

Complement-induced Glomerular Epithelial Cell Injury

Role of the Membrane Attack Complex in Rat Membranous Nephropathy

Andrey V. Cybulsky, Helmut G. Rennke, Irwin D. Feintzeig, and David J. Salant

Evans Memorial Department of Clinical Research and Department of Medicine at University Hospital, Boston University Medical Center, Boston, 02118; Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02115

Abstract

In passive Heymann nephritis (PHN) in rats, antibody (anti-Fx1A) reacts in situ with a glomerular epithelial antigen and induces complement (C)-mediated cell-independent proteinuria. To assess the role of the membrane attack complex (MAC), we determined the need for C8 in the pathogenesis of proteinuria in an autologous-phase model of PHN. Isolated rat kidneys, containing nonnephritogenic, non-C-fixing $\gamma 2$ sheep anti-Fx1A (planted antigen), when perfused in vitro with C-fixing guinea pig anti-sheep IgG and a source of C (fresh human plasma 50% vol/vol in buffer containing bovine serum albumin), developed marked proteinuria after 20 min (0.58 ± 0.08 mg/min · g, $n = 8$) that increased further to 3.20 ± 0.93 mg/min · g after 80 min. In contrast, identical kidneys perfused with antibody and heat-inactivated or C8-deficient human plasma and normal kidneys perfused with antibody and fresh plasma excreted only 0.27 ± 0.03 ($n = 6$), 0.27 ± 0.04 ($n = 5$), and 0.40 ± 0.05 mg/min · g ($n = 6$) after 20 min, and 0.13 ± 0.02 , 0.22 ± 0.03 , and 0.32 ± 0.05 mg/min · g after 80 min, respectively. When C8-deficient plasma was reconstituted with sources of C8 ($n = 3$), proteinuria was restored to the level observed with fresh normal plasma. Differences in protein excretion could not be explained by quantitative differences in glomerular antigen or antibody content. Extensive ultrastructural damage to glomerular visceral epithelial cells was exclusively seen in antigen-containing kidneys perfused with antibody and C8-replete plasma. Thus, glomerular injury in this model results from an antigen-specific, antibody-directed, C8-dependent reaction involving assembly of the MAC. The ultrastructural findings argue in favor of MAC-induced cytotoxicity of the glomerular visceral epithelial cells.

Introduction

In addition to the well-known hemolytic action of the C5b-9 membrane attack complex (MAC)¹ of complement (C), its non-

Address reprint request to Dr. Salant, Renal Section, Boston University Medical Center, 80 E. Concord Street, Boston, MA 02118.

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1. *Abbreviations used in this paper:* C, complement; C8D α - γ , deficiency of the α - γ unit of C8; C8D β , deficiency of the β -unit of C8; CH₅₀, total hemolytic complement activity; C_{inulin}, inulin clearance; EM, electron microscopy; FR_{Na}, fractional reabsorption of sodium; GBM, glomerular basement membrane; HI, heat inactivated; IF, immunofluorescence; IPK, isolated perfused rat kidney; KHB, Krebs-Henseleit buffer; MAC, membrane attack complex of complement; MN, membranous nephropathy; PHN, passive Heymann nephritis; RVR, renal vascular resistance.

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lytic cytopathic effects on nucleated cells are currently receiving considerable attention (1-3). A possible example of the pathophysiological consequences of these effects is provided by observations that glomerular injury in the passive Heymann nephritis (PHN) model of experimental membranous nephropathy (MN) in the rat is C-mediated but leukocyte-independent (4, 5). These findings have recently been extended in a model of MN induced by immunization of normal and C6-deficient rabbits with cationized bovine serum albumin (BSA) (6) and in PHN rats depleted of C6 (7). In both situations, C6-deficient animals failed to develop the features of glomerular injury that occurred in their C-replete counterparts. Thus, the evident lack of chemotactic effects of C in PHN and requirement for C6 in these models suggested a role for the terminal C pathway. Additional circumstantial evidence that the MAC may be responsible for organ damage is provided by several recent immunohistologic and morphologic studies that have documented the presence of terminal C components and the MAC in lesions of patients (8-12) and experimental animals (13-18) with various forms of tissue injury including rat MN (15-18). However, the primary target and mechanisms of C-induced injury that lead to altered glomerular permeability remain unknown and direct proof that the MAC is involved in this process is lacking.

The aim of this study was to obtain definitive functional evidence for or against the role of the MAC in glomerular capillary wall injury using a variant of the rat PHN model. The rationale is based on the fact that membrane-bound C5b-7 has no deleterious effect on cell membrane integrity, whereas binding of C8 to the complex results in membrane damage, a process that is accelerated by the subsequent binding of C9 (19-21). C8 is thus the keystone to formation of a cytopathic complex. Inasmuch as there are no C8-deficient (C8D) rat strains and specific depletion of C8 is difficult to achieve in vivo (Salant, D. J., unpublished observations), we adopted a passive autologous-phase model of PHN in the isolated perfused rat kidney (IPK). The IPK has been successfully used in previous immunologic studies (22-27) and has been accepted as a valid model for investigating changes in glomerular permeability (24-27). In keeping with observations in the autologous phase PHN model in vivo (5, 28), antibody-directed, C-mediated glomerular injury was reproduced in the IPK by perfusing kidneys with antibody and human plasma replete in all C components but not with heat-inactivated (HI) plasma. Furthermore, the absence of injury in kidneys perfused with C8D human plasma and its restoration on reconstituting the C deficiency with a source of C8 established that assembly of the MAC is essential for the development of glomerular injury in rat MN.

Methods

Kidney perfusion and perfusate composition. BSA (Miles Laboratories, Naperville, IL) perfusate was prepared by initially dialyzing a 20% BSA

solution in Krebs–Henseleit buffer (KHB) against 10 vol of KHB for 48 h. The BSA was then diluted to a concentration of 80 mg/ml by additional KHB and amino acids. The final composition of KHB included (in millimoles per liter): Na, 140; K, 4.8; Ca, 3.0; Mg, 1.2; Cl, 119; HCO₃, 25; inorganic phosphate, 1.2; SO₄, 1.2; glucose, 5.0; and amino acids—leucine, 0.8; phenylalanine, 0.64; lysine, 2.0; methionine, 0.66; isoleucine, 0.6; valine, 0.66; histidine, 0.48; threonine, 0.48; tryptophan, 0.14; alanine, 4.0; glycine, 4.6; arginine, 1.0; proline, 0.62; tyrosine, 0.4; cysteine, 1.0; aspartate, 0.4; glutamate, 1.0; asparagine, 0.4; glutamine, 4.0; and serine, 2.0.

Fresh frozen human plasma was provided by Dr. R. Valery of Naval Blood Research Laboratory, Boston, MA. Units of plasma that had been collected into citrate-phosphate-dextrose were pooled, treated with heparin (6 U/ml) and dialyzed for 24 h against 10 vol of KHB without Ca. For some experiments plasma was heat-inactivated at 56°C for 30 min. C8D β plasma was collected by plasmapheresis from an individual with a congenitally dysfunctional C8 molecule and was treated similarly to fresh plasma. The total absence of C8 hemolytic activity in this individual has been fully characterized (29, 30—patient D.T.) and found to be due to deficiency of the β -subunit of the C8 molecule. In one experiment (see below), the C8D β plasma was mixed with serum deficient in the α - γ C8 unit (30, patient R.G.) to reconstitute C8 activity. Prior to use, both BSA-KHB perfusate and plasma were filtered through a 0.45- μ m filter (Millipore Corp., Bedford, MA).

Experiments were carried out in an incubator that maintained a temperature of 37°C. The perfusion circuit was driven by two pumps (Masterflex, Cole-Parmer, Chicago, IL) and consisted of two glass reservoirs, a glass film-oxygenator, an 8- μ m Millipore filter, a flowmeter (Gilmont, Cole-Parmer), and a side-arm pressure gauge, all connected by Tygon^R tubing (Cole-Parmer). Glassware was siliconized and perfusate was gassed with 95% O₂/5% CO₂.

The isolated kidneys were perfused according to Nishiitsutsuji-Uwo et al. (31). Briefly, male Sprague-Dawley rats (CD, Charles River Breeding Laboratories, Wilmington, MA) weighing 275–335 g were anesthetized with an intraperitoneal injection of 3.6% chloral hydrate (1.2 ml/100 g). The right ureter was cannulated with PE-50 tubing after injection of 200 mg of mannitol intravenously. A glass cannula (P. Brooks, Ducklington, England) was passed retrograde through the superior mesenteric artery into the right renal artery without interrupting blood flow. While the kidney was perfused with BSA-KHB, the cannula was tied in place and the kidney and ureter were dissected free and mounted in the incubator.

After 5 min, verapamil (Knoll Pharmaceutical Co., Whippany, NJ) was added to the BSA-KHB perfusate at a concentration of 10⁻⁴ M. Verapamil was required to prevent the development of intense vasoconstriction and renal shutdown, which universally occurred if plasma was added alone. After a further 5 min of perfusion, plasma was added (50% vol/vol) to BSA-KHB. This resulted in a final albumin concentration of 60 mg/ml, and the same electrolyte concentration as in KHB, except for Ca and amino acids that were reduced by 50%. The perfusion pressure was adjusted to, and constantly maintained at 95–99 mmHg. After 10 min of stabilization, antibody was added and measurements were begun. The perfusion lasted 1 or 2 h, with three or six urine collection periods, each 20 min in length.

Measurements. Inulin clearance (C_{inulin}) was measured with [³H]methoxy-inulin, or [¹⁴C]inulin (New England Nuclear, Boston, MA) during experiments involving ¹²⁵I. Perfusate inulin concentration was determined at the midpoint of each collection period. Urine and perfusate samples were placed into scintillation fluid and counted in a beta-scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Protein concentration was determined spectrophotometrically (Gilford Instrument Laboratories, Oberlin, OH) at OD₂₈₀, using a Labtrol (Dade Diagnostics, Agnada, PR) standard. Sodium was measured by a flame photometer (Instrumentation Laboratories, Lexington, MA). Perfusate flow rate was monitored constantly and renal vascular resistance (RVR) was calculated from: average perfusion pressure/perfusate flow rate (mmHg · min · g/ml). C_{inulin} (ml/min · g), urine protein excretion (mg/min · g), and fractional sodium reabsorption (FR_{Na}%) were calculated

from standard formulas. Measurements are expressed as a function of kidney weight.

Production and characterization of antibodies. A sheep was immunized by repeated monthly injections of rat Fx1A, as previously described (4). The γ 2 IgG subclass was isolated by ion-exchange chromatography, heat-inactivated (56°C, 30 min), concentrated, and stored, as described (4). On immunoelectrophoresis the fraction was shown to contain pure γ 2 sheep IgG.

Hartley guinea pigs (Charles River Breeding Laboratories) were immunized by four subcutaneous injections of purified sheep IgG in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich). After 10 wk, antiserum was collected and stored at -70°C until use. The concentration of anti-sheep IgG in whole antiserum was approximately 8.75 mg/ml as determined by reverse radial immunodiffusion (32, 33), using affinity-purified antibody (see below) as the standard.

For iodination, guinea pig anti-sheep IgG was purified by affinity chromatography. Sheep γ 2 IgG was linked to cyanogen bromide (CNBr)-Sephacrose-4B (Pharmacia Fine Chemicals, Piscataway, NJ), as recommended by the manufacturer. After whole antiserum was passed through the column, the bound antibody was eluted with 1.75 M KSCN. The eluted antibody was shown on immunoelectrophoresis to be pure, containing two subclasses of guinea pig IgG. By reverse radial immunodiffusion (32), its relative affinity for sheep IgG was 50% greater than that of whole antiserum.

Quantitation of glomerular antibody binding. The γ 2 fraction of Sh anti-Fx1A (10 mg) and affinity-purified guinea pig anti-sheep IgG (5 mg) were iodinated with 2 mCi of ¹³¹I and 1 mCi of ¹²⁵I, respectively (New England Nuclear), by the chloramine-T method (34). Specific activities were 3.8 × 10⁴ cpm/ μ g of ¹³¹I and 1.2 × 10⁵ cpm/ μ g of ¹²⁵I. The former was diluted with "cold" antibody before injection into rats. Iodinated and noniodinated guinea pig anti-sheep IgG had similar relative affinities for sheep IgG by radial immunodiffusion (32), and had similar indirect immunofluorescence titers against kidney tissue-bound sheep IgG.

To quantitate glomerular antibody binding, perfusate was sampled to measure the concentration of ¹²⁵I-guinea pig anti-sheep IgG and kidneys were perfused with 100 ml of saline at the end of the experiment. In pilot studies it was determined that after this, the nonspecific binding of antibody was insignificant. Glomeruli were then separated by differential sieving (35) and counted for ¹²⁵I and ¹³¹I in a gamma-counter (Packard Instrument Co., Downers Grove, IL, or Searle Analytic Inc., Des Plaines, IL). Counts were corrected for decay, channel spillover, and perfusate antibody concentration. The number of glomeruli isolated from each kidney was determined by visual counting and glomerular binding of γ 2 sheep anti-Fx1A and guinea pig anti-sheep IgG was calculated as described (35) and expressed as micrograms bound per 38,000 glomeruli (the average number of glomeruli per rat kidney).

Hemolytic assays. The total hemolytic complement (CH₅₀) of each pooled plasma preparation was measured by a test tube method according to Kabat and Mayer (36), and was compared to fresh frozen serum of a single normal individual. For determination of C8 activity, sheep erythrocytes, sensitized with C1-7, were prepared by incubating erythrocytes with rabbit anti-sheep hemolysin (A) (Difco, 20 min, 4°C), followed by C8D β plasma (3.0 × 10⁸ cells in 1.5 ml of gelatin-veronal buffer with 0.15 ml of C8D β plasma) for 30 min at 30°C. Then, 0.2 ml of the serially diluted test material was incubated (37°C, 90 min) with 0.2 ml C9 guinea pig (500 CH₅₀ U/ml, Cordis Laboratories, Miami, FL), 0.4 ml of buffer, and 0.2 ml EAC1-7 (1 × 10⁸ cells/ml). After incubation, 2 ml of 0.15 M NaCl was added, the tubes centrifuged (1,800 rpm, 10 min, 0°C) and lysis was determined by measuring absorbance of the supernatant at 415 nm. For each dilution of test material, z values were calculated from $z = -\ln(1 - y)$, where y is the percentage cell lysis (after correction for background lysis) as compared to 100% lysis with distilled water. Values of z were plotted against test reagent dilutions and 1 CH₅₀ U determined from the dilution that gave $z = 0.69$, equivalent to 50% lysis. The concentration of C8 activity in undiluted test reagent was then calculated. Normal titers were 185 CH₅₀ U/ml and 1.9 × 10⁵ C8 U/ml. Verapamil did not have any effect on hemolytic activity.

Tissue processing for immunofluorescence (IF) and electron microscopy (EM). Direct IF was performed on kidney tissue obtained at the end of perfusion and snap-frozen in dry ice-isopentane, sectioned at 4 μ m in a cryostat and fixed in ether-ethanol, as previously described (22). All tissue was stained with fluoresceinated IgG fractions of monospecific antisera, including rabbit anti-sheep IgG, rabbit anti-guinea pig IgG and goat anti-human C3 (Cappel Laboratories, West Chester, PA). Even though guinea pig anti-sheep IgG cross-reacted with the fluoresceinated goat anti-human C3 in Ouchterlony double diffusion in agarose, this did not occur when the guinea pig anti-sheep IgG was tissue-bound.

For EM, kidneys were either perfusion-fixed with 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, or immersion-fixed in paraformaldehyde (2.5 g/100 ml)-glutaraldehyde (2.0 g/100 ml) in 0.1 M sodium cacodylate buffer, pH 7.4, and further treated as previously described (6). At least three kidneys in each group were examined by IF, and two to four kidneys by EM.

Experimental design. Mediation of injury was studied in the first set of experiments, consisting of four groups of kidneys, listed in Table I. 3 d before kidney perfusion, rats in groups I, II, and IV received an intravenous, subnephritogenic dose of non C-fixing γ 2 sheep anti-rat Fx1A (12 mg of IgG fraction) that served as "planted" antigen (5). During the perfusion, all groups received 4.0 ml of guinea pig anti-sheep IgG as HI whole serum, containing 35 mg of antibody. The type of human plasma used is indicated in Table I. Thus, groups I and IV constitute the experimental kidneys, groups II and III being controls.

The second part of the study involved the quantitation of bound glomerular antigen and antibody to evaluate the effects of different perfusion conditions on antibody binding. Antigen was administered as above (8 mg of γ 2 anti-Fx1A, to which 4×10^6 cpm of trace-labeled IgG was added). Plasma perfusions as for groups I, II, and IV were carried out (for 1 h) with tracer antibody alone (30–60 μ g of 125 I-affinity purified guinea pig anti-sheep IgG).

Because the yield of purified C8 isolated from whole plasma is low (37), reconstitution of C8 activity in C8D plasma was performed by two alternate methods. In the first case, two kidneys with planted antigen (as in groups I, II, and IV) were perfused with 50% C8D β plasma and 4.0 ml of fresh guinea pig antiserum (C8R, Table I), as opposed to HI antiserum used in groups I–IV. Fresh guinea pig antiserum provided 35 mg of anti-sheep IgG, as well as sufficient C8 to reconstitute the CH₅₀ of C8D plasma. Two control kidneys with planted antigen were perfused with 4.0 ml of fresh guinea pig anti-sheep IgG without added human plasma (GPS, Table I). Secondly, enough C8D α - γ serum was available to perform one perfusion with HI antiserum and a mixture of C8D β

plasma and C8D α - γ serum (25 ml each). Whereas the individual reagents have no hemolytic or C8 activity, when combined the CH₅₀ and C8 activity are both reconstituted (Table I).

Statistics. Parametric data, including RVR, C_{inulin}, and FR_{Na}, were analyzed by one-way analysis of variance (38) at each time period. Where a significant difference was found, meaningful individual comparisons were made between groups utilizing Scheffe's analysis (38). Nonparametric data (protein excretion, antibody deposition) were analyzed by the Kruskal-Wallis analysis of variance (39), and where significant differences occurred, these were then examined by the Mann-Whitney U test (39).

Results

Hemolytic activity. Fresh-frozen plasma when thawed, pooled, heparinized, and dialyzed contained 90–100% CH₅₀ of fresh human serum. After the addition of fresh plasma to BSA-KHB (50% vol/vol), the CH₅₀ of the mixture was 50% of normal (Table I). This declined by 25% at 60 min of perfusion (i.e., to 37.5% of normal) and a further 25% by 90 min (to 25% of normal). HI plasma, C8D plasma, and HI antisera had no detectable hemolytic activity. The CH₅₀ and C8 titers of the perfusates in all experimental groups are given in Table I.

Protein excretion and function of IPKs. Protein excretion is demonstrated in Fig. 1. The lowest values were found in control groups (II and III), levels that are comparable to those previously described in "normal" IPKs perfused with BSA (24, 26, 40–42). Kidneys in group III had a slightly higher protein excretion than group II, which reached statistical significance after 20 min. Antibody binding in the presence of C (group I) markedly enhanced protein excretion. This increase became significant after 20 min and, by the end of the 2 h, the majority of group I kidneys were massively proteinuric (Fig. 1). Substitution of fresh normal plasma with fresh C8D β plasma (group IV) resulted in only baseline levels of protein excretion that were comparable to control groups (Fig. 1).

Functional data for the experimental groups (I and IV) and controls (II and III) is presented in Table II. RVR was not significantly different between the groups at any time point, except period 6, where it was lower in group II. Among the two control

Table I. Characteristics of Isolated Perfused Kidneys, Perfusate Composition, and Complement Activity

Group	Planted antigen: sheep anti-Fx1A*	Perfusate composition‡		C activity of perfusate§	
		Anti-sheep IgG	Human plasma	CH ₅₀	C8 titer
				% normal	% normal
I (8)	+	HI	fresh normal	50	50
II (6)	+	HI	HI normal	0	not done
III (6)	–	HI	fresh normal	50	50
IV (5)	+	HI	C8D β	0	0
<i>C8 Replacement</i>					
C8R (2)	+	fresh	C8D β	80	15
GPS (2)	+	fresh	—	25	15
C8D β + α - γ (1)	+	HI	C8D β + C8D α - γ	50	20

* 12 mg of IgG given intravenously to rats 3 d before kidney perfusion. ‡ 50% human plasma (vol/vol) in BSA-KHB (100 ml), to which 4.0 ml of fresh or heat-inactivated (HI) guinea pig anti-sheep IgG was added. § CH₅₀ and C8 titer of the final perfusate is expressed as a percentage of the activity in undiluted, fresh, normal human serum. || Number of kidneys studied is in parentheses.

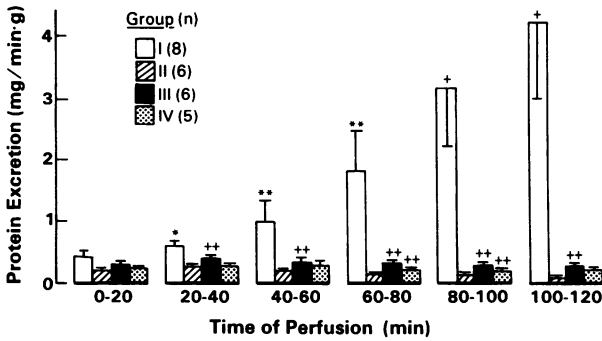


Figure 1. Protein excretion of IPKs. Greatest protein excretion is seen in antigen-containing kidneys, perfused with antibody and fresh plasma (group I). Normal kidneys perfused with antibody and fresh plasma (group III) and antigen-containing kidneys perfused with antibody and HI (group II) or C8D (group IV) plasma displayed baseline proteinuria, although kidneys perfused with fresh plasma (groups III and IV) excreted slightly more protein than group II. Data are presented as the mean \pm SEM. The number of kidneys studied (*n*) is shown in the figure. A significant difference between the four groups was present after 20 min (Kruskal-Wallis one-way analysis of variance). Differences between the groups (Mann-Whitney U test) are as follows: group I vs. II, III, and IV $P < 0.04^*$, $P < 0.025^{**}$, $P < 0.002^+$; group II vs. III and II vs. IV $P < 0.04^{++}$.

groups, HI plasma (group II) resulted in higher C_{inulin} and FR_{Na} than group III, and these were also higher than in groups I and IV (significant during periods 2–4 and 2–6, respectively). Experimental kidneys (group I) showed a lower FR_{Na} than controls and the C8D group (significant during periods 1 and 4–6). Thus, except for FR_{Na} , immunologic reaction in the presence of fresh plasma did not substantially alter kidney function in this model.

Quantitation of antigen and antibody. The purpose of these

experiments was twofold: (a) to determine the variability between kidneys in bound antigen, resulting from an identical intravenous dose of anti-Fx1A, and (b) to establish whether the type of plasma influenced antibody binding when perfused under identical conditions. The RVR and C_{inulin} of kidneys containing planted antigen and perfused with tracer amounts of antibody (data not shown) were similar to those seen in the experiments with the full dose of antibody (groups I, II, and IV, above). The sole exception occurred with fresh plasma perfusions, where RVR was higher with the tracer dose as compared to the full dose of antibody. This resulted in a 2.5-fold higher filtration fraction for trace-dose perfusions, which is still trivial compared to in vivo values ($\sim 4\%$ vs. 20%). It is unlikely that this difference in filtration fraction significantly altered binding; if anything, it could have potentially produced exaggerated values in the tracer group (43). Thus, it is felt that the findings of tracer antibody perfusions are representative of experiments using the full antibody dose.

Whether expressed as the absolute amount of guinea pig IgG bound (microgram/38,000 glomeruli adjusted to a perfusate concentration of 0.33 μg guinea pig IgG/ml) or as a function of the amount of planted antigen in each kidney (guinea pig IgG/sheep IgG), no detectable difference was found between the three groups (Table III). Thus the difference in protein excretion observed with different types of plasma could not be attributed to alterations in glomerular antibody deposition.

Replacement of C8. Two methods of C8 reconstitution were employed. First, fresh guinea pig anti-sheep IgG (rather than HI antiserum as in groups I–IV) was used as a source of C8. When added to perfusate that contained 50% C8D β plasma (group C8R, Table I) it fully reconstituted the CH_{50} , provided 15% of normal C8 activity, and restored the high level of protein excretion (Fig. 2) seen in group I kidneys (Fig. 1). It is likely that the earlier appearance and greater magnitude of proteinuria in group C8R (than group I) is a result of the relatively higher final

Table II. Function of Isolated Perfused Kidneys

Period Time (min)	1 0–20	2 20–40	3 40–60	4 60–80	5 80–100	6 100–120
Group						
I RVR*	4.72 \pm 0.98	4.66 \pm 1.03	4.61 \pm 0.95	4.87 \pm 0.87	5.62 \pm 1.03	6.81 \pm 1.45
$C_{\text{inulin}}\ddagger$	0.16 \pm 0.24	0.35 \pm 0.16	0.49 \pm 0.32	0.38 \pm 0.11	0.36 \pm 0.12	0.37 \pm 0.22
$FR_{\text{Na}}\S$	89.5 \pm 6.2 $\S\S$	95.3 \pm 2.1	94.8 \pm 2.1	90.3 \pm 6.5 $\S\S$	82.4 \pm 9.5	74.4 \pm 13.5
II RVR	4.22 \pm 0.69	3.83 \pm 0.74	3.67 \pm 0.77	3.66 \pm 0.89	3.68 \pm 0.93	3.61 \pm 0.88 [¶]
C_{inulin}	0.37 \pm 0.30	0.86 \pm 0.15 ^{††}	0.86 \pm 0.06 ^{**}	0.69 \pm 0.23	0.49 \pm 0.20	0.35 \pm 0.20
FR_{Na}	98.4 \pm 0.6	98.7 \pm 1.13	98.5 \pm 1.2 ^{††}	98.6 \pm 0.7 ^{††}	98.1 \pm 1.6	97.9 \pm 2.1
III RVR	5.21 \pm 1.04	5.23 \pm 1.33	5.17 \pm 1.48	5.59 \pm 1.97	5.99 \pm 2.34	6.74 \pm 2.82
C_{inulin}	0.13 \pm 0.08	0.50 \pm 0.19	0.43 \pm 0.14	0.52 \pm 0.18	0.42 \pm 0.10	0.38 \pm 0.11
FR_{Na}	91.9 \pm 6.2	97.3 \pm 1.2	95.5 \pm 2.1	94.7 \pm 3.1	93.4 \pm 3.5	91.7 \pm 4.1
IV RVR	4.40 \pm 0.75	4.05 \pm 0.69	4.16 \pm 0.67	4.29 \pm 0.54	4.56 \pm 0.43	4.93 \pm 0.53
C_{inulin}	0.67 \pm 0.18	0.73 \pm 0.07	0.64 \pm 0.10	0.52 \pm 0.06	0.48 \pm 0.03	0.44 \pm 0.11
FR_{Na}	97.2 \pm 0.9	95.3 \pm 1.1	95.1 \pm 0.8	95.3 \pm 1.2	94.4 \pm 2.5	93.5 \pm 2.0

All values are expressed as mean \pm SD. * RVR, renal vascular resistance (mmHg \cdot g \cdot min/ml). $\ddagger C_{\text{inulin}}$, inulin clearance (ml/min \cdot g). $\S FR_{\text{Na}}$, fractional reabsorption of sodium (%). ^{||} $P < 0.05$. [¶] $P < 0.025$. ^{**} $P < 0.01$. ^{††} $P < 0.005$, II vs. I, III, and IV. ^{\S\S} $P < 0.05$. ^{|||} $P < 0.005$, I vs. II, III, and IV.

Table III. Quantitation of Antigen and Antibody Deposition in Glomeruli

Plasma perfusate (n)*	Antibody: Guinea pig anti-sheep IgG	Antigen: Sheep anti-Fx1A	Antibody / Antigen × 10 ³
	μg/38,000 glomeruli	μg/38,000 glomeruli	
Fresh (4)	0.32±0.05 (0.26–0.38)	14.9±2.4 (11.6–17.1)	22.2±0.5 (17.8–29.2)
HI (5)	0.28±0.14 (0.12–0.40)	16.1±5.7 (9.9–23.0)	20.8±13.3 (5.8–33.4)
C8D β (4)	0.26±0.03 (0.23–0.30)	14.5±1.6 (12.9–16.6)	18.6±3.7 (14.1–21.7)

All values are expressed as mean±SD with range in parentheses below. There are no significant differences between the three groups in any variable. * Number of kidneys studied is in parentheses.

CH₅₀ of this perfusate (Table I). The enhanced protein excretion was not due to an independent effect of fresh guinea pig antiserum because antiserum added alone to plasma-free perfusate (group GPS, Table I and Fig. 2) produced baseline proteinuria.

Replacement of C8 was also accomplished by combining two complementary C8D reagents that independently do not have hemolytic or C8 activity (i.e., C8D β plasma and C8D α-γ serum). When 25% vol/vol of each was added to the perfusate, the CH₅₀ was reconstituted to 50% of normal and the C8 titer to 20% (Table I). Perfusate with reconstituted C8 activity produced enhanced proteinuria (Fig. 2) that was unequivocally higher than C8D β perfusions (group IV, Figs. 1 and 2) and was in the range of group I kidneys.

IF and EM findings. Antigen-containing kidneys perfused with guinea pig anti-sheep IgG (groups I, II, and IV) demonstrated positive IF staining of the glomerular capillary wall for

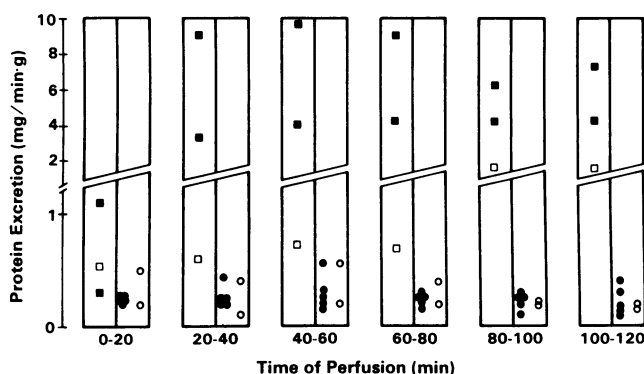


Figure 2. Replacement of C8. All kidneys contained planted antigen, and were perfused with guinea pig anti-sheep IgG. Protein excretion was markedly enhanced to levels comparable with group I when C8 activity was reconstituted in C8D β plasma perfusate with fresh guinea pig antiserum (C8R, ■) (see text for details). Control perfusions with fresh guinea pig antiserum added to plasma-free perfusate (GPS, ○) resulted only in baseline proteinuria which is similar to that of perfusions containing no C8 activity, i.e., HI guinea pig antiserum and C8D β plasma (group IV, ●). Perfusion with HI antiserum plus the combination of C8D β plasma and C8D α-γ serum also induced enhanced protein excretion (□).

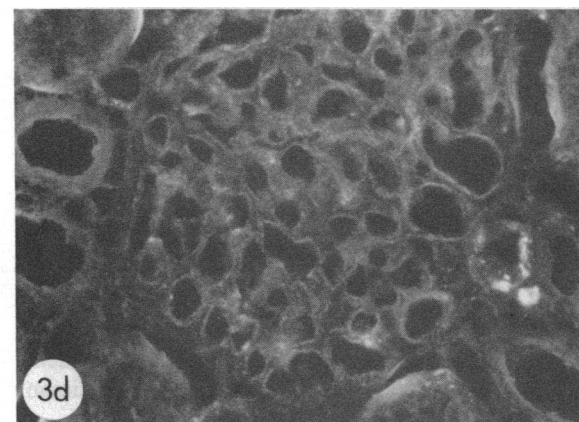
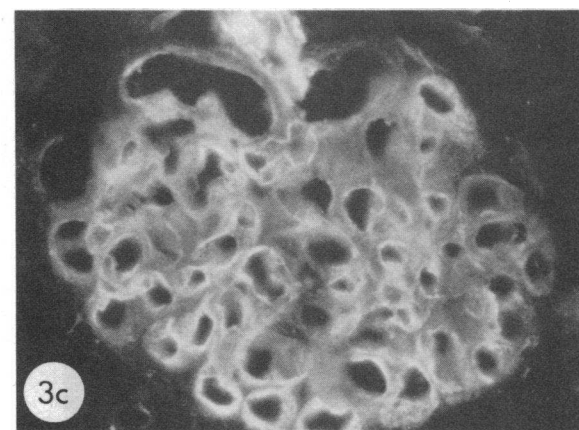
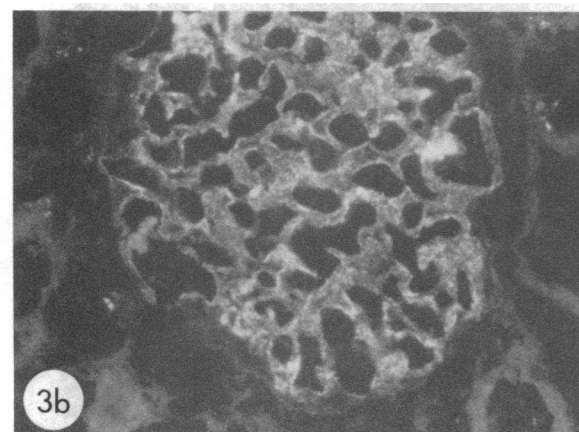
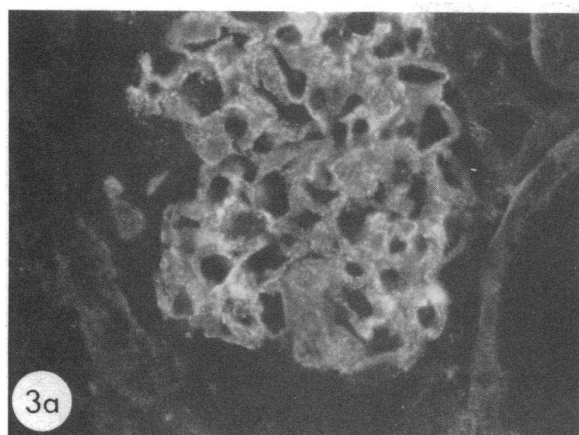
sheep IgG and guinea pig IgG (Fig. 3 a and b) in a granular pattern. No difference in intensity was detected between the groups. Positive C3 staining of similar intensity and distribution was seen in kidneys perfused with fresh normal plasma (group I) (Fig. 3 c) and C8D plasma (group IV). There was also some C3 staining of vessel walls. No staining for C3 was visible in group II kidneys perfused with HI plasma (Fig. 3 d), and no glomerular staining for any component was present in group III kidneys that contained no planted antigen.

Except for rare damage to endothelial cells, EM of group III controls without planted sheep anti-Fx1A (Fig. 4) demonstrated good preservation of glomerular architecture after 2 h of perfusion. Subepithelial electron dense deposits were present in all glomeruli from animals injected with sheep anti-Fx1A (groups I, II, and IV). Kidneys perfused with HI (II) or C8D (IV) plasma showed well-preserved glomerular epithelial cells, with only minimal focal effacement of podocytes overlying larger electron dense deposits (Fig. 5). In contrast, heavily proteinuric kidneys in groups I and C8R showed diffuse epithelial cell changes (Fig. 6) characterized by massive effacement and simplification of foot processes, microvillous degeneration, vacuolization of the cell body, and focal retraction with complete denudation of the GBM. In such areas electron-dense deposits often persisted, together with small, membrane-bound vesicles (Fig. 6 b and c) attached to the surface of the GBM facing the urinary space. Occasionally, capillary loops completely devoid of an epithelial cell layer could be observed in heavily proteinuric kidneys (Fig. 7). Signs of overt epithelial cell necrosis were invariably seen in association with this latter finding. These observations indicate that marked epithelial cell injury occurs near the site of antibody deposition only when conditions are suitable for assembly of the MAC.

Discussion

This study establishes, for the first time, that C8 can mediate injury in an intact organ system through assembly of the cytopathic MAC. Utilizing a cell-free, plasma-perfused isolated rat kidney model, we demonstrated that enhanced glomerular permeability, induced by subepithelial in situ immune complex formation, is dependent on the presence of C8. These conclusions are supported by the following results. Marked proteinuria was observed in antigen-containing kidneys perfused with C-fixing antibody and fresh plasma. No increment in protein excretion occurred when normal (antigen-free) kidneys were perfused with antibody and fresh plasma or antigen-containing kidneys were perfused with antibody and HI or C8D plasma. Severe proteinuria developed when total C activity was restored to C8D plasma by reconstitution with C8. Furthermore, the absence of injury in kidneys perfused with HI and C8D plasma could not be accounted for by qualitative and quantitative differences in glomerular deposits of antigen or antibody as compared to kidneys perfused with fresh plasma. Because C8 is essential for C-mediated membranolytic and has no other defined role, it is reasonable to conclude that injury in rat MN is mediated by the MAC.

From these studies it seems most likely that proteinuria in rat MN is the result of a structural or compositional alteration in the glomerular capillary wall. No consistent hemodynamic change was observed that could account for the time-related progressive increase in proteinuria in kidneys perfused with an-



tibody and a complete source of C. In contrast, ultrastructural observations in these experiments suggest that antibody-directed, C-induced injury is directed at the glomerular visceral epithelial cells. Foot process effacement, separation of podocytes from the GBM, and formation of membrane-bound vesicles were clearly related to the presence of C hemolytic activity in the perfusate and the development of massive proteinuria. These changes have not been seen in IPKs made proteinuric by other C-independent means (25, 26) indicating that they are unlikely to be nonspecific effects of proteinuria.

The probability of C-induced injury to the epithelial cell membrane in rat MN is consistent with the morphologic evidence of epithelial cell injury discussed above, with the known membranolytic action of the MAC, and with the location of electron-dense deposits in this lesion. Immunohistologic studies of rat serum sickness indicate that immune deposits produced by antibody reacting with exogenous antigen in the subepithelial space induces MAC assembly in the epithelial plasma membrane (14). It is probable that the MAC is similarly located in Heymann nephritis, where formation of subepithelial immune deposits is likely due to antibody-modulated capping and shedding of endogenous epithelial cell antigens (44, 45). Furthermore, as the MAC requires a phospholipid layer for insertion, the GBM is an unlikely target for the MAC because it does not normally contain phospholipids (46). Assembly of the MAC in the epithelial cell plasma membrane is also in keeping with current knowledge of its membranolytic action. This involves the formation of transmembrane channels (47) and rearrangement and/or loss of phospholipids (48, 49) with subsequent loss of cell membrane integrity. In contrast to C-dependent erythrocyte lysis that is an all-or-nothing "one-hit" phenomenon (47), lysis of several types of nucleated cells appears to require the formation of multiple transmembrane channels (1). The absence of total lysis of epithelial cells in experimental MN is compatible with established sublytic cytopathic effects of the terminal C pathway on nucleated cells, which involve the release of intracellular contents (1), including reactive oxygen species (50) and prostaglandins (2, 3, 51). Further investigation is necessary to determine the precise location of the MAC and whether altered glomerular permeability in experimental MN is the direct effect of epithelial cell injury leading to focal sieving defects or an indirect consequence of the release of other mediators of basement membrane or cellular injury.

One might speculate as to why these florid epithelial cell alterations are not routinely seen in vivo in experimental or human MN. In the IPK model described here immunopathologic changes that develop over several days in PHN (5, 35), weeks in active Heymann nephritis (52), and months or years in human MN, are condensed into a period of 2 h. During this time an amplified immunologic attack is directed at the glomerulus that may have little opportunity or capacity to regenerate and repair epithelial cell damage and clear the debris. This explanation implies that the findings in the IPK are an exaggeration and acceleration of a process that occurs in vivo but is not usually

Figure 3. Immunofluorescence microscopy of representative glomeruli. Antigen-containing kidneys perfused with antibody and fresh plasma (group I) or C8D plasma (group IV) showed glomerular capillary wall staining for sheep IgG, guinea pig IgG, and rat C3 (as in panels a, b, and c respectively), whereas such kidneys were negative for rat C3 when perfused with HI plasma (group II) (as in panel d) ($\times 360$).

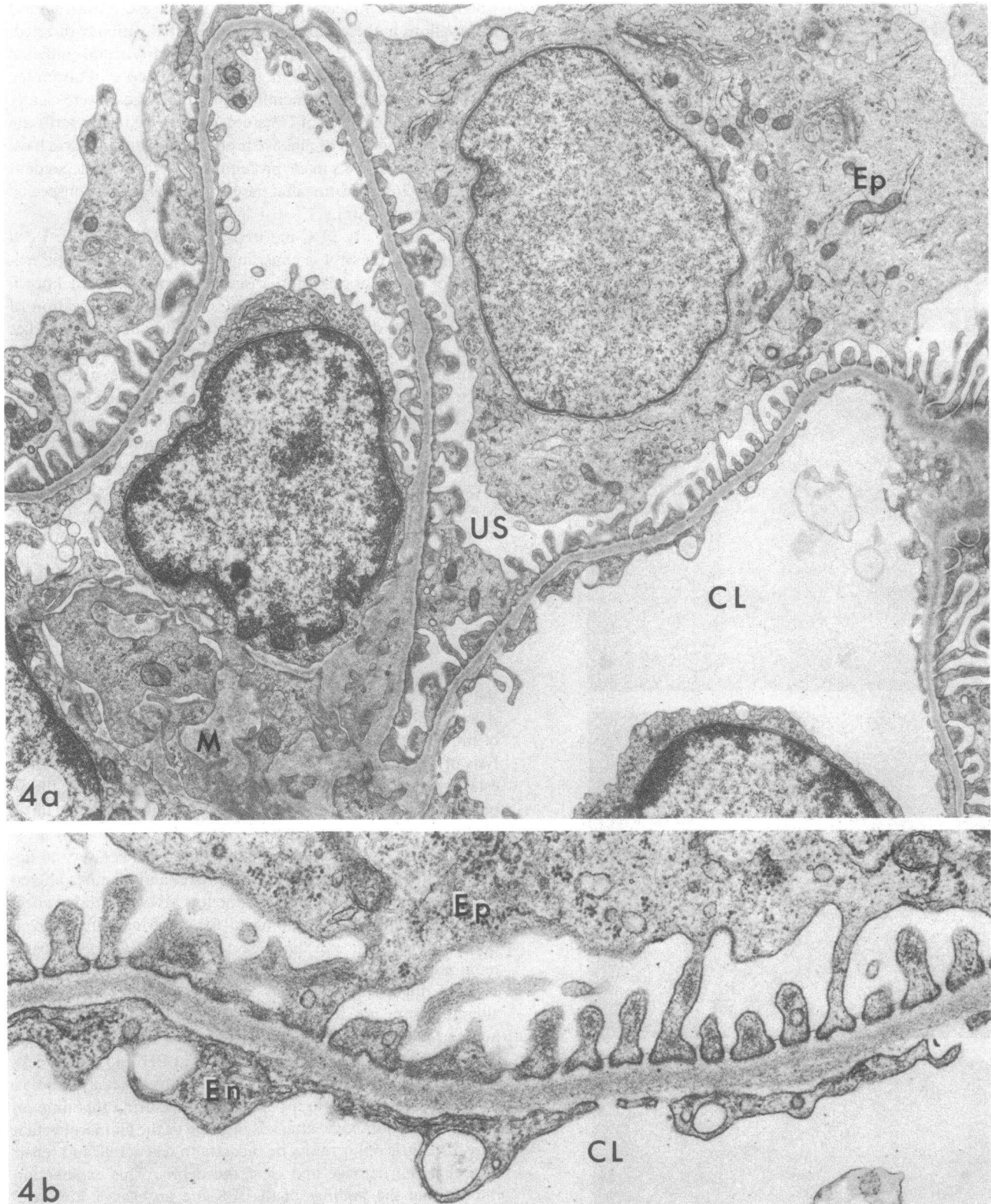


Figure 4. Ultrastructure of group III kidneys. (a) Two intact capillary loops with adjoining mesangium (M) are depicted ($\times 11,250$). (b) Details of the glomerular capillary wall. Electron-dense deposits are absent ($\times 35,000$). Ep, glomerular epithelial cells; US, urinary space; CL, capillary lumen; En, endothelium.

visible. Actually, published observations suggest the possibility of epithelial injury in the Heymann models of rat MN (53, 54). In addition, vesicular bodies resembling those seen in this study

and possibly representing membrane fragments have been identified within subepithelial electron densities in cases of human MN (55).

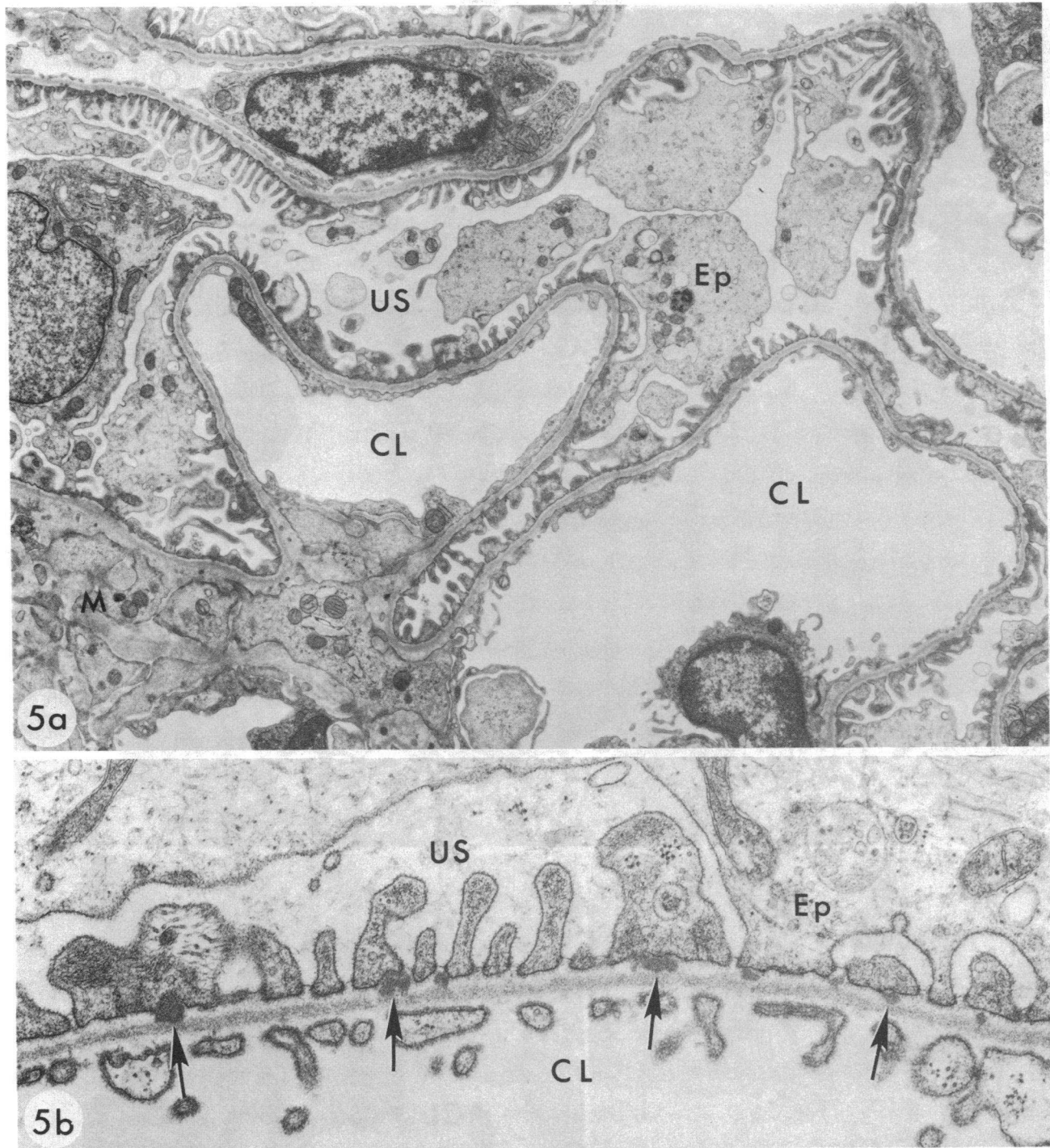


Figure 5. Ultrastructure of groups II and IV kidneys. (a) The general architecture of the glomerulus is well preserved, with minimal, focal obliteration of foot processes (group II kidney) ($\times 8,400$). (b) Glomerular capillary wall of group IV kidney. Small, discrete electron-dense

deposits (arrows) are present in the subepithelial space. Notice preservation of the diaphragms in numerous epithelial slits, separating adjacent processes ($\times 35,000$). Ep, glomerular epithelial cells; US, urinary space; CL, capillary lumen; M, mesangium.

Although the plasma-perfused IPK proved invaluable in defining the role of C8 in rat MN, one needs to recognize certain potential limitations of the model. First, C_{inulin} and sodium reabsorption in the IPK are subnormal as compared to normal kidneys in vivo and function declines further with time (41). Nevertheless, in our plasma-perfused kidneys, it was possible to

achieve adequate and relatively stable function for 2 h, with preservation of normal glomerular histology. C_{inulin} and sodium reabsorption compared favorably with IPKs perfused with plasma-free perfusates of similar oncotic pressure (41) even though vascular resistance was somewhat elevated and perfusion flow rate proportionately reduced, especially in the presence of

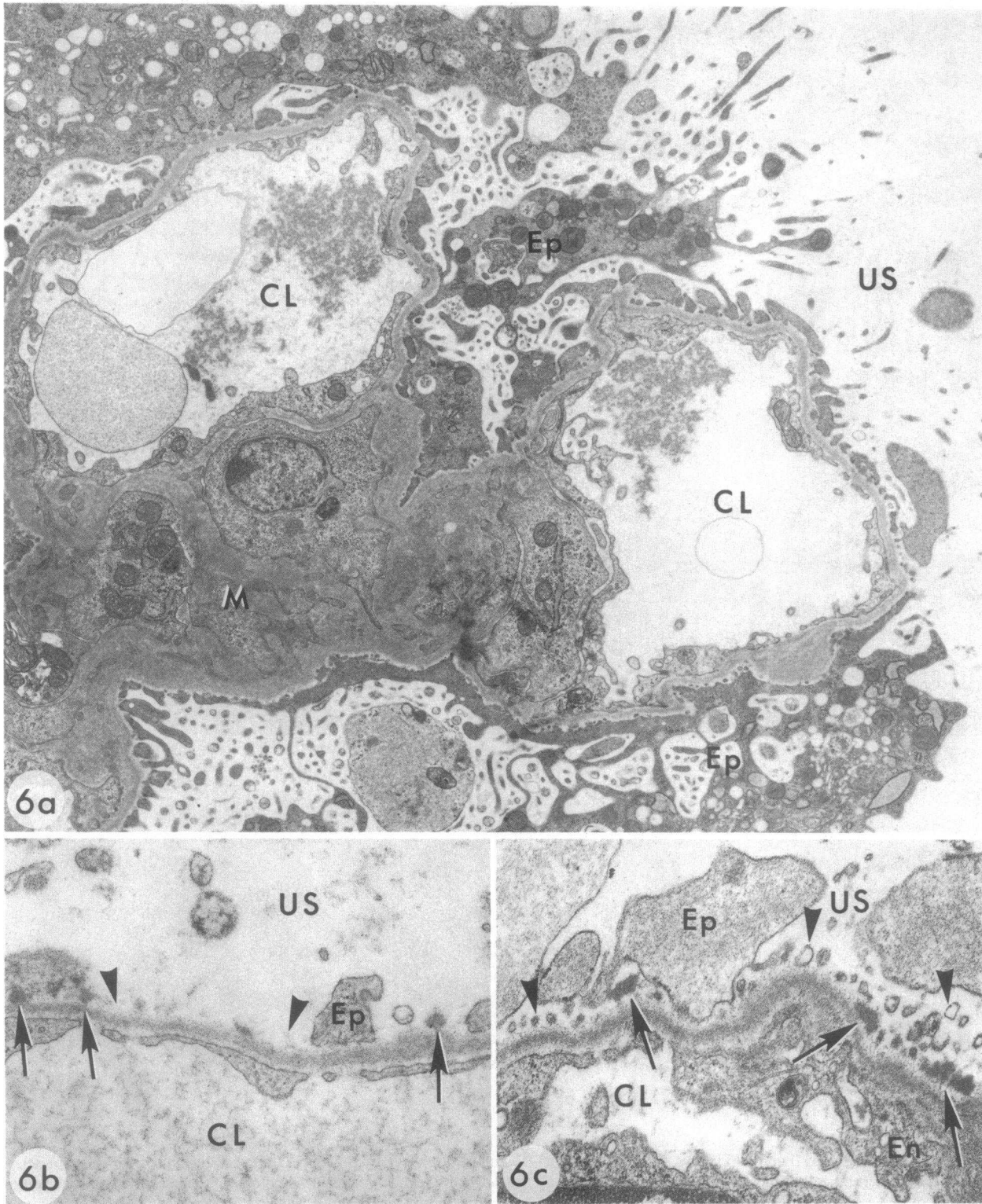


Figure 6. Ultrastructure of kidneys from groups I and C8R. (a) Glomerular capillary loop showing striking epithelial cell abnormalities, with massive effacement of interdigitating foot processes, microvillous transformation and vacuolization ($\times 11,250$). (b) Details of the glomerular capillary wall, showing complete denudation of the basement membrane (arrowheads). Notice also the presence of electron-dense deposits on the epithelial side of the basement membrane (arrows) ($\times 25,000$). (c) Retraction of epithelial cells and formation of small, membrane-bound vesicles (arrowheads) in a C8R kidney; electron-dense deposits (arrows) are present in the lamina rara externa ($\times 24,500$). Ep, epithelial cell; US, urinary space; CL, capillary lumen; M, mesangium; En, endothelium.

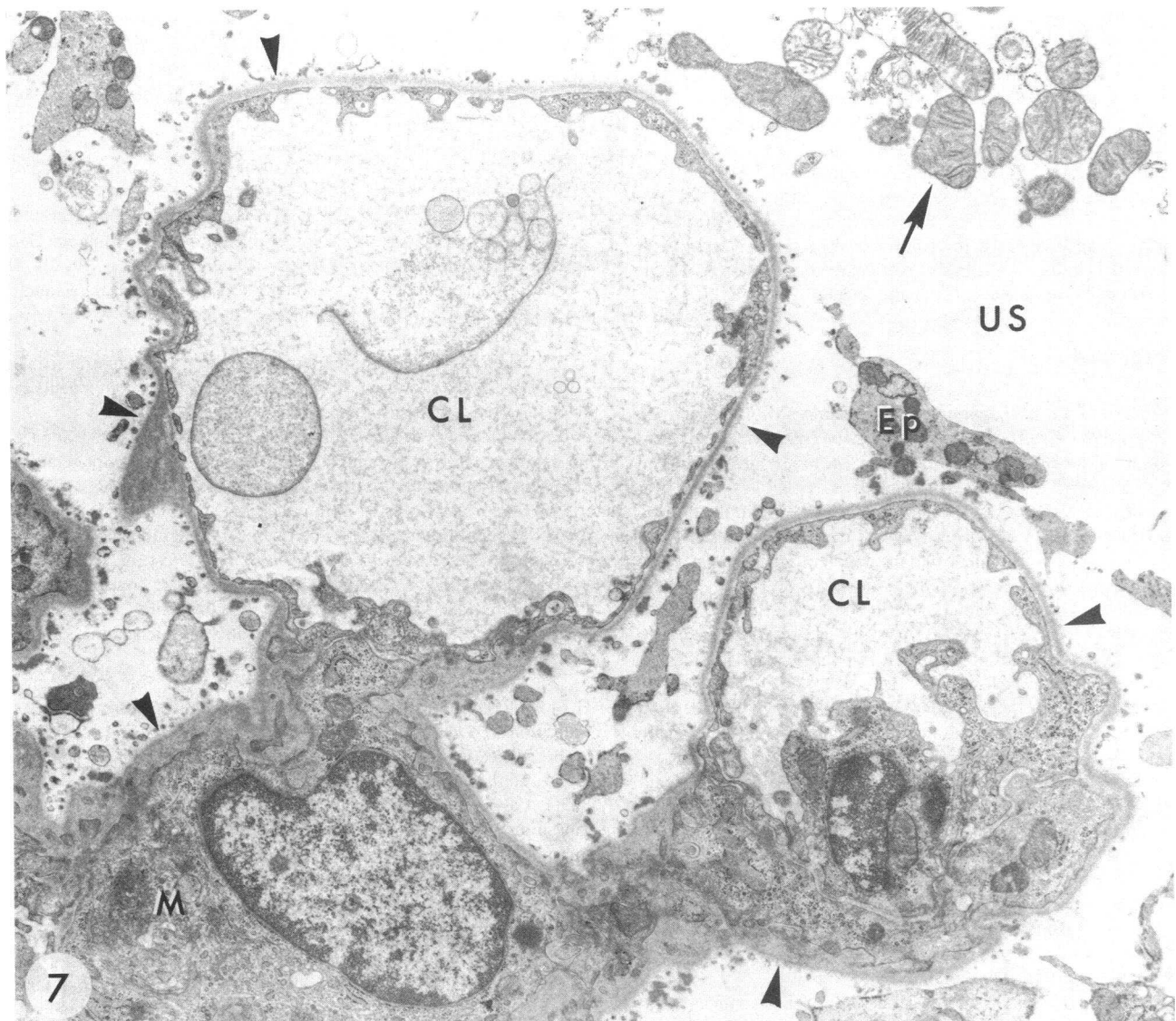


Figure 7. Ultrastructure of group I kidneys. Two capillary loops are completely devoid of glomerular epithelial cell layer. Notice the numerous small membrane-bound vesicles attached to the basement membrane (*arrowheads*). Loose cell organelles in the urinary space

(*arrow*) are indicative of cell disintegration and necrosis ($\times 11,250$). Ep, epithelial cell; US, urinary space; CL, capillary lumen; M, mesangium.

fresh plasma (Table II). This vasoconstriction is at least partly due to angiotensin II generation upon addition of plasma angiotensinogen in the presence of high concentrations of renin (56). It was largely countered by the addition of verapamil in doses that did not independently affect proteinuria or C_{inulin} in studies reported by others (24). Another potential disadvantage to the IPK is the high basal excretory rate of protein (24, 26, 40–42). A substantial proportion of this proteinuria is of glomerular origin (24) even though glomerular ultrastructure is normal (25). Despite this basal proteinuria we were readily able to detect an alteration in glomerular permeability in experimental kidneys perfused with fresh plasma as early as 20 min after the addition of antibody.

In addition to our own observations in rat MN, there now exists a substantial body of information suggesting that C components may directly contribute to cell injury and induce various

forms of tissue damage without invoking the cooperation of classical inflammatory cells. For instance, experimental hyperacute cardiac allograft rejection is C-mediated but cell-independent (57, 58). C6 deficiency partly protects rabbits from cardiac xenograft rejection (59) and heterologous anti-GBM nephritis (60), antibody-directed bone resorption is indirectly mediated by terminal C components (51) and C exacerbates proteinuria when rat kidneys are perfused *in vitro* with anti-GBM antibody in the absence of cells (27). Thus, this study not only provides compelling functional and structural evidence that the MAC is responsible for proteinuria in a model that closely resembles a common form of chronic renal disease in man, it also lends strong support to the notion that the MAC may be responsible for tissue injury in several other clinical and experimental settings. To the best of our knowledge, it is also the first study to indicate that the MAC is responsible for epithelial cell injury.

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