The Chemically Isolated Lamina Densa of the Renal Glomerulus[1] (37231)

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Renal glomerular capillaries have a basement membrane (GBM) which is made up principally of a lamina densa (LD). The LD, in turn, is bordered by laminae rarae. The plasma membranes of the endothelial cells and of the podocytes of the visceral epithelial cells are adherent to the laminae rarae. The filtration slits between the podocytes and the nonadherent surfaces of the plasma membranes are covered with a glycocalyceal coat. In the preparation of GBM by the common procedure of sonic vibration of isolated renal glomeruli, the LD is apt to be contaminated by cellular membranes, their glycocalyces and by adherent laminae rarae. The difficulties in obtaining a pure preparation of GBM, namely LD, have been dealt with recently by Mohos and Skoza (1) and by Westberg and Michael (2). Nonetheless, the chemical and antigenic properties of such sonically vibrated preparations have been assumed to represent those of LD. Thus, it has been assumed that both sialoglycoprotein and the "nephrotoxic" antigen are components of LD. These assumptions have been questioned. It has been demonstrated histochemically that sialic acid-containing glycoproteins are present in the glycocalyces, to some extent in the laminae rarae but not in LD (3–8). It has also been proposed that the "nephrotoxic" antigen is a sialoglycoprotein which is associated with the cellular membranes and not with the LD (9, 10).

It was found in earlier studies that LD, free of impurities, could be isolated by chemical means (11, 12). Such isolated LD was used in the present studies to attempt to resolve some of the above differences.

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Materials and Methods. Glomeruli were isolated from canine kidneys and GBM prepared from them according to previously described methods (13). Preparations with 3% or more tubular contaminants were discarded. The GBM obtained by sonic vibration of the glomeruli in 0.15 *M* saline was washed 4 to 5 times with 0.15 *M* NaCl by centrifugation at 1800*g* and at 4°. It was then washed four times with deionized distilled water. Samples were taken for electron microscopic examination. The rest was lyophilized and stored at —15°. In addition, isolated glomeruli in 0.15 *M* NaCl were packed by centrifugation. The supernatant was thoroughly drained. The glomeruli were dispersed in a freshly prepared 60% (w/w) aqueous solution of trichloroacetic acid (TCA) (Mallinckrodt Chemical Works, reagent grade) as described previously (11, 12). The following modifications were introduced. After exposure of the glomeruli at 25° for 4 to 5 hr, the preparation was centrifuged at 20,000*g* for 24 min in an International centrifuge, Model B-35. The sediment was resuspended in fresh 60% TCA utilizing a vortex mixer and respun at 1800*g* in a refrigerated centrifuge. This process of packing and resuspension was repeated until the supernatant no longer turned turbid when water was added to it. After the last centrifugation most of the supernatant TCA was discarded and distilled water was added dropwise from a burette to the glomeruli which were kept dispersed by means of a 15 mm Teflon-coated stirring bar and a magnetic mixer. The treated glomeruli were dialyzed overnight against running tap water in the cold. Samples of the material were fixed for electron microscopy. The rest of the material was lyophilized and stored at —15°.

Electron microscopy. Samples of each preparation of vibrated GBM and TCA-treated glomeruli (TCA LD) were placed in Karnovsky's formaldehyde–glutaraldehyde fixative (14), stained with colloidal iron (15), further fixed in osmic acid and embedded in Epon. Sections were cut on a Sorvall "Porter-Blum" ultramicrotome (Models MT-2 and MT-2B), placed on parlodion and carbon-coated copper grids and stained with uranyl acetate and lead citrate. The grids were examined with a Hitachi HS-7S.

Analytical methods. Total nitrogen was determined by micro-Kjeldahl. Two milligrams of the lyophilized samples were used for amino acid determination with norleucine as an internal standard. The samples were hydrolyzed with 1 ml peroxide-free 6 *N* HCl at 110° for 24 hr under nitrogen in sealed tubes. The hydrolysates were repeatedly lyophilized to remove acid and subsequently analyzed on a Spinco automatic amino acid analyzer 120° (Beckman Instruments, Palo Alto, CA) according to the procedure of Spackman, Stein and Moore (16). No correction was made for amino acid loss during hydrolysis. Hydroxyproline was determined independently by the colorimetric method of Neuman and Logan (17). Sialic acid was determined by the thiobarbituric acid methods of Warren (18) and Aminoff (19) and by the resorcinol method of Svennerholm (20). Hexosamines were quantitated by the method of Elson and Morgan (21) as described by Blix (22) utilizing the correction technique of Ogston (23). The individual hexoses were determined by gas–liquid chromatography according to Lehnhardt and Winzler (24). Fucose was determined by the method of Dische and Shettles (25). For total phospholipids, control lyophilized whole glomeruli were used for comparison with TCA LD. The extraction was performed in a semimicro-Soxhlet apparatus with boiling chloroform–methanol (1:1) for 24 hr. The extracts were evaporated to dryness under a stream of nitrogen and redissolved in chloroform–methanol (2:1). This solution was washed with 0.1 *M* potassium chloride as described by Folch, Lees and Sloane-Stanley (26). The chloroform-rich phase was evaporated to dryness. The lipids were redissolved in benzene. Phosphorus was determined by the procedure of Kirkpatrick and Bishop (27) as modified by Henderson (28) in which the amounts of reagents and digestion mixture are doubled.

Immunization. Rabbits were injected intramuscularly with either 3 mg of lyophilized GBM or 3 mg of lyophilized TCA LD in aluminum hydroxide jelly (13). The rabbits were bled after 21 days. Dogs were injected intravenously with 3 ml of rabbit antiserum/

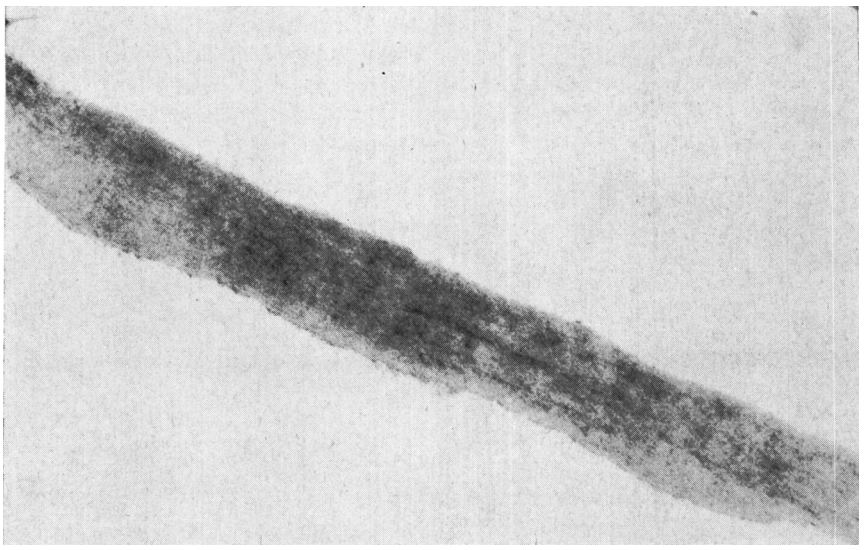

FIG. 1. The ultrastructural appearance of lamina densa isolated by treatment with 60% TCA. Note absence of colloidal iron uptake. ×90,000.

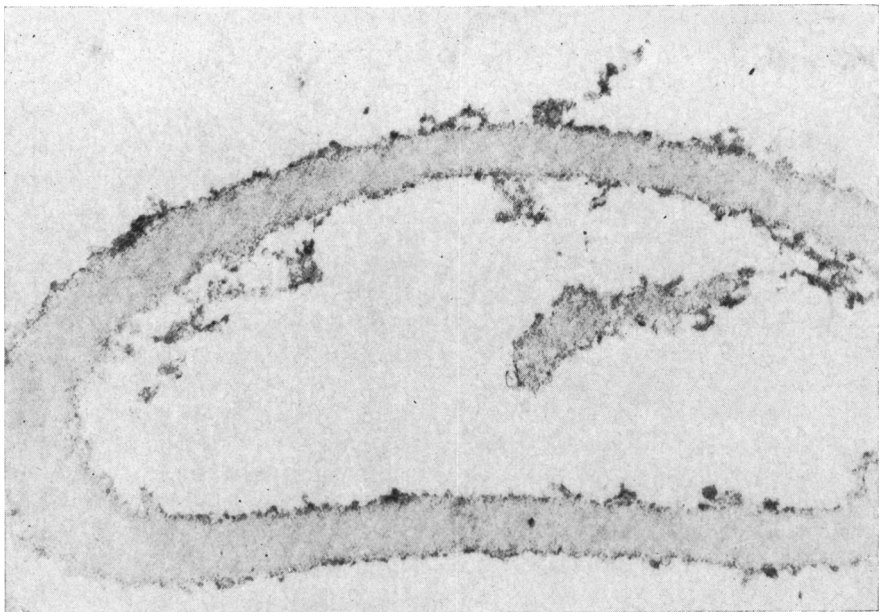

FIG. 2. The ultrastructural appearance of glomerular basement membrane prepared by sonic vibration of isolated glomeruli. Note adherent colloidal iron-positive material. ×95,000.

kg of body weight. Urines were examined daily for protein with Exton's reagent (29). The dogs were sacrificed 5 days after injection. The kidneys were examined microscopically.

Results. Electron microscopy. Careful survey of all samples revealed that the glomeruli after TCA treatment were no longer in the form of tufts or loops but appeared as fragments of smooth-surfaced LD (Fig. 1). There was no colloidal iron-staining material within or over the surface of these fragments. By contrast, vibrated GBM showed positive colloidal iron-staining material over the rough surfaced LD (Fig. 2). This material presumably represented adherent laminae rarae and cellular membranes.

Analytical data. The data for the carbohydrate composition of TCA LD and vibrated GBM are presented in Table I. The total amount of carbohydrate was the same in both types of preparations, namely 9.6%. Sialic acid was present in TCA LD but in reduced amount. Accompanying this reduction in sialic acid, there were also lesser amounts of mannose, galactose and glucosamine. Fucose, however, was the same in both preparations. The elevated level of glucose in TCA LD is not readily explained. However, it may be due to the occasional falsely high values of glucose obtained with gas–liquid chromatography. It is not likely to be due to the linkage of glucose and galactose to an increased amount of hydroxylysine of the collagenous moiety of TCA LD (30), since hydroxylysine was actually decreased (Table II). The total amount of carbohydrate for TCA LD should therefore have been less than 9.6%. These findings would suggest that there is a lower percentage of the heteropolysaccharide or sialoglycoprotein in LD than in vibrated GBM (31).

TABLE I. Carbohydrate Composition of Vibrated GBM and TCA Isolated Lamina Densa.

	Vibrated GBM	TCA LD
	(g/100 g membrane)	
Fucose	0.58	0.60
Mannose	1.04	0.76
Galactose	2.80	2.47
Glucose	2.31	3.82
Glucosamine[a]	1.93	1.35
Sialic acid	1.03	0.60
Total	9.69	9.60

[a] Galactosamine was not detected in either preparation.

There were distinctive differences in the amino acid composition of the two preparation (Table II). There was an appreciable reduction in the number of residues of the following amino acids in TCA LD compared with vibrated GBM: hydroxyproline, methionine, tyrosine, phenylalanine and hydroxylysine. No half-cystine could be detected in TCA LD. These differences are brought out in Table III where comparison is made with the values for calf skin collagen as reported by Piez and Gross (32). There are striking reductions in the aromatic, hydroxyamino and sulfur-containing amino acids as well as in hydroxylysine in TCA LD compared with vibrated GBM. In fact these reduced values approximate those for collagen. However the other groups of amino acids in TCA LD conform more closely with those for vibrated GBM. This seemingly erratic removal of amino acids from TCA LD can best be ascribed to the etching and solvent action of 60% TCA leading to the disruption of both certain peptide bonds of the protein moieties and the linkages of the collagenous component with the heteropolysaccharide or sialoglycoprotein.

TABLE II. Composition of Vibrated GBM and TCA Isolated Lamina Densa.[a]

	Vibrated GBM	TCA LD
	Residues/1000 total amino acid residues	
Hydroxyproline	67.3	47.6
Aspartic acid	72.4	78.7
Threonine	40.4	42.1
Serine	51.3	58.4
Glutamic acid	101.6	114.5
Proline	59.6	68.8
Glycine	192.1	204.5
Alanine	57.3	82.6
Valine	44.8	58.9
Methionine	15.1	6.7
Isoleucine	31.9	35.4
Leucine	68.8	77.1
Tyrosine	18.4	7.1
Phenylalanine	31.2	10.1
Hydroxylysine	24.5	9.7
Lysine	33.1	27.6
Arginine	49.4	54.3
Half-cystine	19.9	—

[a] The estimated amount of nitrogen for vibrated GBM was 12.1%, the determined amount was 15.2%; for TCA LD the estimated amount was 12.0% and the determined amount 13.3%.

TABLE III. Comparison of the Amino Acid Patterns of Vibrated GBM, TCA Isolated Lamina Densa and Skin Collagen.

	Vibrated GBM	TCA LD	Skin collagen
	Residues/1000 total amino acid residues		
Glycine	192	205	320
Imino acids	127	117	232
Hydroxyamino acids	202	165	158
Acidic amino acids	174	193	117
Basic amino acids	127	109	89
Aromatic amino acids	50[a]	17[a]	16
Sulfur-containing amino acids	35	7	4.3
Hydroxylysine	25	10	7.4

[a] Tryptophan not included.

Whole glomeruli contained 49.3 μg total phospholipid/mg of dried material. By contrast TCA LD had less than 1.53 μg/mg of dried material or virtually no phospholipids.

Antigenicity of the preparations. The rabbit antisera to both TCA LD and vibrated GBM produced proteinuria when injected into dogs. From a zero base-line level over a 3 day preinjection period, the protein content of the urine for the first 5 days following injection ranged between 0.02 and 0.21 g/24 hr/kg of body weight for the antiserum to vibrated GBM and 0.07–0.64 for the antiserum to TCA LD. The kidneys 5 days after injection of the antiserum to TCA LD were pale and studded with petechiae. Microscopically they presented the classical changes of an exudative and proliferative glomerulonephritis (Fig. 3). These changes were more severe than those obtained with the antiserum to vibrated GBM.

Discussion. Sixty percent trichloracetic acid will dissolve isolated renal glomeruli. The rate of solution is a function of time and temperature. Of all the components of the glomerulus, the LD is the most resistant to the action of the acid. One can therefore isolate LD by employing a temperature of 25° and an exposure of 4 to 5 hr. The freedom of contamination of the isolated LD by cellular membranes, glycocalyces and laminae

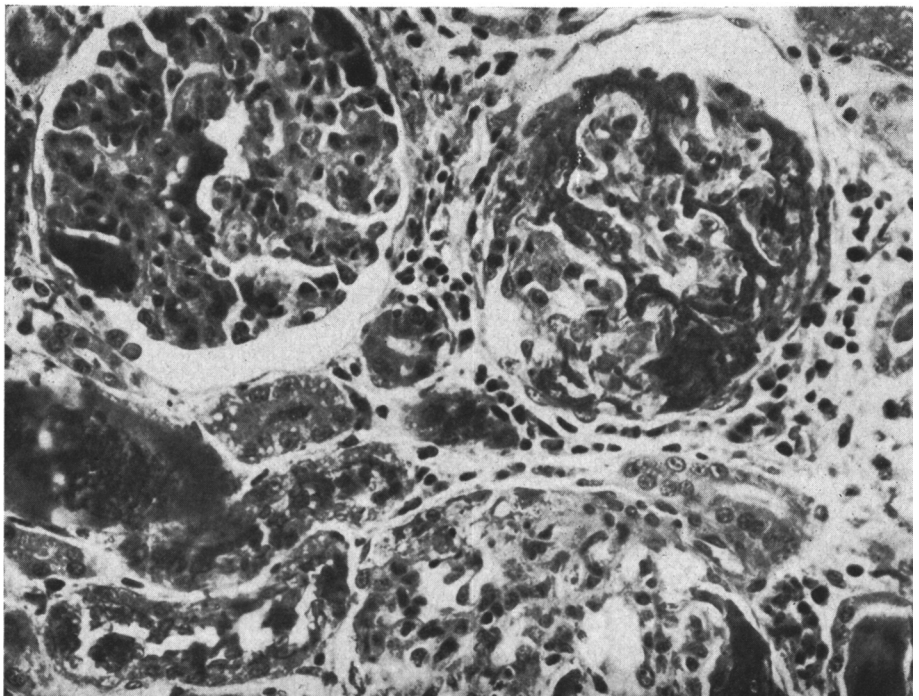

FIG. 3. A high power view of a section of kidney from a dog injected with the antiserum to lamina densa obtained by trichloroacetic acid treatment of isolated glomeruli. There is a rich fibrinous exudate between the tufts and within the capsule of the glomeruli. There are early proliferative capsular changes. The glomerulus to the left is hypercellular. There are hemorrhagic casts in a tubule to the left of the photograph and in one at the bottom to the right. Hematoxylin–eosin ×430.

rarae is attested to by (a) the electron microscopic appearance of the recovered product, (b) the absence of staining for acid glycoproteins with colloidal iron, (c) the virtual absence of phospholipids. By contrast, preparations of vibrated GBM have material attached to the LD which takes the colloidal iron stain. In keeping with this the sialic acid content of such preparations is higher at 1.03% than TCA LD with 0.6%. Likewise the total phospholipid content of vibrated GBM, an index of contamination with cellular membranes, was reported to be 0.5% by Spiro (31) and 1.0% by Westberg and Michael (2). The present value for whole glomeruli was 4.9% and for TCA LD less than 0.1%.

Isolated TCA LD, though made up physically of noncontaminated structural units presents distorted chemical values probably due to the etching effects of the strong acid upon the LD. The present values are therefore probably not representative of the intact LD. Nevertheless, there can be no doubt that LD contains a sialoglycoprotein and most likely the bulk of the collagen. The failure of LD to take up colloidal iron may be due to the probable lesser amount of sialoglycoprotein in LD than in the glycocalyces and the interaction of the sialoglycoprotein with the collagen of the LD. There also can be no doubt that LD contains the "nephrotoxic" antigen. In fact, TCA LD was as or more immunogenic than vibrated GBM.

The present information on LD makes it all the more desirable to determine the chemical composition and antigenic properties of the laminae rarae and the glycocalyces. It is not known whether the laminae rarae contain a collageneous component and, if so, how much. They probably do contain sialoglycoprotein since they bind some colloidal iron. The glycocalyces are undoubtedly very rich in sialoglycoproteins. It is unlikely

that they contain collagen. There is evidence based on the localization of specific antibodies to the nephrotoxic antigen that the latter is probably present in the laminae rarae (33–35). There is, however, no clear evidence that the glycocalyces contain the antigen.

Summary. The lamina densa (LD) of glomerular basement membrane (GBM) isolated by chemical means contains both a sialoglycoprotein and the "nephrotoxic" antigen.

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