human neoantigen(s) suggests that MAC determinants are phylogenetically conserved, a property of proteins and polypeptides of biological importance. The consistent association of tissue injury with MAC localization in both experimental and human glomerulonephritis supports the hypothesis that the MAC is an *in vivo* mediator of membrane toxicity. Numerous cell- and serum-derived mediators, such as proteolytic enzymes, kinins, coagulation factors, lymphokines, and toxic oxygen metabolites (53-56) have been demonstrated within inflammatory reactions. Examination of both physiologic and anatomic parameters will be required to establish the *in vivo* pathogenetic significance of the MAC, other mediators, and their interaction which may either augment or inhibit tissue injury.

Summary

The role of the membrane attack complex (MAC) as a mediator of renal tissue injury was evaluated in rats affected by bovine serum albumin (BSA)-induced immune complex glomerulonephritis. Immunofluorescence studies revealed concurrent deposits of IgG, BSA, C3, and the MAC along glomerular capillary walls, although the MAC manifested a more restricted distribution than that observed for immune complexes. Immunoelectron microscopic techniques were utilized to demonstrate immune complexes, C3, and the MAC within dense deposits in the subepithelial aspect of the basement membrane. Visceral epithelial foot processes were fused in areas overlying large dense deposits and exhibited intense staining for the MAC, lesser reactivity for C3 but IgG was absent from the foot process membranes. Smaller granular deposits of immune complexes, C3, and the MAC were observed in the subendothelial region of the lamina rara interna and the lamina densa. Immune complexes may activate the classical complement pathway causing diffuse injury to the glomerular basement membrane (GBM), allowing subepithelial accumulation of

complexes. These observations implicate the MAC as a mediator of GBM and juxtaposed podocyte membrane injury, thereby contributing to disruption of the glomerular filtration barrier.

IgG and C3 were demonstrated within tubulointerstitial regions on the surface of collagen fibers in close proximity to the tubular basement membrane (TBM) of proximal convoluted tubules. Within the TBM, C3 localization was prominent with diminished reactivity for the MAC, but IgG was not detectable. The demonstration of C3 and scant MAC deposits in the TBM of nonimmunized control rats without evidence of interstitial IgG and C3 deposits suggests that both nonimmune and immune processes play a role in the pathogenesis of extraglomerular lesions.

Evidence derived from these morphologic studies indicates that the MAC is associated with injury to the GBM, foot process membranes of visceral epithelium, and the TBM. Further experiments designed to selectively enhance or inhibit the deposition of MAC and assess consequent renal dysfunction are required to substantiate hypotheses concerning the *in vivo* membranolytic potential of the MAC in experimental immune complex glomerulonephritis.

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