PROTEINURIA AND STRUCTURAL ALTERATIONS IN RAT GLOMERULAR BASEMENT MEMBRANES INDUCED BY INTRAVENOUSLY INJECTED ANTI-LAMININ IMMUNOGLOBULIN G*

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Nephrotoxic nephritis (NTN)[1] is a biphasic disease produced by the intravenous injection of heterologous antisera raised against a variety of complex antigens and tissues (reviewed in 1). In the heterologous phase, the injected nephrotoxic antibodies (NTAb) bind to the glomerular basement membrane (GBM) and cause a dose-dependent proteinuria (2) that is usually accompanied by C3 deposition (3, 4), polymorphonuclear leukocyte (PMN) infiltration (4), endothelial swelling, and widening of the GBM (5, 6). During the subsequent autologous phase, the host animal develops a humoral response against the kidney-bound NTAb (7). This results in the binding of host IgG to the GBM, additional C3 deposition, and the infiltration of mononuclear cells into glomeruli (8), leading to further proteinuria.

The antigenic components within the GBM that bind NTAb have not been clearly identified. We attempted to produce NTN with antibodies directed solely against the basement membrane glycoprotein laminin (9). Laminin is isolated (9) from the Englebreth-Holm-Swarm (EHS) sarcoma, a murine tumor that also secretes basement membrane collagen (10). Additionally, laminin has been shown by immunological methods to exist in a variety of basement membranes, including the GBM (11–17). Thus, with affinity-purified sheep anti-laminin IgG, we sought to determine (*a*) the distribution and amount of kidney-bound anti-laminin after intravenous administration into rats and (*b*) what effects anti-laminin would have in vivo on the normal structure and function of the glomerulus.

Materials and Methods

Purification and Characterization of Laminin. The EHS chondrosarcoma was maintained by serial subcutaneous passage in Swiss-Webster mice. Laminin was purified by a modification of

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[1] *Abbreviations used in this paper:* DAB, diaminobenzidine; EHS, Englebreth-Holm-Swarm sarcoma; GBM, glomerular basement membrane; NTAb, nephrotoxic antibodies; NTN, nephrotoxic nephritis; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; SαL, affinity-purified sheep anti-laminin IgG; SαT, sheep anti-tumor IgG after the immunoadsorption of SαL; TBM, tubular basement membrane.

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the method of Timpl et al. (9). The 0.5 M NaCl tumor extract was applied to DEAE-cellulose equilibrated in deionized 2 M urea in 0.05 M Tris, pH 8.6, and the column was washed with the same buffer. A fraction of the bound proteins containing laminin was then eluted with the addition of 0.15 M NaCl in buffer and chromatographed on agarose A 1.5 M (Bio-Rad Laboratories, Richmond, CA) to obtain pure laminin. Electrophoresis was carried out in 5% polyacrylamide tube gels (18). The amino acid composition of the purified laminin was generously determined by Dr. Steven Krane of the Massachusetts General Hospital, Boston, MA. Laminin was also tested for cross-reactivity with anti-guinea pig fibronectin IgG that was a gift from Dr. Heinz Remold of our department.

Preparation of Sheep Anti-Laminin IgG (SαL). Two male sheep were immunized against an EHS sarcoma homogenate emulsified in Freund's adjuvant, and IgG was purified by standard procedures. The monospecific anti-laminin fraction was obtained by passing the IgG over a column of cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) to which laminin had been coupled. The sheep IgG that failed to bind to laminin-Sepharose (SαT) was pooled and used in control experiments. Bound sheep anti-laminin (SαL) was eluted with 1 M propionic acid, pH 2.6, and dialyzed against phosphate-buffered saline (PBS). The SαL was centrifuged to remove aggregates, and the range of isoelectric points of the SαL was determined by agarose gel isoelectrophoresis as suggested by LKB (LKB, Stockholm, Sweden).

Distribution of Intravenously Injected SαL by Immunofluorescence and Immunoelectron Microscopy. The distribution of kidney-binding sites for SαL after intravenous injection was examined by indirect immunofluorescence microscopy. 12 rats weighing 150–200 g received injections of 0.04–4 mg of SαL in 1–2 ml of PBS. A total of six animals that received injections of normal sheep IgG (2), SαT (2), saline (1), or that were sham operated (1) served as controls. 1–24 h after the injection, kidneys were removed and frozen sections 4–6 μm thick were cut at −20°C and air dried. Sections were incubated with fluorescein-conjugated rabbit anti-sheep IgG (N. L. Cappel Laboratories, Inc., Cochranville, PA) and washed with PBS.

The distribution of kidney-bound SαL was also determined by direct immunoelectron microscopy. SαL was conjugated to horseradish peroxidase (HRP) (type VI; Sigma Chemical Co., St. Louis, MO) (19), dialyzed against PBS, and the isoelectric range was measured as above. SαT was also conjugated to HRP and used in control experiments. Six male Sprague-Dawley rats, 125–175 g, were anesthetized, and 1-ml solutions of SαL-HRP that contained 0.65 mg SαL bound to 0.5 mg HRP were injected intravenously. SαT-HRP of the same concentration was injected into four additional animals. 1–2 h after injection, the left kidney of each animal was fixed *in situ* (20) with 1% formaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 (21). Sections of cortex 40-μm thick were incubated in 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2 (22), pH 6.0, washed in buffer, and post-fixed in 2% OsO_4 in 0.1 M phosphate, pH 7.3. Thin sections were stained for 1 min with lead citrate (23).

Quantitation of Intravenously Injected SαL Bound to the Kidney. SαL was radiolabeled with ^{125}I using the Iodo-gen reagent (Pierce Chemical Co., Rockford, IL) (24), and unbound ^{125}I was removed by gel filtration. Concentrations of 0.04–4 mg of SαL that contained 100,000 cpm of [^{125}I]SαL were injected intravenously into 23 150-g Sprague-Dawley rats. 1 h later, samples of blood and both kidneys from two animals were removed, weighed, and counted for ^{125}I. The amount of SαL bound per kidney was determined by the following formula: (mg SαL injected per animal) (percent of injected SαL in kidney)/(weight of kidney), and expressed as μg SαL/g kidney tissue. The remaining 21 animals were killed and counted 4 d later.

Evaluation of Rats That Received Intravenous SαL Injections. To evaluate the effect of intravenously injected SαL on kidney function, rats that had received injections of 0.5–4 mg of SαL or SαT were maintained for 24-h periods in metabolic cages up to 10 wk after injection. The amount of urinary protein (Table I) was determined by the Biuret assay, and differences between the SαL and SαT rats were analyzed for statistical significance by a one-tailed Student's *t* test. Rats were killed at intervals (Table II), and their kidneys were examined for the continued presence of SαL and also for the deposition of rat C3 and rat IgG by indirect immunofluorescence. In addition, sera taken at the time of killing was tested for the presence of rat anti-sheep IgG by double immunodiffusion.

Kidneys from rats that had received injections of SαL or SαT up to 10 wk previously were

TABLE I

*Urinary Protein Excretion (mg/24 h ± SEM)**

SαL injected	Day								
	1	5	7	14	21	28	42	50	70
mg									
0‡	5.8 ± 0.5 (11)	8.4 ± 0.9 (5)	7.1 ± 0.1 (2)	11 ± 0.2 (2)	8.8 ± 2.4 (6)	6.3 (1)	11.7 ± 1.2 (3)	5 ± 0.4 (2)	15 (1)
0.5–1.6	4.9 ± 0.2 (17)	6.2 ± 2.2 (3)	—	—	5 ± 0.3 (9)	—	—	5 ± 0.1 (4)	—
4.0	7.4 ± 1.9 (7)	13.5 ± 4.2 (4)	9.3 ± 0.6 (3)	9.8 ± 1.3 (3)	16.4 ± 1.1 (3)	16.6 ± 2.3 (3)	27.7 ± 5.3 (6)	—	28.7 ± 0.9 (3)

* The number of rats measured at each time point is shown in parentheses.
‡ All rats in this category received 0.5–4.0 mg SαT.

TABLE II

Number of Animals Sampled for Immunofluorescent and Electron Microscopy

Treatment	Time of sampling								
	1–2 h	1 d	5 d	14 d	21 d	28 d	42 d	56 d	70 d
0.5–1.6 mg SαL	4 (8)	—	(4)	4	5	2	—	2	—
1.5 mg SαT	1 (5)		(2)	1	1	1	—	1	—
4 mg SαL	—	2	—	—	—	—	3	—	3
4 mg SαT	—	1	—	—	—	—	1	—	1
Uninjected	2								

Kidneys from animals that were examined by immunofluorescent microscopy only are shown in parentheses. All others were examined by both fluorescence microscopy and electron microscopy.

also evaluated at intervals by electron microscopy (Table II). Blocks of kidney cortex that were fixed in aldehydes as described above were post-fixed in 1% OsO_4 in veronal-acetate buffer, pH 7.4, and stained en bloc with uranyl acetate (25). Thin sections were stained for 5 min each with uranyl acetate and lead citrate (23).

Results

Characterization of Laminin and SαL. The yield of laminin was ~90 mg from 100 g wet weight of the EHS tumor. The reduced protein migrated as two prominent bands of ~440,000 and 220,000 M_r in sodium dodecyl sulfate gels (Fig. 1), as previously reported (9). Other clearly defined species were not seen, even when the gels were grossly overloaded with 300 μg of protein. When unreduced, laminin barely entered a 5% polyacrylamide gel.

The amino acid composition of this laminin preparation is shown in Table III and is similar to that published previously (9), although we detected higher amounts of half-cysteine and isoleucine, a lower amount of glycine, and no hydroxylysine. Both laminin preparations differ considerably from the amino acid composition of collagen type IV (26) and fibronectin (27) (Table III). Finally, IgG that was purified from antisera against fibronectin failed to bind purified laminin, as shown by others (9, 11).

Monospecific sheep anti-laminin IgG (SαL) was purified by immunoabsorption of sheep anti-tumor IgG on laminin-Sepharose. By Ouchterlony immunodiffusion, SαL gave a single precipitin band of identity when developed against either pure laminin or the crude laminin extract (data not shown). The isoelectric points of SαL ranged from 4.6–7.1 (Fig. 2), as did the majority of IgG in the sheep anti-tumor preparation.

Localization of Kidney-binding Sites for Intravenously Injected SαL. Within 1 h after injection of SαL, sheep IgG was localized in both GBM and TBM by immunofluo-

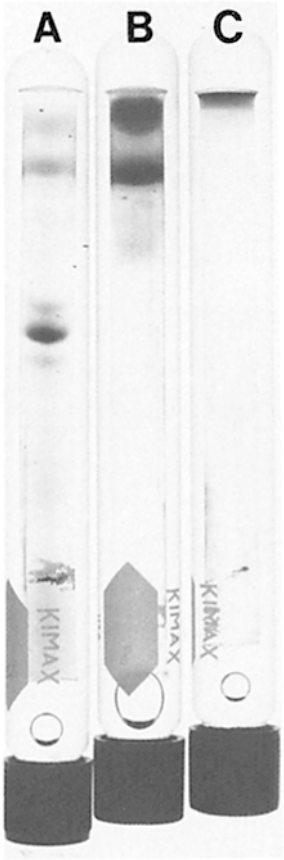


FIG. 1. 5% polyacrylamide gels of laminin preparations. Tube A contains reduced proteins eluted from the DEAE column in 0.15 M NaCl, 2 M Urea, and 0.05 M Tris, pH 8.6. Tube B contains reduced laminin obtained at the end of purification. The upper band is ~440,000 mol wt and the lower is 220,000 mol wt. Smearing below the lower band is only seen when high sample loads; in this case 300 μg is applied to the gel (compare with tube A). Other molecular weight species are not seen. Tube C contains unreduced laminin (300 μg) that barely enters the gel.

rescence (Figs. 3 and 4). The GBM in all glomeruli stained intensely but the binding of SαL to the TBM was focal and occurred only in rats that received injections of >0.5 mg SαL (Fig. 4). Throughout the GBM and when present in the TBM, the binding was linear in all 12 rats examined. Binding to the tubular epithelium was not observed, and SAL was not present within the urinary spaces. When normal sheep IgG or SαT was injected into each of six rats, no binding to any kidney structure was seen.

The distribution of SαL was also examined by electron microscopy. 1–2 h after the injection of SαL-HRP conjugates (pI 4.6–7.1; Fig. 2), peroxidase reaction product was found throughout the full thickness of the GBM and mesangial matrix (Fig. 5). Endocytic uptake of SαL by mesangial, endothelial, or epithelial cells was not observed, however. Peroxidase was also not seen within the urinary space nor within tubular epithelial cells. SαL-HRP was bound, however, to some areas of the TBM. In

TABLE III
Amino Acid Composition

	Laminin	Laminin*	Human fibronectin‡	Type IV collagen§
Asp	125	109	92	53
Thr	52	58	97	34
Ser	72	77	68	57
Glu	117	122	116	102
Pro	49	53	76	65
Gly	79	93	80	281
Ala	72	76	43	44
Half-Cys	52	30	26	7
Val	40	48	81	28
Met	12	14	11	13
Ile	67	42	44	25
Leu	83	92	57	52
Tyr	30	27	45	9
Phe	29	31	27	29
Lys	48	52	36	11
His	23	24	21	11
Arg	49	50	52	32
Hyl	0	2	0	37
3-Hyp	0	0	0	6
4-Hyp	0	0	0	104
Trp	ND‖	ND	ND	ND

* From reference 9.
‡ From reference 27.
§ From reference 26.
‖ Not determined.

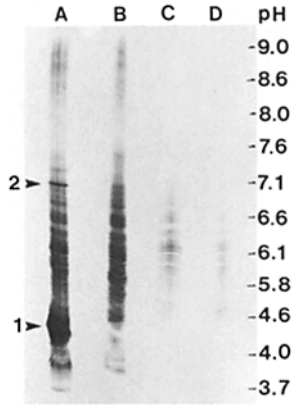


FIG. 2. Isoelectric focusing in agarose. Lane A, sheep anti-tumor homogenate serum; lane B, anti-tumor homogenate IgG; lane C, SαL; lane D, SαL-HRP. The isoelectric points of SαL and SαL-HRP range from 4.6–7.1. Sheep albumin (1) and hemoglobin (2) serve as internal standards.

control animals that received injections of SαT-HRP, peroxidase was not present within the GBM, mesangial matrix, (Fig. 6) or TBM.

Quantitation of Kidney-bound [^{125}I]SαL. When injected intravenously into rats, [^{125}I]SαL rapidly bound to the kidney, and ~1.6% of an administered dose was measured within each kidney after 1 h. 4 d later, the amount of kidney-bound [^{125}I]-SαL was ~2% of the injected dose over the range of 0.04–0.95 mg and equaled

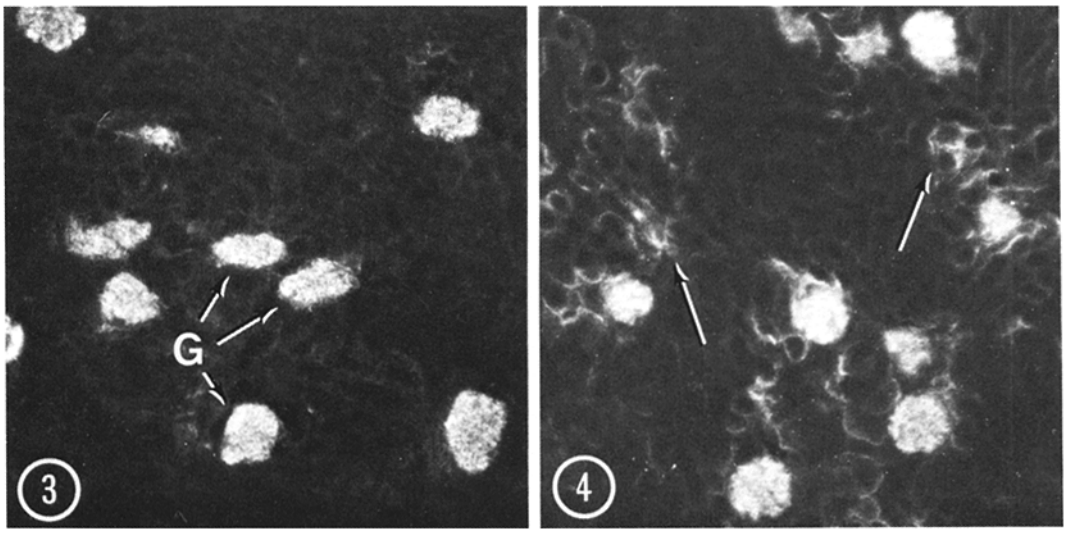


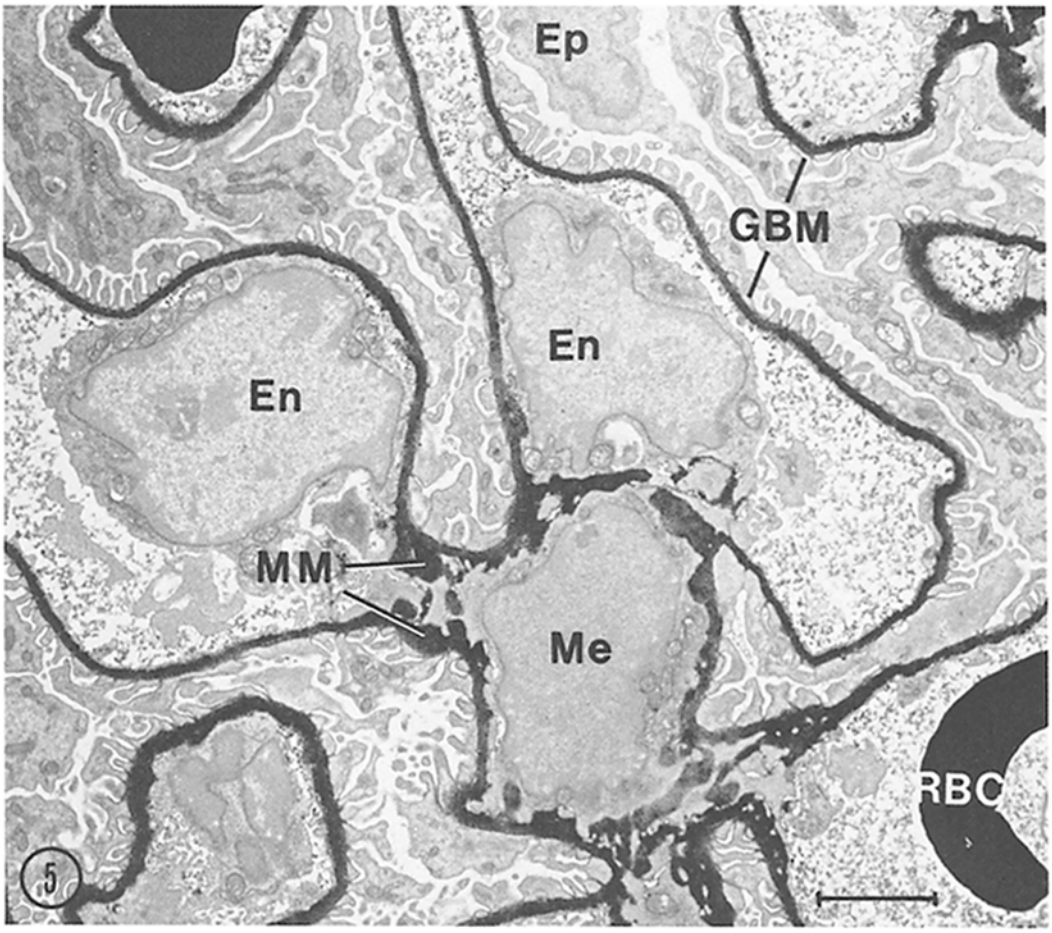
FIG. 3 and 4. Fluorescence micrographs of the renal cortex from rats that had received intravenous injections of SαL 60 min previously. The frozen sections were stained with fluorescein rabbit anti-sheep IgG. Fig. 3 is taken from a rat that had received 0.4 mg SαL. The glomeruli (G) stain intensely but little staining of the TBM is evident. Fig. 4 is taken from an animal that had received 4 mg SαL. The glomeruli fluoresce intensely and areas of the TBM (arrows) also stain for sheep IgG at this higher dose (compare with Fig. 3).

0.65–19 μg/g kidney (Fig. 7). In contrast, when 4 mg of [^{125}I]SαL was injected, 62 μg/g kidney or 1.5% of the administered dose was bound. The amount of unbound [^{125}I]SαL within the renal circulation was calculated to be <3% of the bound SαL and therefore was ignored.

Acute Effects of SαL. Rats that received 0.5–1.6 mg SαL intravenously had the same urinary protein concentration as normal and SαT-injected rats (4.9–8.4 mg/24 h) for the first 5 d after injection (Table I). Those animals that received 4 mg SαL averaged 7.4 mg during the first 24 h and 13.5 mg/24 h 5 d later, but these values did not differ significantly from the controls ($P > 0.05$).

Immunofluorescent examination of kidneys from all animals that received SαL yielded similar results for the distribution of SαL, rat C3, and rat IgG from 1 h to 5 d after injection. Over this time period, SαL remained bound to the GBM and, in doses above 0.5 mg, to the TBM in a linear pattern. Rat C3 was present within some glomeruli in a few animals but was completely absent in most (Fig. 8 a, b). Portions of the TBM stained for C3 in all animals (Fig. 8 b). In contrast, homologous rat IgG was not present in the GBM or TBM of any rat. Control rats receiving SαT did not have renal-bound sheep IgG or rat IgG, but rat C3 was again occasionally found bound to the TBM. Electron microscopic examination revealed normal glomerular architecture and no evidence of cellular infiltration 1 h–1 d after the injection of SαL or SαT.

Long-Term Effects of SαL. At longer intervals after SαL injection, all rats appeared healthy, and their kidneys were grossly normal. Proteinuria was not observed in any rat receiving <1.6 mg SαL up to 50 d after injection (Table I). In contrast, rats that received 4 mg SαL became mildly proteinuric within 3 wk (16.4 mg/24 h). Mean



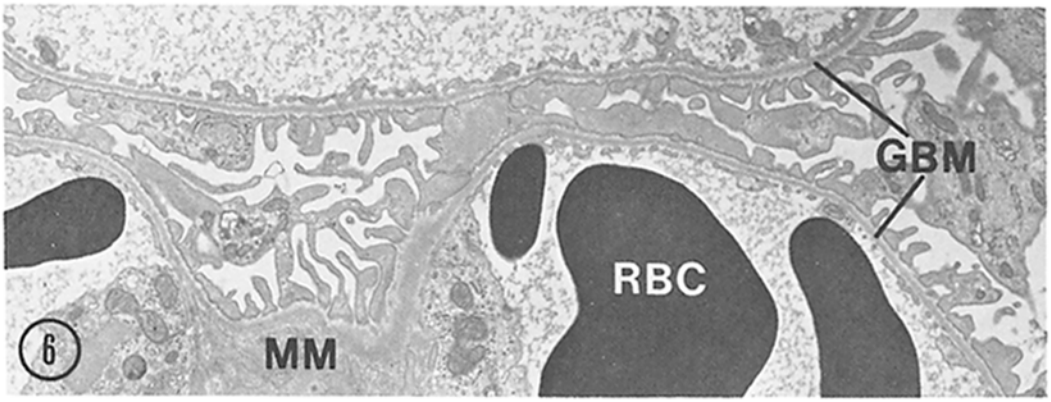


FIG. 5. Electron micrograph of a portion of a glomerulus from a rat that received an intravenous injection of SαL-HRP 1 h before killing. Peroxidase reaction product is present throughout the full thickness of the GBM and mesangial matrix (MM). The endocytic uptake of HRP by mesangial (Me), endothelial (En), or epithelial cells (Ep) is not seen. Erythrocytes (RBC) stain intensely for HRP because of the peroxidatic activity of hemoglobin. Magnification, 7,600×. Scale bar, 2 μm.

FIG. 6. Glomerulus from a control rat that had received SαT-HRP 1 h before fixation. Peroxidase is absent from the GBM and mesangial matrix (MM). Deeply stained erythrocytes (RBC) assure adequate penetration of H_2O_2, DAB, and OsO_4. Magnification, 7,600×.

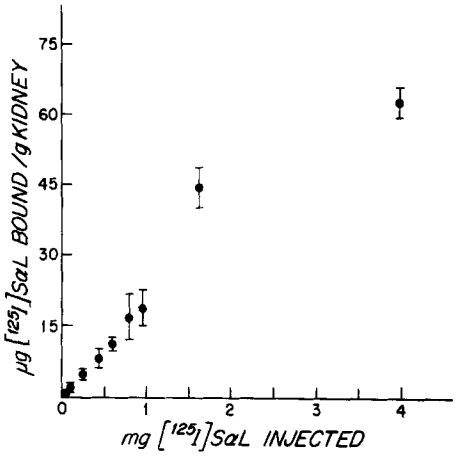


FIG. 7. Plot of [^{125}I]SαL binding to the kidney 4 d after injection. The amount of kidney-bound SαL increases proportionally to the injected dose over the dose range of 0.04–0.95 mg and averages ~2%. The amount of kidney-bound SαL at 4 mg, however, is 1.5% of the injected dose.

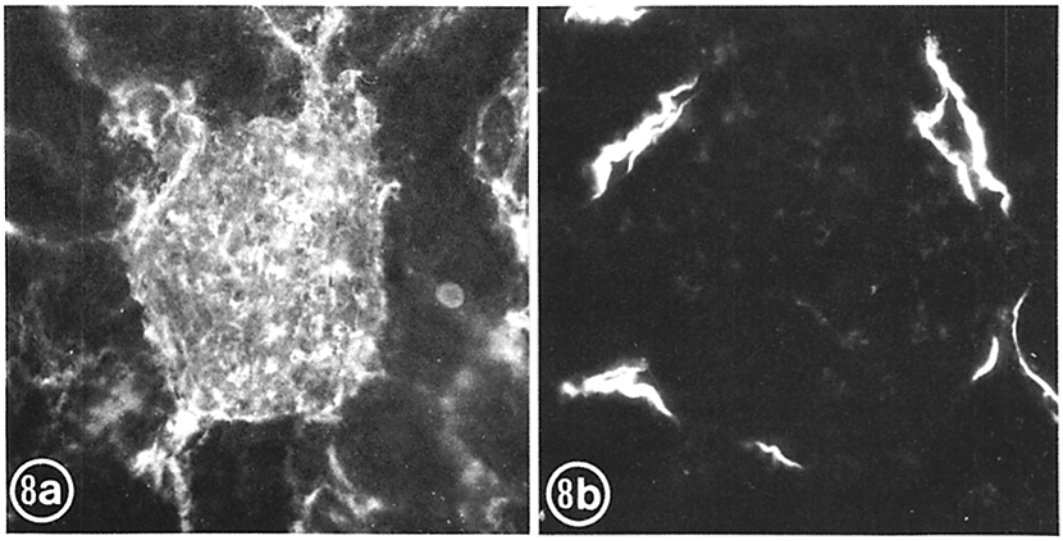


FIG. 8. Frozen sections from the kidneys of SαL-injected rats that were stained with fluorescein-goat anti-rat C3. (a) kidney from a nonproteinuric rat 5 d after the injection of SαL. The glomerulus and adjacent TBM fluoresce intensely. (b) in contrast, the animal shown was injected with 4 mg SαL 10 wk previously and was proteinuric. There is only trace staining of the glomerulus and intense focal staining of the TBM. Because similar staining was also seen in normal rats, SαT-injected rats, and most rats within the first 5 d after SαL injection, this pattern probably represents background. (a) Magnification, 350×; (b) Magnification, 500×.

urinary protein excretion increased to 27.7 mg/24 h at 6 wk, and this condition persisted until 10 wk (28.7 mg/24 h). The differences between the proteinuric and control rats were statistically significant ($P < 0.05$) at the 3- and 6-wk time points and, when the urinary protein readings from the rats that received 4 mg SαL at all time points were pooled and compared with the controls, over the entire study ($P < 0.001$).

Kidneys were examined as before for the presence of sheep IgG, rat C3, and rat IgG. Large amounts of sheep IgG remained bound in the GBM and TBM until killing in all animals that received SαL injections. However, the distribution of bound SαL changed over time, and the amount of SαL present in the peripheral GBM appeared to diminish progressively (compare Fig. 9 a and 9 b). In contrast, mesangial areas stained intensely for sheep IgG 10 wk after SαL injection (Fig. 9 b), whereas only moderate mesangial staining was noted in animals that were examined after 1 d (Fig. 9 a). Rat C3 was again observed bound to the GBM and TBM in some but not all rats that received SαL, as noted in the acute time period. No correlation was seen between C3 deposition and proteinuria, however, because most mildly proteinuric animals lacked C3 in their glomeruli and some nonproteinuric rats had focal C3 deposition. Homologous rat IgG, in contrast, was not detected bound to the GBM or TBM at any time in any animal throughout the experiment. In addition, sera from the proteinuric animals failed to immunoprecipitate SαL or normal sheep IgG. At all time points, neither sheep nor rat IgG was present in the kidneys from control rats that received SαT. Variable, focal staining of the TBM for rat C3 was again observed.

Kidneys from 19 rats that had received injections of 0.5–4 mg SαL 2–10 wk previously (Table I) were examined by electron microscopy. In all of these, the glomeruli contained focal abnormalities that were not evident when examined by light microscopy and not present in control or normal rats.

The most prominent and frequent change seen in all animals that received SαL

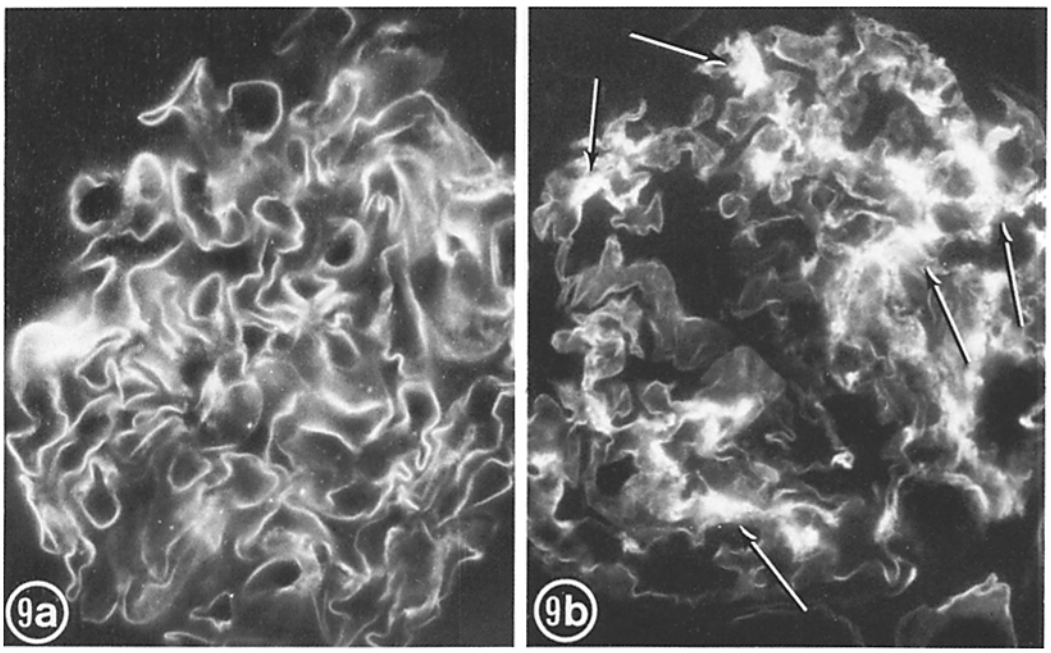


FIG. 9. Fluorescence micrographs of glomeruli from rats that received 4 mg SαL and that were stained for the presence of sheep IgG. (a) 1 d after SαL injection, sheep IgG is distributed in a linear pattern throughout the GBM. (b) 10 wk after SAL injection, the GBM does not stain with the same intensity as seen in part a. In contrast, the mesangial regions of the glomerulus (arrows) fluoresce intensely, suggesting the SαL moves from the GBM to the mesangium with time. Magnification in a and b, 900×.

was GBM thickening. Flocculent, subendothelial deposits were seen in the GBM 2 wk after the injection of SαL, and by 6 wk, focal areas of greatly increased width measuring as much as 1.5 μm (normal is 0.15 μm; ref. 20) were present in the peripheral capillary (Fig. 10 a). These deposits contained 250 Å fragments of membrane, and projections of the endothelial cytoplasm extended into them (Fig. 10 a). In addition to deposits within the lamina rara interna, the width of the lamina densa was often increased (Fig. 10 a), and in some areas the lamina densa was split or reduplicated into as many as four distinct layers (Fig. 10 b). The lamina rara externa, in contrast, appeared normal in all animals, and subepithelial deposits were not seen.

The second most common finding was the presence of small numbers of mononuclear cells within the glomerular capillary lumen. This was seen in all SαL-injected rats and appeared as early as 2–3 wk after injection. The mononuclear cells were often attached to the peripheral capillary wall at the point of subendothelial deposits (Fig. 11). Pseudopodia extended beneath the endothelium, and vesicles containing electron-dense material were present within the monocyte cytoplasm adjacent to the deposits. In other areas, larger pseudopodia extended for several microns beneath the endothelium and appeared to displace the endothelium from the GBM (Fig. 12). This might have resulted in the complete removal of portions of the endothelium because areas of the GBM were seen that were denuded of endothelium (Fig. 13). PMN were rarely observed within glomeruli at any time point and were never seen attached to the capillary wall.

Minor changes were seen in the mesangium and glomerular epithelium. Electron-dense deposits were not present within the mesangial matrix. At 6 and 10 wk after the injection of 4 mg SαL, however, some glomerular capillaries in all rats were filled with mononuclear cells, but a clear distinction between mesangial cells and infiltrating mononuclear cells was not possible (Fig. 14). In the epithelial cells, absorption droplets or lysosomes were present in the cytoplasm (Fig. 15), slit diaphragms were displaced (Figs. 10 b and 11), and surface microvilli protruded into the urinary space (Fig. 10 a).

Normal, uninjected animals and animals injected with SαT had none of the changes described above. In addition, the morphology of the tubular epithelium and the ultrastructure of the TBM appeared normal in all SαL- and SαT-injected animals.

Discussion

These studies demonstrate that, after intravenous injection into rats, affinity-purified SαL rapidly bound throughout the GBM. The bound SαL, at concentrations that appeared to saturate the glomeruli, failed to cause significant proteinuria within the first 5 d. Rats that received a single injection of 4 mg SαL, however, gradually developed a mild proteinuria over a 3–10 wk period that appeared to be glomerular in origin because structural changes were confined to the glomerulus. The proteinuria might have been caused by an irregular, increased thickening of the GBM, mononuclear cell infiltration, or the stripping of the endothelium from the GBM. However, these lesions did not result from a host humoral response typical of the autologous phase of NTN (3, 7) because rat IgG was not present within the GBM and rat anti-sheep IgG was not detected in the serum of affected animals. These results therefore resemble the proteinuria and structural changes seen in the autologous phase of NTN, but dissociate these changes from the earlier or heterologous phase and the subsequent humoral response.

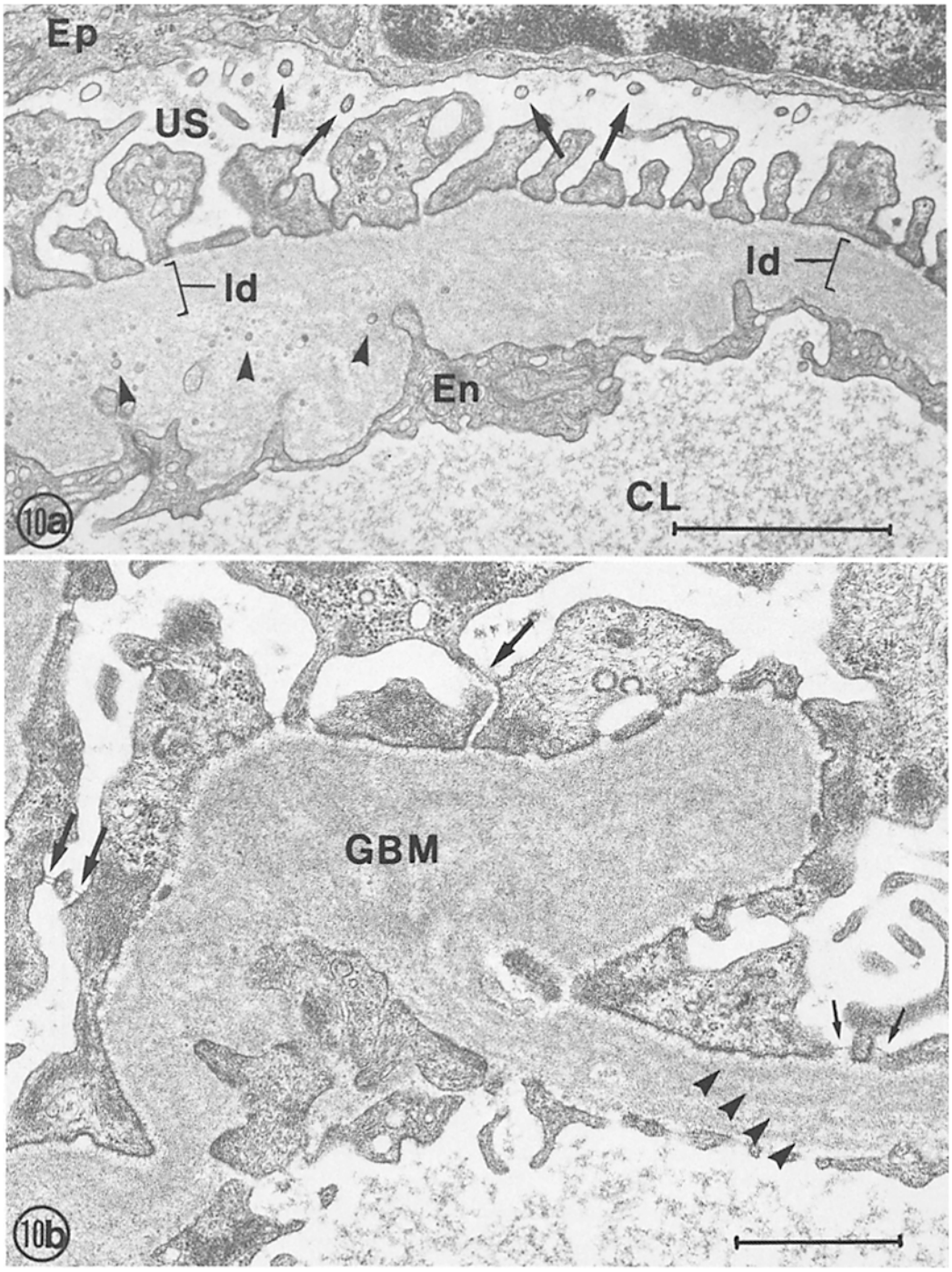


FIG. 10. Examples of thickened GBM from rats that received 4 mg SαL 6 wk before killing. (a) the endothelium (En) has few fenestrations and extends spike-like projections between subendothelial deposits. The deposits contain spherical membrane fragments (arrowheads). The lamina densa (l d) is abnormally wide, measuring 270 nm. Microvilli (arrows) extend from the epithelium (Ep) into the urinary space (US). CL, capillary lumen. Magnification, 31,500×. Scale bar, 1 μm. (b) a large knuckle of thickened GBM is seen in the center of the picture (GBM), and the GBM is split into four distinct layers on the right (arrowheads). Some of the epithelial slit diaphragms (large arrows) are displaced from the GBM. Normally located diaphragms are on the right of the micrograph (small arrows). Magnification, 24,000×. Scale bar, 1 μm.

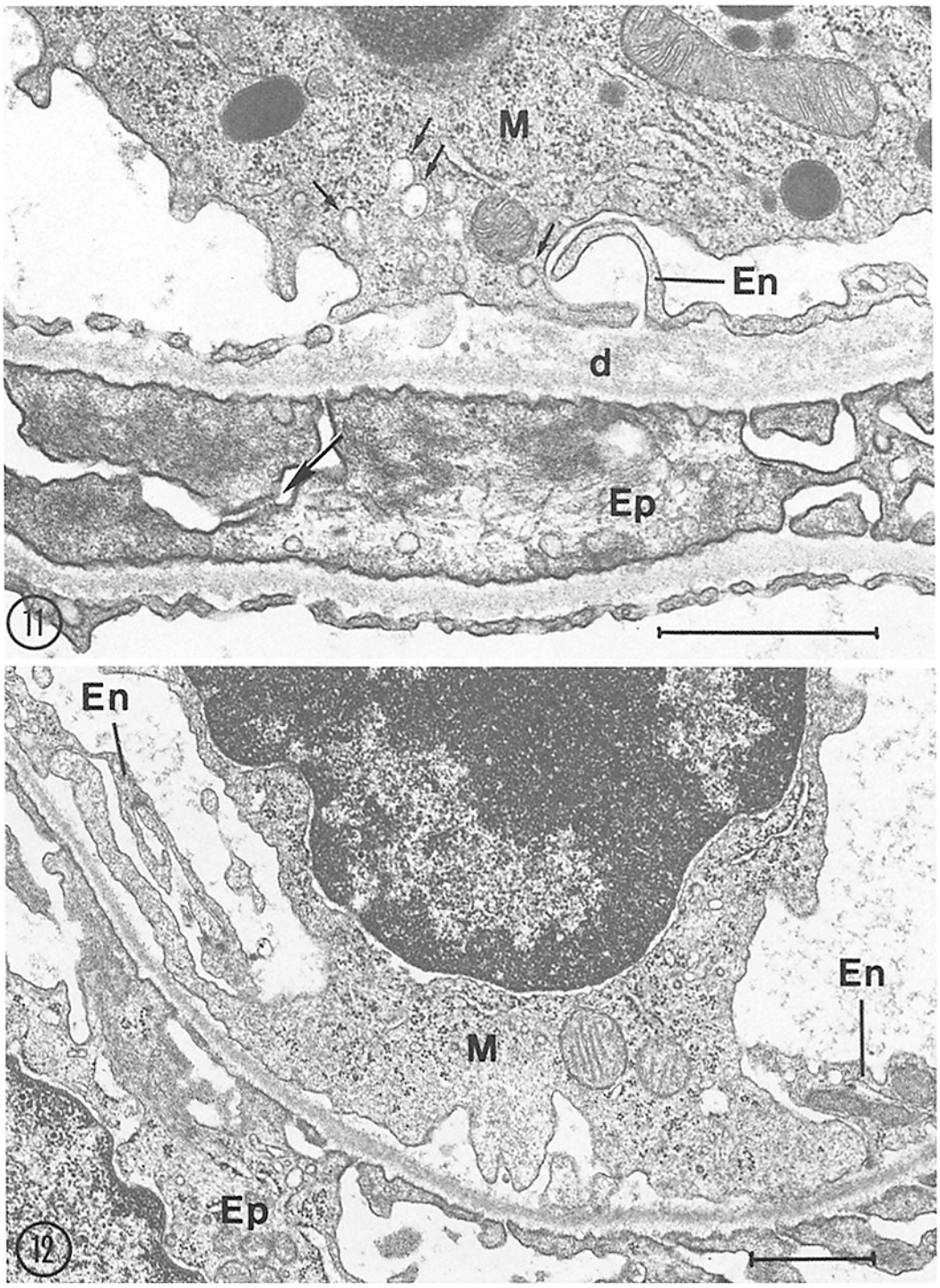
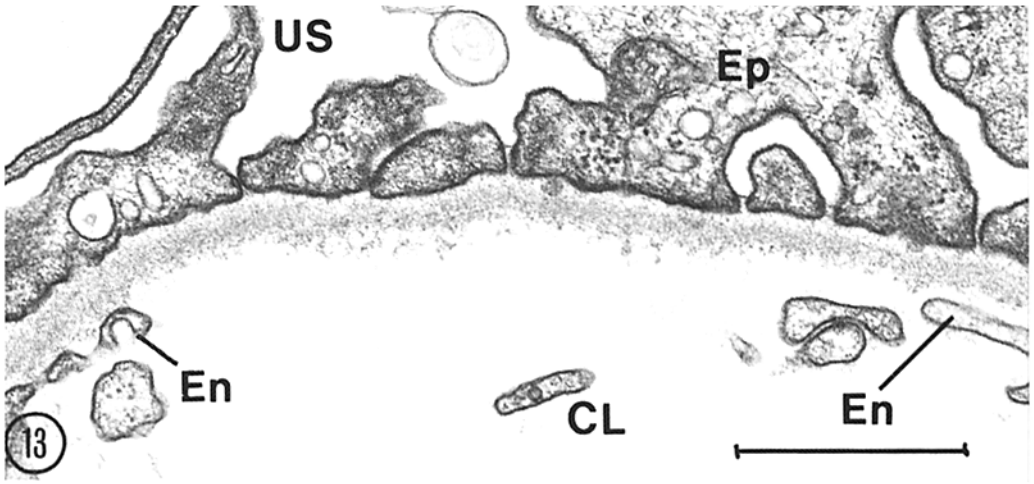


FIG. 11. Monocytic cell attached to the peripheral capillary wall from a rat injected with 1.6 mg SαL four wk before killing. The monocyte (M) is extending a pseudopod beneath the endothelium (En) and abutting on the subendothelial deposit (d). Small intracellular vesicles (small arrows) within the monocyte contain material similar in electron density to the deposit. There is a displaced slit diaphragm (arrow) between two epithelial cells (Ep). Subendothelial deposits are not observed within the apparently normal GBM at the lower part of the micrograph. Magnification, 32,500×. Scale bar, 1 μm.

FIG. 12. Mononuclear cell (M) attached to the GBM from a rat that received 4 mg SαL 10 wk earlier. Long pseudopodia have inserted beneath the endothelium (En) and have displaced a broad region of the endothelium from the GBM. Ep, epithelium. Magnification, 18,500×. Scale bar, 1 μm.



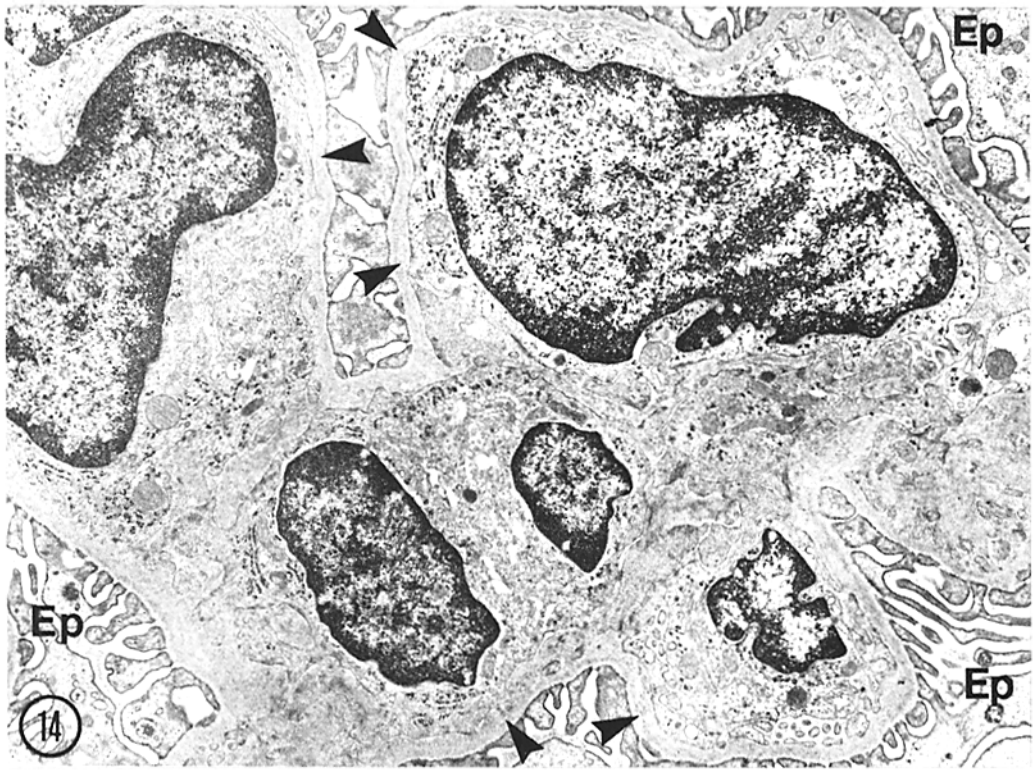


FIG. 13. Peripheral glomerular capillary from a rat that received 4 mg SαL 6 wk before killing. The endothelial cell layer (En) is absent from the GBM over a span of >3 μm. This is possibly because of the earlier infiltration of mononuclear cells, such as is shown in Figs. 11 and 12. US, urinary space; CL, capillary lumen. Magnification, 31,000×. Scale bar, 1 μm.

FIG. 14. Portion of a glomerulus from a rat that received an injection of 4 mg SαL 10 wk before killing. The capillary loops are congested with large, mononuclear cells. These cells abut the GBM in several broad areas (arrowheads). Ep, epithelium. Magnification, 8,300×.

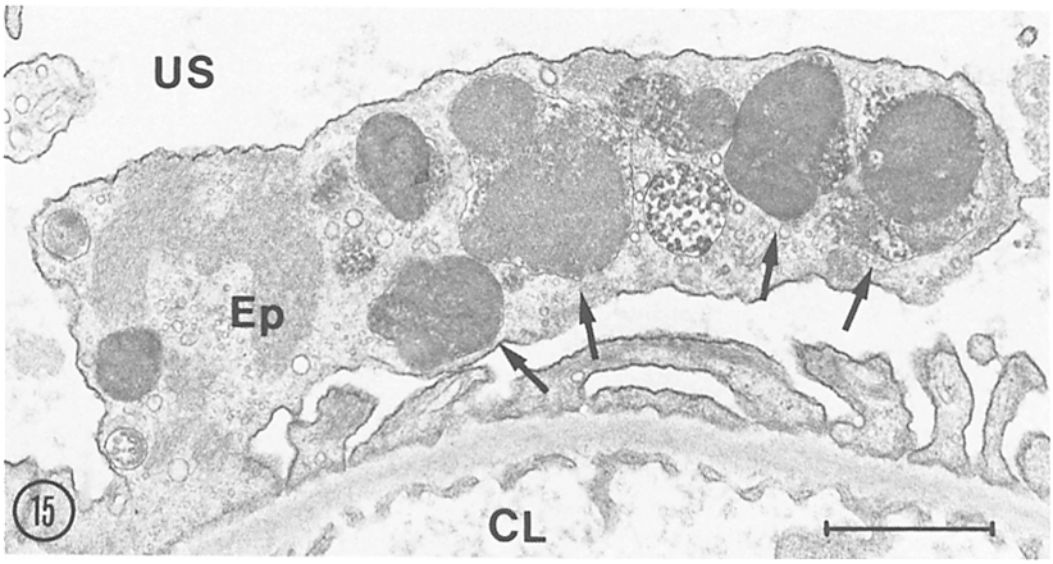


FIG. 15. Micrograph from a proteinuric rat that had received 4 mg SαL 10 wk previously. Numerous absorption droplets or lysosomes (arrows) are present within the epithelial cell (Ep). CL, capillary lumen, US, urinary space. Magnification, 22,000×. Scale bar, 1 μm.

The binding of SαL to the GBM, TBM, and mesangium was highly immunospecific because antibody from the same sheep IgG preparation that failed to bind laminin-Sepharose (SαT) did not bind to any renal structure. Nonspecific ionic interactions of SαL with the fixed negative charges of the GBM (28), such as that seen in the glomerular binding of cationized ferritin (29, 30), were also unlikely because the isoelectric points of SαL measured from 4.6–7.1. In addition, SαL bound very rapidly to kidney basement membranes because 80% of the amount bound at 4 d was bound within the 1st h after injection. A similar rapid binding is seen for heterologous nephrotoxic antibodies (NTAb) (2) that are directed against complex mixtures of kidney antigens.

The binding of monospecific SαL to the kidney differed from the binding of polyspecific NTAb in several respects, however. First, the binding of SαL was more diverse because, at injected doses above 0.5 mg, SαL bound to both GBM and TBM, whereas NTAb apparently binds exclusively to the GBM, even at doses that saturate the kidney (1, 2). Second, glomerular laminin apparently became saturated with SαL at injected doses of 0.5 mg, because, at high doses, SαL also bound to the TBM without an apparent increase in the immunofluorescence of the glomeruli. Third, if the glomerular binding was indeed saturated at 0.5 mg of injected SαL, the maximum amount of SαL bound to glomeruli at this and higher doses was 10 μg/g kidney. This amount is far less than the 175 μg of glomerular-bound NTAb thought necessary to cause immediate proteinuria (1, 2) and might explain why injections of up to 4 mg SαL did not induce acute damage. However, the fact that SαL did not consistently induce complement deposition in the GBM or stimulate PMN infiltration, both of which have been shown to cause proteinuria after the injection of NTAb (1, 3, 4), might be equally important. The lower levels of SαL binding and variable C3 deposition might also have accounted for the failure to generate subendothelial

deposits, GBM thickening, and endothelial exfoliation in the first few days after injection, as occurs in the heterologous phase of NTN (5, 6).

The etiology of the proteinuria in rats that received 4 mg SαL and started 3–6 wk after the injection of 4 mg SαL is unclear. The lesion was not caused by the glomerular deposition of homologous rat anti-sheep antibody because rat IgG was not found in the GBM and rat anti-SαL was not present in the sera. Proteinuria was probably not caused by complement either because there was no change in the amount of glomerular C3 detected subsequent to the acute period and because most proteinuric animals had no complement in their glomeruli. The reason for proteinuria, however, was most probably due to changes that occurred within the glomeruli because the epithelial cells had absorption droplets (31–33), microvilli formation (34), and displaced slit diaphragms (33, 35), all of which are changes seen in other glomerular proteinurias.

The two most likely explanations for the development of proteinuria are GBM injury and mononuclear cell infiltration with subsequent endothelial stripping. Both lesions were seen 2–3 wk after SαL injection at about the time of the onset of proteinuria. However, both lesions were also present, although qualitatively less severely, in nonproteinuric rats that received doses of <4 mg SαL. Thickening of the GBM was caused by both the formation of amorphous subendothelial deposits and an increase in the breadth of the lamina densa. The composition of the subendothelial deposits is unknown but they clearly did not contain rat IgG or rat C3, both of which are believed to occur in similar deposits that are seen in NTN (6). The deposits might have contained, however, SαL, laminin, or other GBM components in addition to cell debris. The reason for thickening of the lamina densa is also unknown. Perhaps the cross-linking of laminin molecules by SαL decreased the normal degradation of GBM components and resulted in focal areas of increased thickness.

Monocytes have been recently associated with proteinuria in several examples of glomerulonephritis. Proteinuria can be prevented by the administration of anti-macrophage serum (36), and mononuclear infiltrates are correlated with proteinuria in both serum sickness (37) and an accelerated model of NTN (8). Monocytes might endocytose deposits from beneath the endothelium and improve glomerular filtration, as suggested by others (38). On the other hand, the adherence of mononuclear cells to the GBM might damage the capillary wall because in our experiments the endothelium was lifted off of the GBM by the cells and, in areas, was completely removed. A similar process occurs in the heterologous phase of NTN where PMN infiltrate beneath the endothelium and partially remove it, although this defect is repaired within a few days (39, 40).

The failure of up to 4 mg SαL to stimulate an anti-sheep IgG response in the rat was surprising. This might have been due to the removal of SαL aggregates by centrifugation before injection because monomeric IgG is a poor immunogen (41). Alternatively, because we used affinity-purified antibody rather than a mixed antibody preparation, the injected SαL might have combined with laminin in renal and extrarenal sites, and adequate amounts of free sheep IgG might not have been available for the appropriate stimulation of a humoral response. However, others (7) who have injected 3.5 mg polyspecific NTAb have observed the development of a host humoral response, although the injection of 1.5 mg NTAb or less was deemed inadequate to stimulate host antibody formation. In the absence of a humoral

response, perhaps the injection of 4 mg SαL was sufficient to stimulate a cell-mediated (delayed-type) reaction within glomeruli (42) that might have contributed to the functional and structural changes that we observed.

These experiments demonstrate that laminin is distributed throughout the GBM and mesangial matrix. This finding is in partial agreement with the work of others who have localized laminin variously to the mesangium (11–13, 15), the lamina rarae (13, 14), the lamina rara interna and lamina densa but not the lamina rara externa (12), as well as to Bowman's capsule (11–13, 15) and the TBM (11–13, 15, 43). Apart from species differences and the use of direct vs. indirect labeling techniques, the discrepancies that exist between all of these studies are probably also the result of the method of antibody delivery, i.e., the in vivo permeability of the GBM to immunological tracers might differ from the permeability in vitro. Finally, our experiments indicate that SαL and presumably the laminin to which it is bound are cleared from the peripheral GBM by a gradual translocation into the mesangium. This is the same pathway proposed for normal GBM turnover on the basis of silver nitrate-labeling studies (44).

Summary

Antibodies against laminin, which is a defined glycoprotein of basement membranes, were produced in sheep and affinity purified by immunoadsorption on laminin-Sepharose (SαL). When injected intravenously into rats, SαL rapidly bound in a linear pattern to the glomerular basement membrane (GBM) in the peripheral and mesangial regions of all glomeruli, and, when >0.5 mg SαL was injected, to some tubular BM as well. 1–2 h after the injection of conjugates of horseradish peroxidase (HRP) and SαL, HRP reaction product was present throughout the full thickness of the GBM and mesangial matrix. [^{125}I]SαL binding to the kidney in vivo increased linearly over the dose range of 40–950 μg of IgG and accounted for ~2% of the injected dose/g kidney. When 4 mg of [^{125}I]SαL was injected, 1.5% or 62 μg/g kidney was bound. Proteinuria did not develop within 7 wk of injection in rats that received 0.5–1.6 mg of SαL. In contrast, all animals that received injections of 4 mg of SαL gradually became proteinuric within 3–6 wk. Thickening, reduplication, and flocculent subendothelial deposits were observed in the GBM of these animals. In addition, mononuclear cells adhered to the GBM and infiltrated beneath the endothelium. However, the deposition of rat C3 was infrequently observed, and rat IgG was not seen in the glomeruli of any rat that received SαL. 10 wk after injection, much greater amounts of SαL appeared within the mesangium than the peripheral GBM. These results demonstrate that the interaction of SαL with the GBM, possibly in concert with infiltrating mononuclear cells, gradually altered the structure and permeability characteristics of the glomerulus independent of a host anti-SαL humoral response.

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