

Distribution of Basement Membrane Antigens in Glomeruli of Mice With Autoimmune Glomerulonephritis

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Glomerulonephritis was induced in mice by the repeated injection of human glomeruli or purified glomerular basement membrane. The glomerular basement membranes of nephritic animals were observed to develop subepithelial extensions, "spikes." Although normally Type IV collagen is found throughout the full thickness of base-

ment membranes, the "spikes" reacted with antibodies to laminin but not with antibodies to Type IV collagen. It is proposed that in murine autoimmune glomerulonephritis, the visceral epithelial cells produce an excess of laminin. (Am J Pathol 1986, 122:36-49)

THE BIOCHEMICAL composition of the glomerular basement membrane (GBM) has been studied intensively, and several components have been identified, including laminin, Type IV collagen, heparan sulfate proteoglycan, entactin, nidogen and amyloid P.¹⁻⁹ The distribution of basement membrane components has been established in normal glomeruli,^{1,2,4,6,7,9,10-14} in several types of human¹⁵⁻²² and of experimentally induced^{8,23} glomerular diseases using specific antibodies. These studies, combined with data obtained from glomerular cells grown *in vitro*, provide a foundation for exploring the role of individual GBM components in pathologic conditions.

Patients with Stage 2 and 3 membranous glomerulonephritis show as a characteristic abnormality of the glomerular capillary wall, the presence of basement membrane "spikes" between subepithelial immune deposits.²⁴ The pathogenesis of this disease is usually ascribed to antigen-antibody complexes formed *in situ* or in the circulation.²⁵ Recently, however, Bolton and co-workers²⁶ observed that mice immunized with human GBM preparations developed glomerular lesions characterized by diffuse and unusually large spikes not separated by immune deposits. This finding is consistent with the notion that this glomerular disease is due to the interaction of circulating antibodies with "structural antigens of the GBM."²⁶ The purpose of our study was to establish the distribution of two well-defined

basement membrane components, Type IV collagen and laminin, in the glomeruli of mice with this form of anti-GBM antibody-mediated glomerulonephritis. The results indicate that laminin, but not Type IV collagen, is a major component of the spikes.

Materials and Methods

Animals

Female Swiss-ICR mice (20-25 g) were purchased from West Seneca Laboratory (West Seneca, NY) and allowed free access to food and water.

Antigen Preparation

Normal human kidneys were obtained from autopsies performed within 12 hours on accident victims and stored at -60 C until used. Glomeruli were isolated according to the method of Krakower and Greenspon.²⁷ After isolation, glomeruli were dialyzed against distilled

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water and lyophilized (glomerular fraction). Part of this fraction was further processed for separation of the GBM from cellular components, with a modification of the detergent method described by Meezan et al.²⁸ Glomeruli were treated with 4% deoxycholate containing 0.1% sodium azide for 3.5 hours at room temperature with constant stirring. After centrifugation at 10,000 rpm for 10 minutes, the supernatant was dialyzed against distilled water and lyophilized (cellular fraction). The sediment was washed three times with distilled water and lyophilized (GBM fraction). The protein content of each fraction (weight percent) was determined by the modification of Lowry's method.²⁹

Immunization

Sixteen mice were immunized with the glomerular fraction, 5 mice with the GBM fraction, and 5 mice with the cellular fraction. The initial immunization consisted of subcutaneous injection at multiple sites of a total of 0.75 mg of protein, in 0.075 ml phosphate-buffered saline (PBS) mixed with 0.075 ml of Freund's complete adjuvant (FCA), (Difco Laboratories, Detroit, Mich). Further immunizations were carried out by subcutaneous injections of the same antigenic mixture every 2 weeks until the mice died or were sacrificed (24 weeks after immunization). Seven control mice were given injections, following the schedule described above, of a mixture of 0.075 ml of PBS and FCA.

Antibodies

For immunofluorescence microscopy, rabbit antibodies directed against mouse Type IV collagen, laminin and fibronectin were used. For immunoelectron microscopy, the Fab fragment of monospecific rabbit antibodies directed against mouse Type IV collagen or fibronectin or against rat laminin were used. The purification, characterization, and specificity of these antibodies has been described previously.^{3,4,6,12,30,31} Fluorescein isothiocyanate (FITC)-conjugated goat antibodies to rabbit IgG, mouse IgG, or mouse IgM and mouse C3 were purchased from Cappel Laboratories (Cochranville, Pa). FITC-conjugated goat anti-rabbit IgG did not react with mouse IgG when tested by direct immunofluorescence on kidney sections of mice with nephritis. Goat anti-rabbit Fab and rabbit Fab-PAP were prepared as previously described.^{12,32,33}

Urinary Protein Excretion and Blood Sampling

Mice from each group were placed every 2 weeks in metabolism cages, and 24-hour urine specimens were collected. The protein excretion was determined by the

biuret method. Blood samples were taken from the retroorbital plexus, before immunization, 8 weeks after initial immunization, and at the time of sacrifice. The sera were tested for the presence of basement membrane antibodies by indirect immunofluorescence microscopy with frozen sections of normal Swiss-ICR mouse kidney as substratum.

Elution of Immunoglobulins From Kidney Tissue

Elution was carried out by treatment of renal homogenates with citrate buffer at pH 3.2 or with glycine buffer at pH 2.2, as previously described.³⁴

Light and Electron Microscopy

For light microscopy, kidney tissue was fixed in 10% buffered formalin overnight and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) or with periodic acid-Schiff (PAS) reagent. Some blocks were embedded in methacrylate, and 1- μ sections were reacted with H&E and Jones' silver methanamine. For electron microscopy small pieces of cortex were fixed in a mixture of paraformaldehyde and glutaraldehyde,³⁵ postfixed in 1% osmium tetroxide, and embedded in Epon 812-Araldite. Thin sections were stained with uranyl acetate and lead citrate. For silver staining, tissue blocks were fixed in osmium tetroxide and embedded in Epon 812-Araldite. Thin sections were oxidized by periodic acid, reacted with a mixture of silver nitrate, hexamethylene tetramine, and sodium borate, and fixed with sodium thiosulfate.³⁶

Immunofluorescence Microscopy

Sections of frozen or paraffin-embedded kidney tissue were used for the study. Frozen sections were processed as described in previous publications.³⁷ Paraffin sections were deparaffinized with xylene and ethanol, washed in distilled water for several minutes, incubated in 0.1% trypsin (Cat. No. 610-5095, GIBCO Laboratories, Grand Island, NY) and 0.05% CaCl₂ in PBS at 37 C for 15, 30, 60, 120, 180, and 240 minutes. After washing in distilled water and in PBS the sections were stained for mouse IgG, Type IV collagen, laminin, and fibronectin. All the sections were studied with a Leitz Ortholux microscope equipped with epifluorescence optics and appropriate filters.

Immunoelectron Microscopy

The kidneys of anesthetized animals were perfused with 4% formaldehyde via the abdominal aorta for 10 minutes according to previously described methods.^{4-6,12}

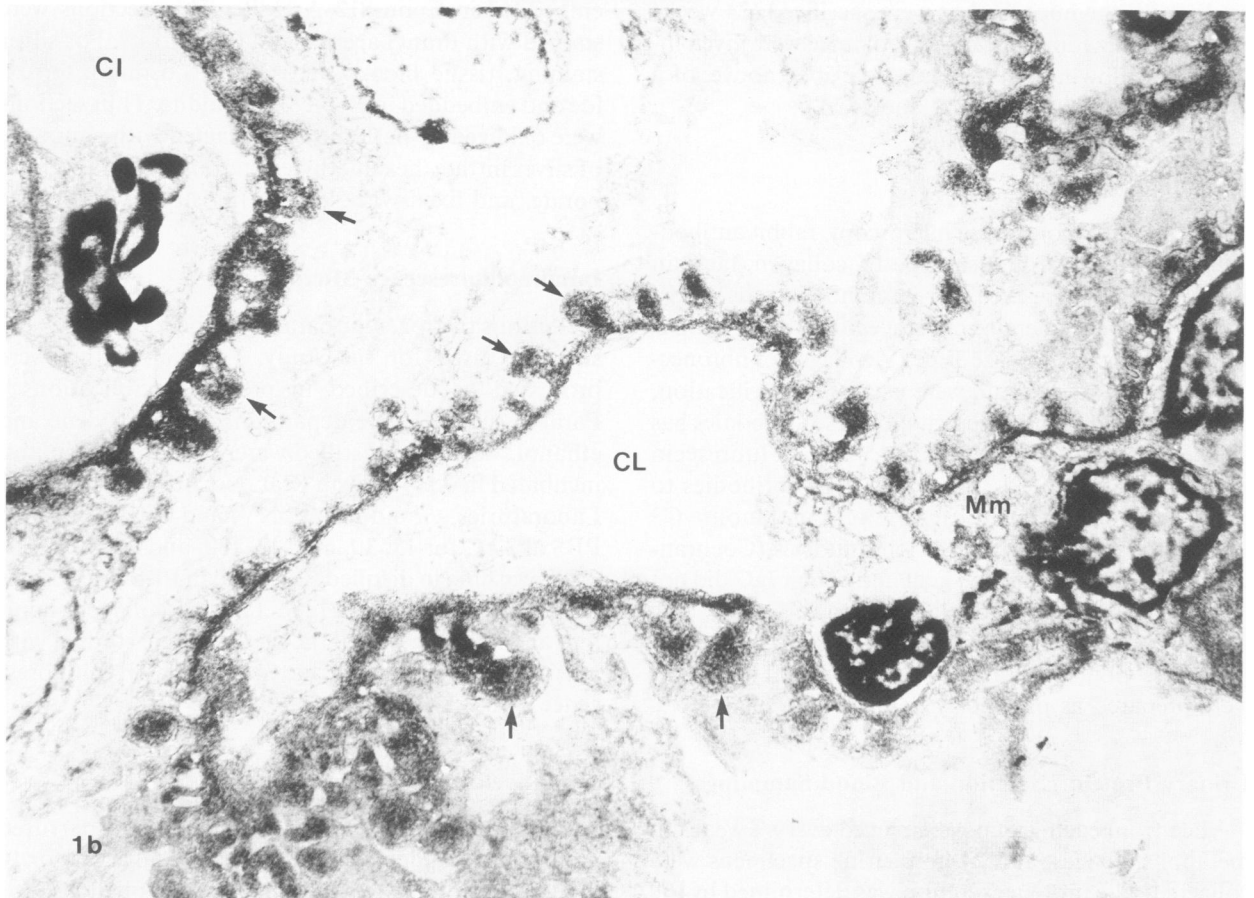
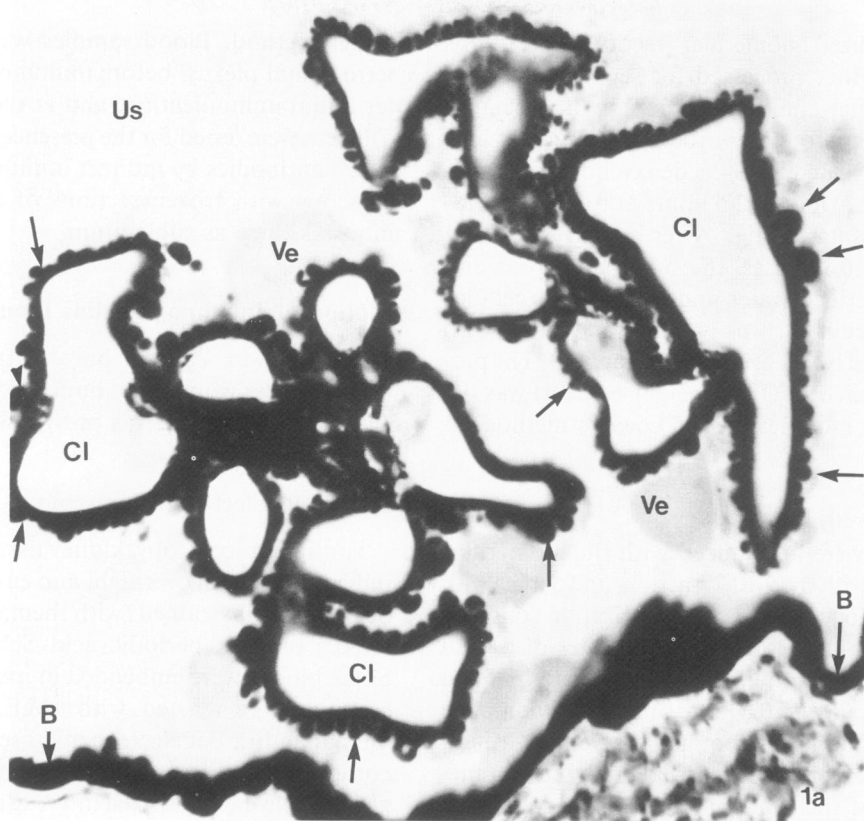




Figure 1—Glomeruli from mice immunized with the glomerular fraction. Arrows point to some argyrophilic spikes. **a**—Methacrylate-embedded section reacted with Jones' silver methanamine. The GBM of every capillary loop contains multiple spikes. ($\times 750$) **b**—Epon-embedded section reacted with silver. The spikes are located at the epithelial side of the GBM. ($\times 3000$) **c**—Standard electron micrograph of the glomerular capillary wall. Note the absence of subepithelial foreign electron-dense deposits between the spikes. ($\times 12,000$) Abbreviations used in all figures: *B*, Bowman's capsule; *Cl*, capillary lumen; *GBM*, glomerular basement membrane; *En*, endothelial cell; *Mc*, mesangial cell; *Mm*, mesangial matrix; *N*, nucleus; *Pe*, parietal epithelial cell; *RER*, rough endoplasmic reticulum; *Tc*, tubular cell; *TBM*, tubular basement membrane; *Us*, urinary space; *Ve*, visceral epithelial cell.

After perfusion, the kidneys were removed, minced into $1 \times 1 \times 0.5$ cm blocks, and postfixed in 4% formaldehyde at 4 C with continuous stirring for 3 hours. After fixation, the blocks were rinsed in multiple changes of PBS with 4% sucrose at 4 C overnight. After a final 1-hour wash in PBS, with 4% sucrose and 7% glycerol, the tissues were snap-frozen in methylbutane precooled to liquid nitrogen temperature. Six-micron sections were cut in a cryostat and allowed to dry on albumin-coated slides. Sections were sequentially reacted with sodium borohydride, normal goat serum, normal rabbit serum, or primary antibody (Fab). The localization was completed with goat anti-rabbit Fab, Fab-PAP, 2.5% glutaraldehyde, glycine, and diaminobenzidine, according to previously described methods.^{12,33} Sections were reacted with 1% OsO₄, dehydrated in ethanol, and embedded in plastic via propylene oxide. Ultrathin sections were examined and photographed without further staining.

Results

Proteinuria

About 20 weeks after immunization the mice receiving either glomerular or GBM fractions developed significant proteinuria (>20 mg/24 hours). Two of the 5 mice immunized with cellular fraction and one of the

7 control mice had transient proteinuria during the course of the experiment.

Circulating Antibodies

Sera from mice immunized with the glomerular or GBM fraction bound in a linear pattern to mouse glomerular and tubular basement membrane. Some sera of mice treated with the glomerular fraction also reacted with the brush border of proximal tubules. The antibody titers were low (1:1–1:4). Sera from mice immunized with the cellular fraction reacted with the brush border of mouse proximal tubules and, possibly, with some cellular components of mouse glomerular capillary wall. Sera from mice given injections of PBS and FCA alone did not react with mouse kidney.

Histologic Findings

In immunized mice the most characteristic light- and electron-microscopic lesion of the glomerular capillary wall was the diffuse presence of GBM spikes toward the epithelium (Figure 1). This change was more pronounced in mice immunized with the glomerular or GBM fraction than in those immunized with the cellular fraction. In addition, fibrocellular crescents were observed in a few of the mice given the glomerular or GBM

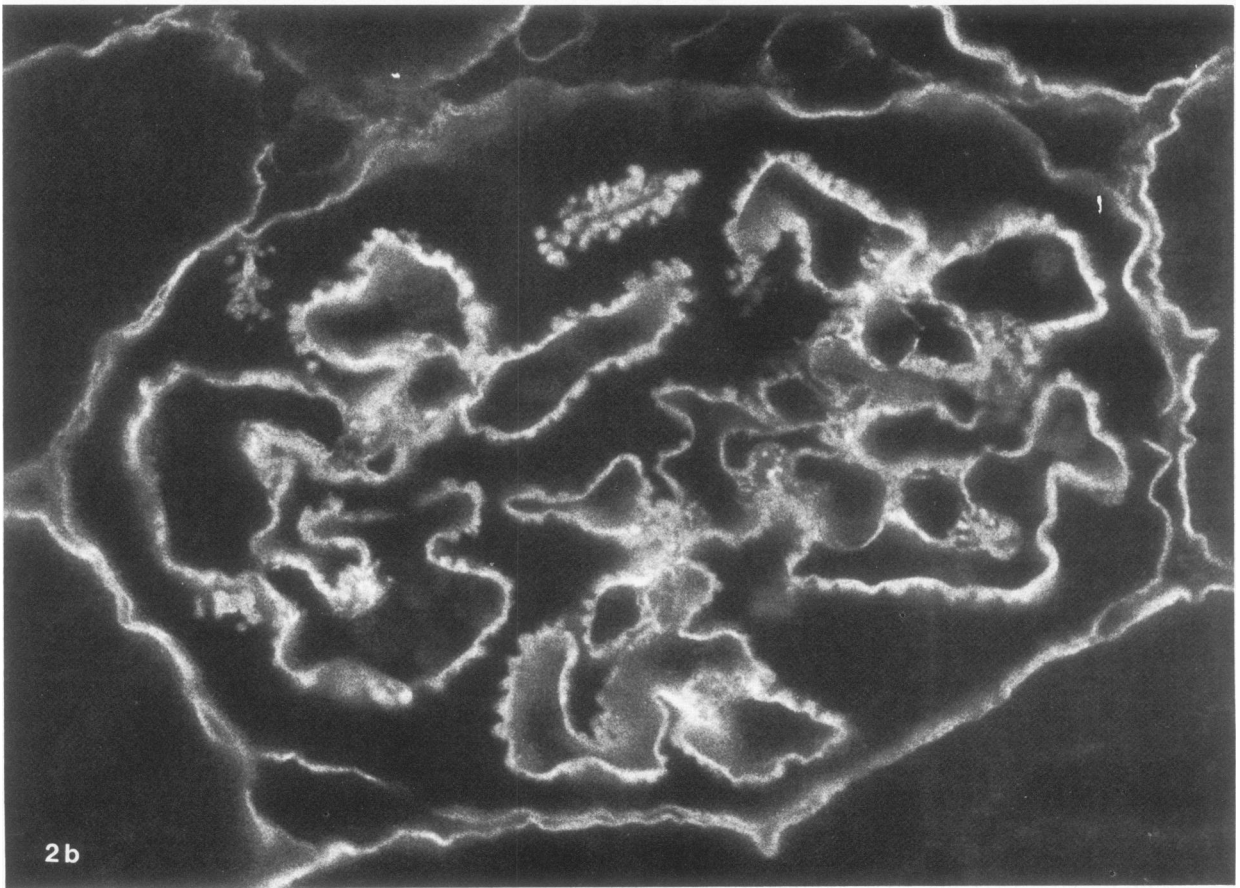
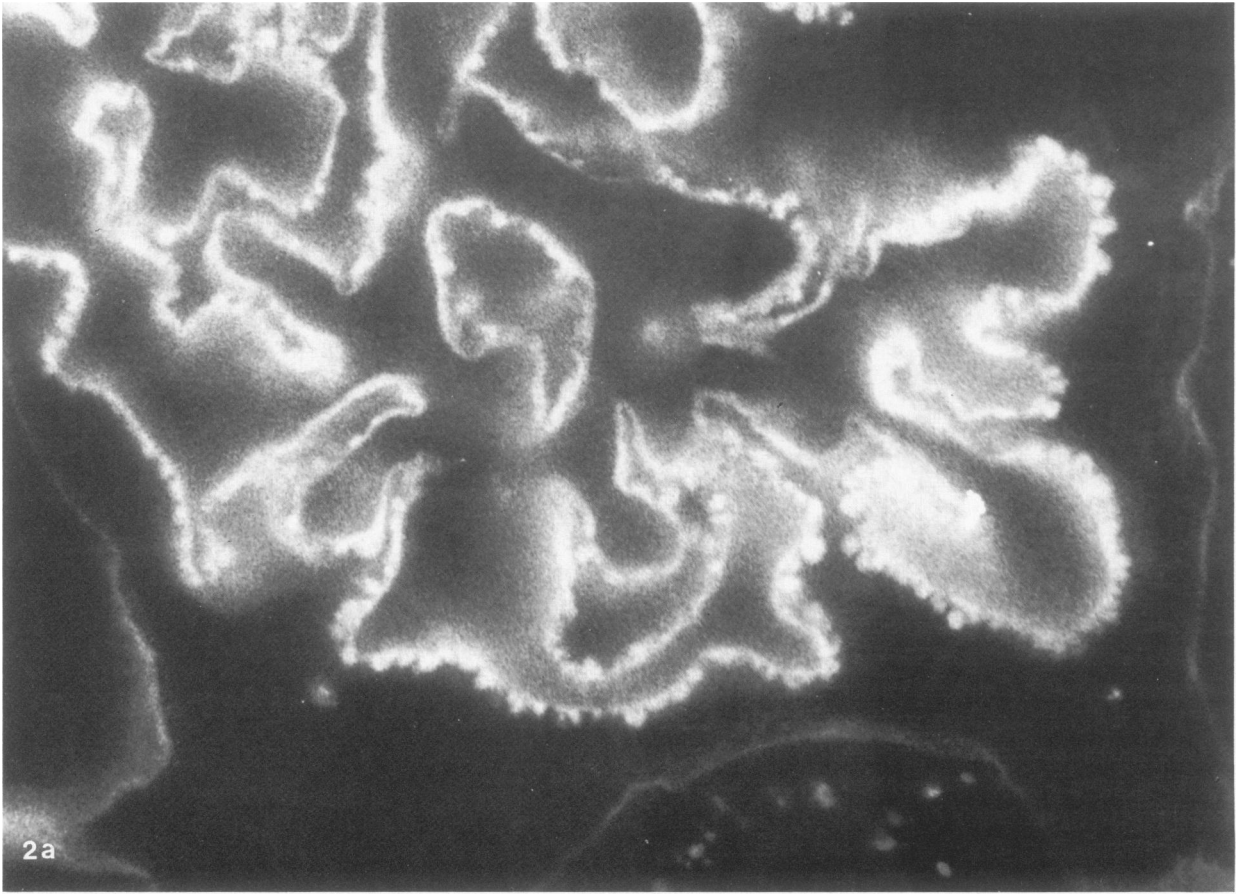
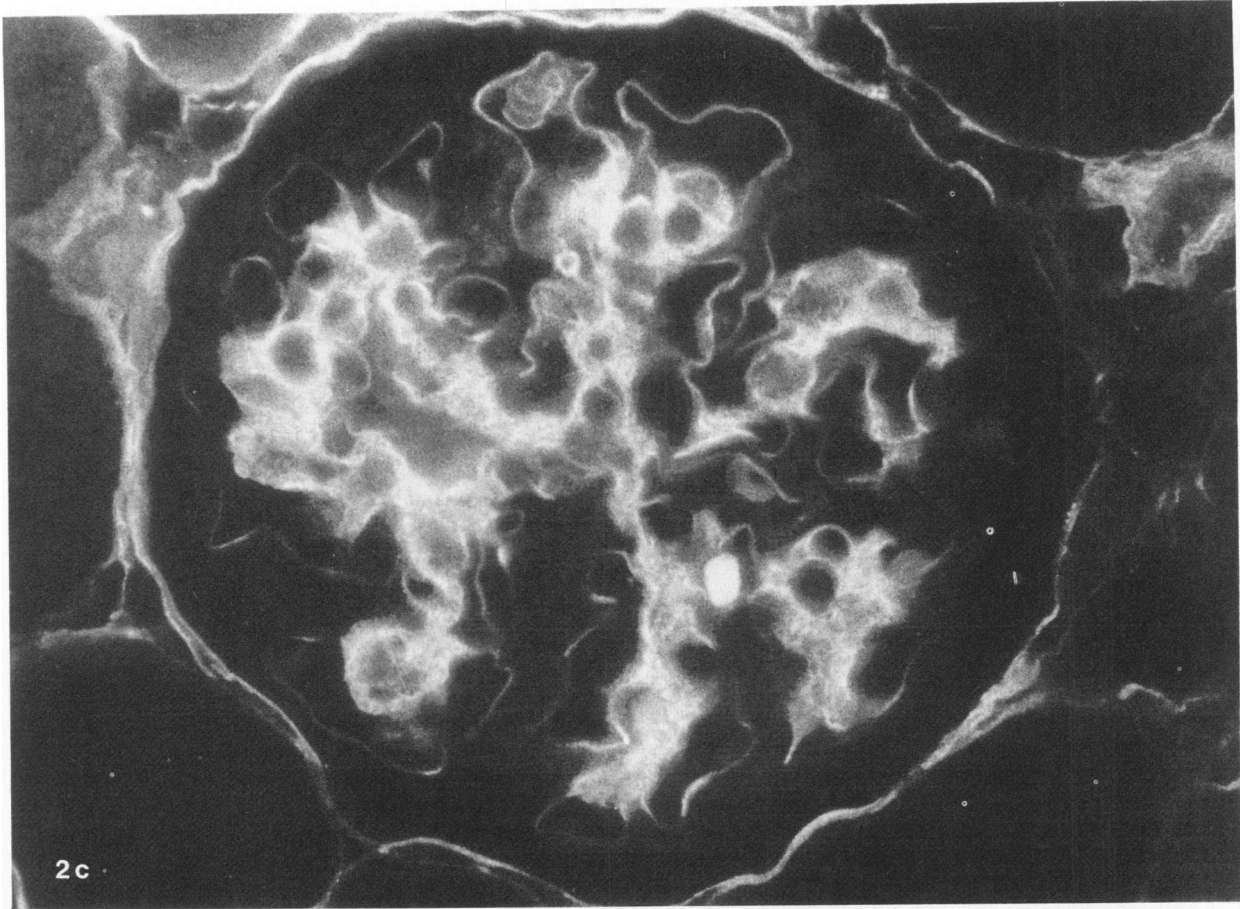
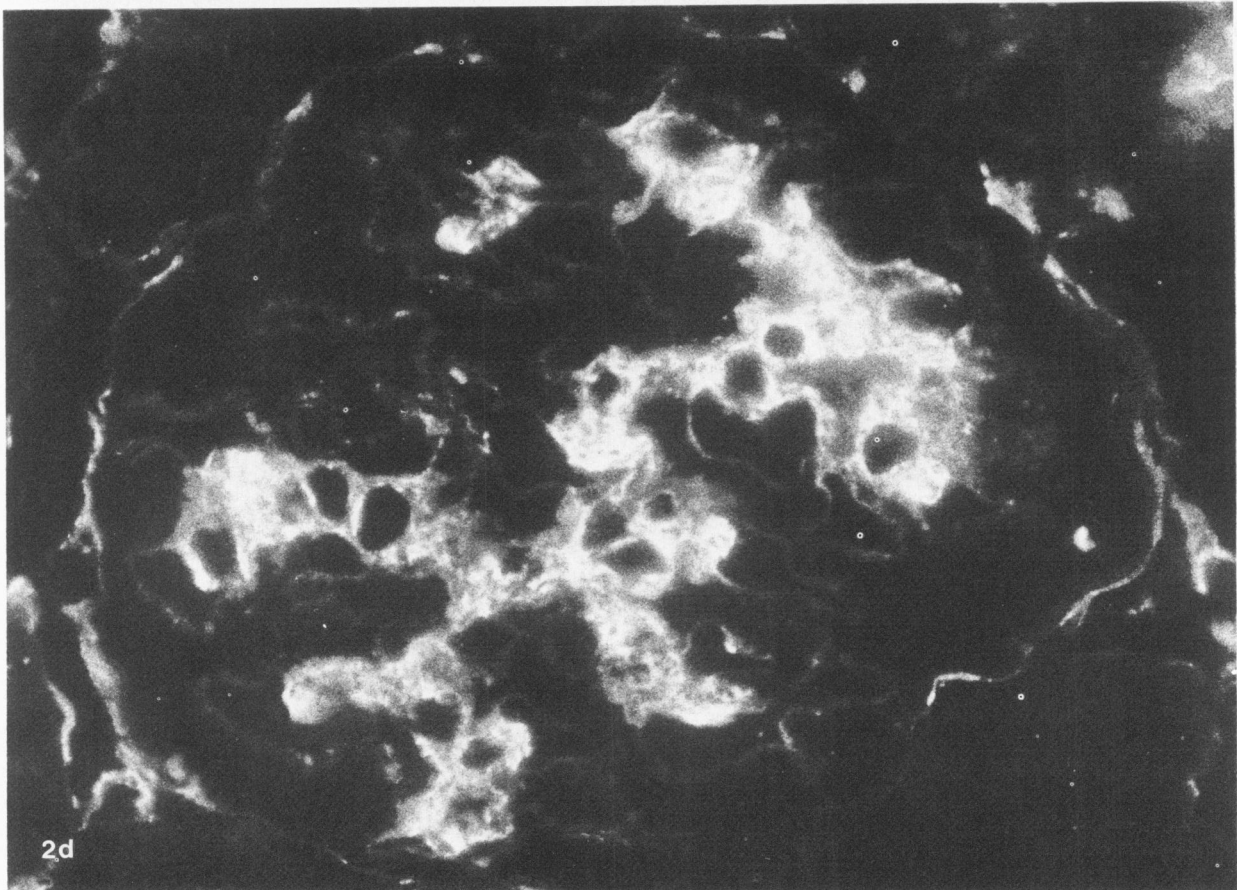


Figure 2—Immunofluorescence microscopy of glomeruli from animals given the glomerular fraction, 24 weeks after immunization. **a**—Mouse IgG. Linear deposits of IgG are demonstrable along the GBM; the spikes contain IgG deposits. ($\times 1600$) **b**—Laminin. Laminin is present in Bowman's capsule, mesangial matrix, and glomerular and tubular basement membrane. In addition, laminin is prominent in all spikes. ($\times 1000$) **c**—Type IV



2c



2d

collagen. Type IV collagen is demonstrable in Bowman's capsule, mesangial matrix, and glomerular and tubular basement membrane. Notice that the spikes do not contain demonstrable type IV collagen (compare with b, laminin). ($\times 1000$) d—Fibronectin. Fibronectin is present in mesangial matrix. The GBM and the spikes have only occasional, irregular, weak reactivity. ($\times 1000$)

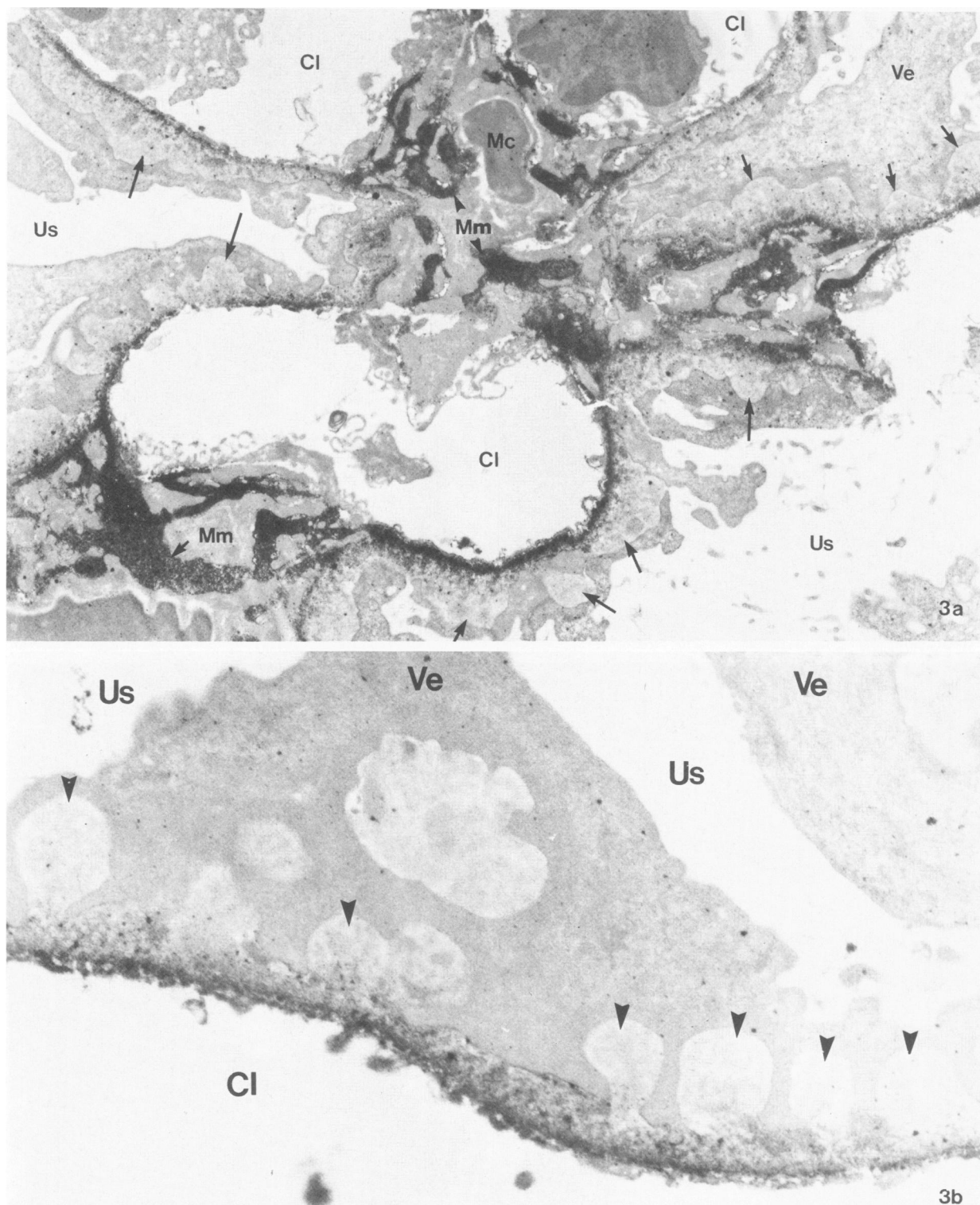


Figure 3—Electron immunohistochemical localization of Type IV collagen in glomeruli of animals given the glomerular fraction, 17 weeks after immunization. **a**—Type IV collagen is demonstrable in mesangial matrix and along the GBM, in a linear pattern. The spikes (*arrows*) are negative (compare with Figure 4a). ($\times 8000$) **b**—Detail of capillary loop. Type IV collagen is present in the lamina rara interna and lamina densa and only weakly in the lamina rara externa. The spikes (*arrowheads*) lacking demonstrable Type IV collagen are surrounded by epithelial cytoplasm. ($\times 22,000$)

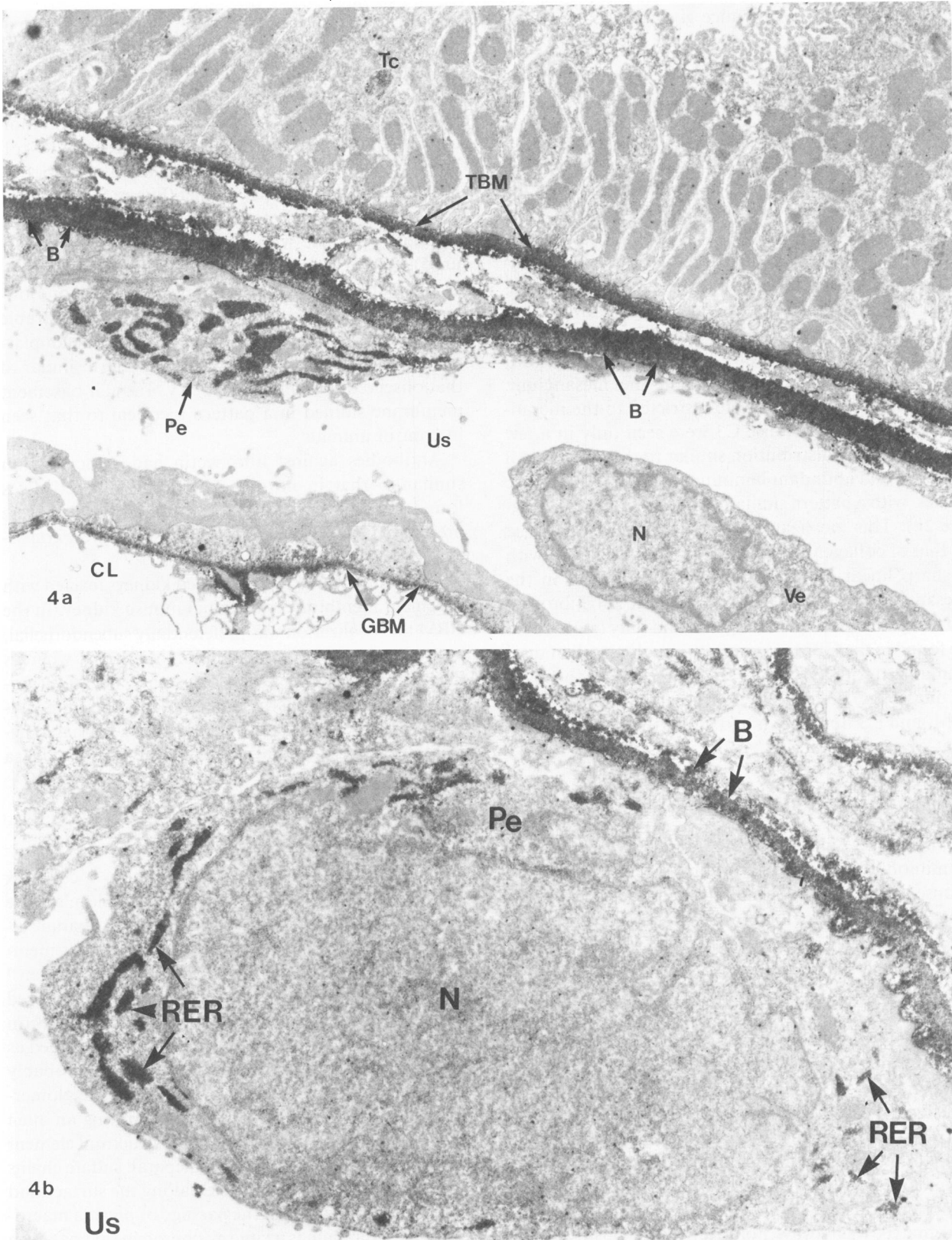


Figure 4—Electron immunohistochemical localization of Type IV collagen in glomeruli of mice immunized with the glomerular fraction, 17 weeks after immunization. **a**—The tubular basement membrane and Bowman's capsule are homogeneously positive. In the GBM only the lamina rara interna and densa react with the antibody. A parietal epithelial cell contains Type IV collagen antigens in the cytoplasm. ($\times 8500$) **b**—Detail of a parietal epithelial cell. The cisternae of the rough endoplasmic reticulum contain Type IV collagen antigens. ($\times 17,000$)

fraction. The kidneys of mice given FCA alone were consistently normal.

Immunofluorescence Findings

The results obtained with sections of frozen or paraffin-embedded kidney tissue were comparable, although superior resolution was obtained with paraffin sections. The glomeruli of mice given FCA alone had minimal deposits of mouse IgM in the mesangium. Laminin and Type IV collagen were seen in a linear pattern in the GBM. Fibronectin was present in the mesangium and only in trace amounts in the GBM.

Mice immunized with the glomerular or GBM fraction had diffuse deposits of mouse IgG in the GBM, in the spikes (Figure 2a), and in the mesangium. Deposits of mouse IgM were restricted to the mesangium. Deposits of mouse C3 were seen only in a few mice and had a distribution similar to that of IgG. All the mice had abundant laminin in the GBM and in the spikes, with a pattern similar to that of mouse IgG (Figure 2b). This localization of laminin was in contrast to that of collagen Type IV, which was detectable, with a sharp linear distribution, in the GBM and in the mesangium, but not in the spikes (Figure 2c). Fibronectin was present in the mesangium, and only traces could be found in the GBM (Figure 2d). The glomeruli of mice immunized with the cellular fraction had linear deposits of mouse IgG in the GBM. Laminin, collagen Type IV, IgM, and C3 fibronectin had a distribution similar to that of control mice.

Antibodies Eluted From Nephritic Kidneys

The eluates, containing IgG, were tested by indirect immunofluorescence microscopy, using Swiss-ICR mouse kidney as substratum. The IgG reacted in a sharp linear pattern with glomerular and tubular basement membrane.

Immunoelectron Microscopy

Type IV collagen and laminin had different localizations in mice immunized with the glomerular or the GBM fraction. Antibodies against Type IV collagen had a discrete localization in the GBM. The reaction product was prominent in the lamina rara interna and in the lamina densa and less intense or absent from the lamina rara externa (Figures 3 and 4). The spikes were almost invariably negative (Figures 3 and 4). The mesangial matrix was always homogeneously positive (Figure 3). The parietal cells of Bowman's capsule reacted with the antibody (Figure 4a), and it was obvious at higher magnification that the reaction product was pres-

ent in the cisternae of the rough endoplasmic reticulum (Figure 4b). The staining of tubular basement membrane (Figure 4a) and Bowman's capsule (Figure 4) was identical to that seen in control animals.

Antibodies against laminin had a localization radically different from that of antibodies to Type IV collagen. The GBM reacted with anti-laminin antibodies in a diffuse, homogeneous manner (Figure 5). The spikes (negative with anti-Type IV collagen antibodies) were invariably positive with anti-laminin antibodies (Figure 5). The mesangial matrix was homogeneously positive (Figure 5a). In addition, epithelial cells of Bowman's parietal layer frequently had demonstrable laminin antigens in the cisternae of the rough endoplasmic reticulum (data not shown) in a pattern similar to that observed for collagen Type IV. Tubular basement membrane stained in a pattern identical to that seen in control animals.

Antibodies against fibronectin had a localization similar to that in normal animals. Fibronectin was found in the mesangial matrix, and only traces were present in the GBM (Figure 6). The spikes were consistently negative (Figure 6).

Antibodies eluted from diseased kidneys reacted with basement membranes of normal mouse kidney. In the GBM the localization was preferentially subendothelial, in the lamina rara interna and lamina densa, whereas the lamina rara externa was consistently negative (Figure 7a). In tubular basement membranes the eluted antibodies bound in a homogeneous manner, with a somewhat preferential localization in the lamina densa (Figure 7b).

Discussion

The glomerular basement membrane is a complex structure composed of a variety of macromolecules. The presence of laminin,^{6,7} Type IV collagen,^{8,9} heparan sulfate proteoglycan,^{2,8} and entactin⁶ in basement membranes has been convincingly demonstrated. Nidogen³⁸ and amyloid P component¹ have also been proposed as basement membrane components. The fibronectin traces often found in GBM and originally described as another basement membrane component^{10,39} probably represent plasma fibronectin "trapped" in the glomerular filter.^{12,32,40,41} Type IV collagen, forming an open network, is assumed to be the major structural element of the basement membrane. The heparan sulfate chains of the proteoglycan are arranged along the surface and create an ionic barrier to the passage of plasma macromolecules. Laminin is a large glycoprotein that has been proposed to bind to Type IV collagen and heparan sulfate proteoglycan and also to cell surface receptors. Nidogen and entactin, possibly related proteins, are

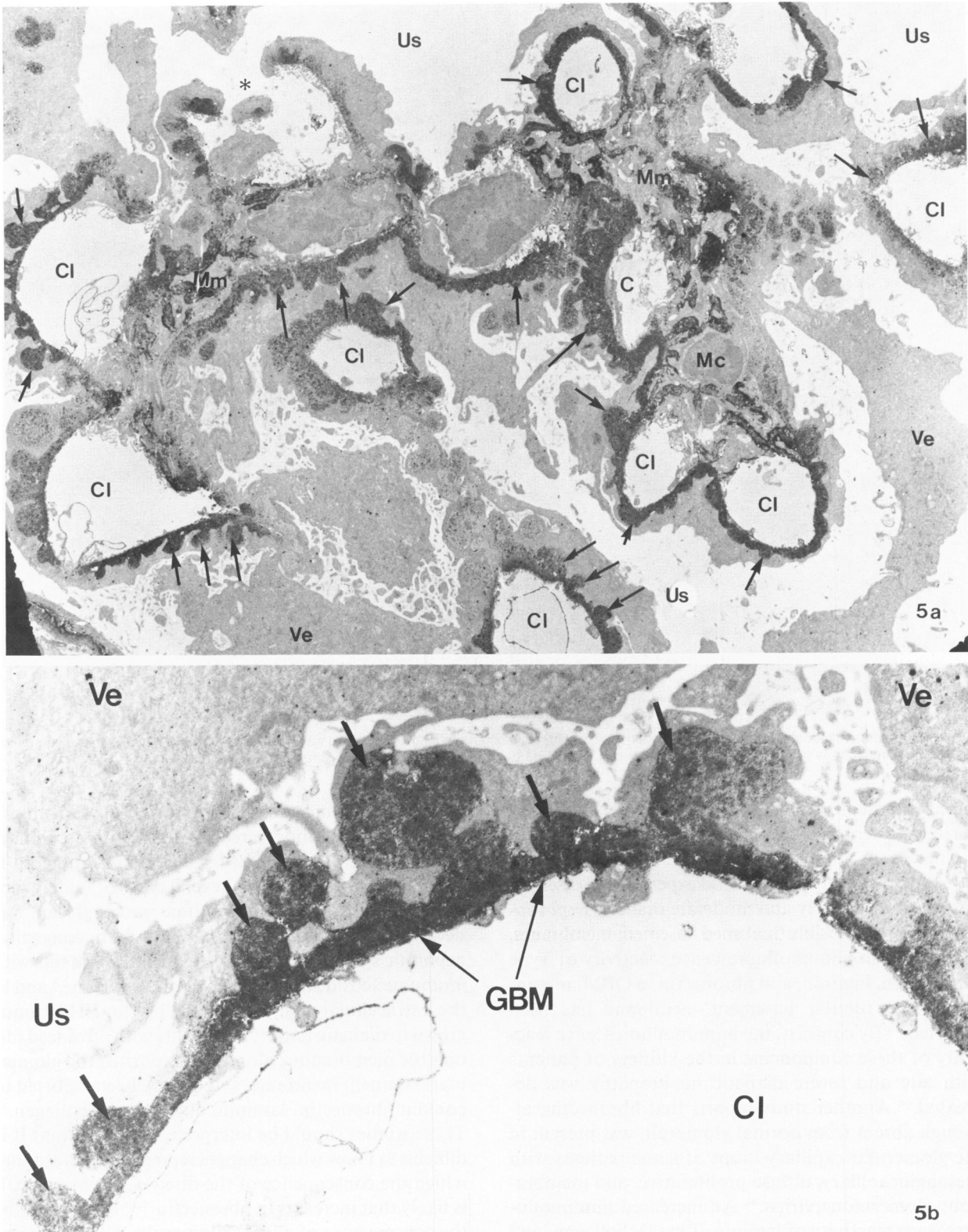


Figure 5—Electron immunohistochemical localization of laminin in glomeruli of mice immunized with the glomerular fraction, 17 weeks after immunization. **a**—Laminin antigens are present in mesangial matrix, capillary loops, basement membrane, and in all spikes (arrows) (compare with 3a). The breaks in the glomerular capillary wall (asterisk) are an artifact of freezing. ($\times 5000$) **b**—Detail of a capillary loop. The full thickness of the GBM reacts with the antibody. The spikes are intensely positive for laminin, but not as homogeneously as the GBM. ($\times 15,000$)

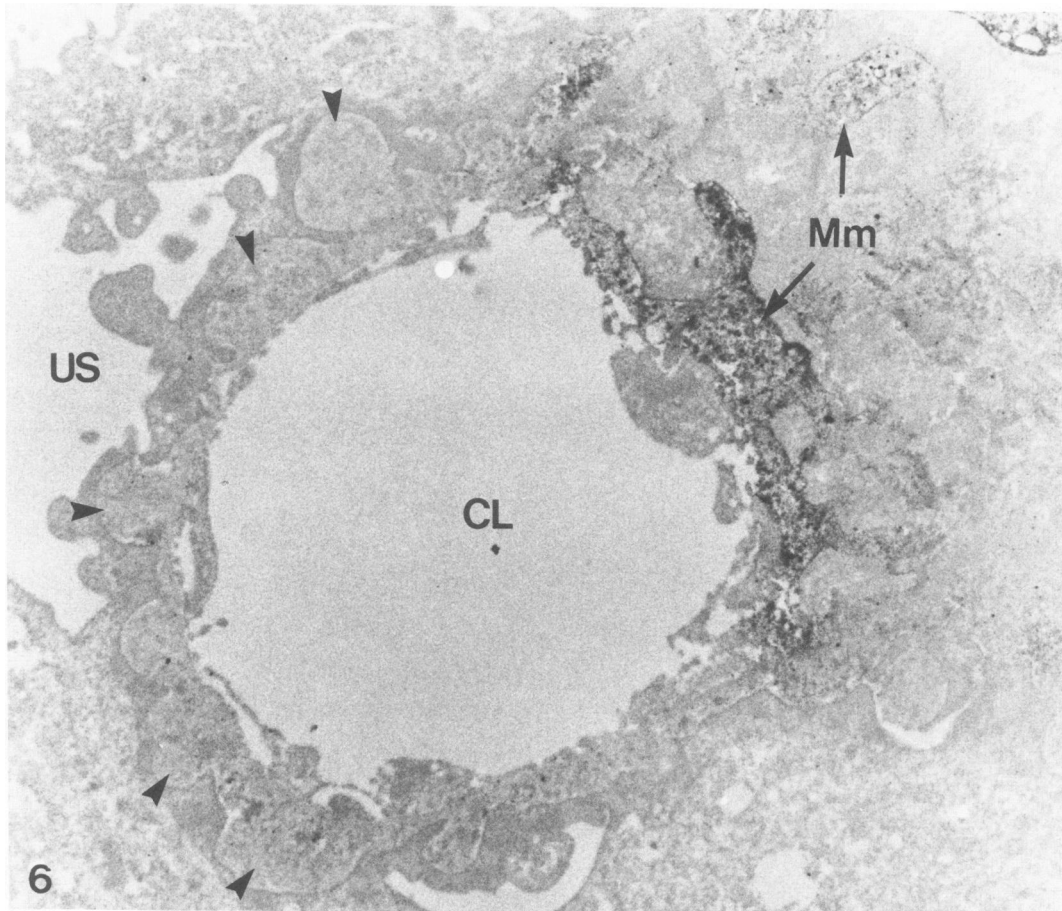


Figure 6—Electron immunohistochemical localization of fibronectin in glomeruli of mice immunized with glomerular fraction, 17 weeks after immunization. Some fibronectin is present in the mesangial matrix. The GBM and the spikes (arrowheads) are negative. ($\times 15,000$)

found in basement membranes but are relatively minor constituents.

Changes in some basement membrane components have been reported in human and experimental diseases. For instance, in early and moderate diabetic nephropathy, along with visibly thickened basement membranes, an increase in immunofluorescence reactivity of Type IV collagen, laminin, and fibronectin in GBM, mesangium, and tubular basement membrane has been reported.¹⁶ By contrast, the immunofluorescence reactivity of these components in the kidneys of patients with late and severe diabetic nephropathy was decreased.¹⁶ Another study reports that fibronectin, although absent from normal glomeruli, was present in the glomerular capillary loops of some patients with mesangiocapillary, diffuse proliferative, and membranous glomerulonephritis.⁴² An increased immunofluorescence reactivity of laminin, Type IV collagen, and fibronectin has been reported in glomeruli of patients with preeclampsia and hypertensive syndromes of pregnancy.¹⁷ Type IV collagen and laminin were present in the mesangium of patients with focal glomeruloscle-

rosis.¹⁸ The glomerular distribution of Goodpasture's antigen,²¹ of the antigen recognized in human kidneys by rabbit anti-human GBM sera,²⁰ and of amyloid-P component¹⁵ was altered in several types of human glomerulonephritis. Heparan sulfate proteoglycan was decreased in glomeruli of patients with congenital nephrotic syndrome,²² in diabetes mellitus,¹⁹ in rats with aminonucleoside induced nephrotic syndrome,⁸ and in the extracellular matrix secreted by the EHS tumor grown in diabetic mice.⁴³ In patients with advanced idiopathic membranous glomerulonephritis, the glomerular basement membrane spikes have been reported to contain fibronectin, laminin, and Type IV collagen.¹⁸ These studies should be interpreted with caution. It is difficult to know which changes represent the cause, and which the consequence of the disease. For instance, it is likely that increases in fibronectin^{17,42} may simply be the consequence of a leaky glomerular filter. Decrease of one component may result in increased compensatory deposition of others. Such a mechanism has been proposed to explain the decrease of heparan sulfate proteoglycan seen in diabetic mice and the subsequent

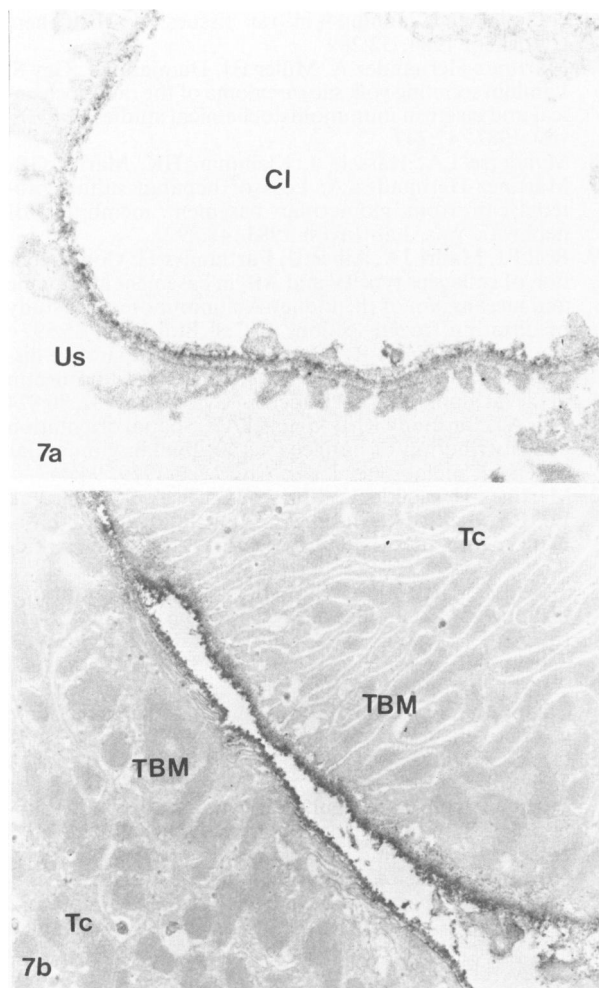


Figure 7—Electron immunohistochemical localization in normal mouse kidney of antibodies eluted from kidneys of mice immunized with the glomerular fraction. **a**—In the GBM the eluted antibodies localize in a linear pattern. They localize preferentially in the lamina rara interna and lamina densa. The lamina rara externa is consistently negative with these antibodies. ($\times 10,000$) **b**—In the tubules the eluted antibodies localize in all basement membranes. The reactivity is more intense in the lamina densa. ($\times 7500$)

thickening of the basement membrane.⁴³ At present, the mechanisms involved in basement membrane assembly are unknown. Complex mechanisms must exist to regulate the assembly of at least five macromolecules into the structure that we call basement membrane; however, the exact arrangement of molecules as determined by their relative affinity, binding constants, and covalent cross-linking remains unknown.⁵ Interpretation of the changes seen in disease is hampered by the lack of this essential information.

It is worth noting that in our studies we found no obvious morphologic abnormality in the glomerular endothelial cells, whereas the visceral epithelial cells undergo significant changes, of which the most prominent is the effacement of foot processes. This morphologic

change may be indicative of an altered function of the podocytes. Many of the findings in this study can be interpreted in this light. In the normal murine GBM, Type IV collagen is found throughout the full thickness of the GBM, with a preferential distribution in the lamina densa.^{8,10} In the mice with autoimmune glomerulonephritis, Type IV collagen could not be demonstrated in the lamina rara externa. This redistribution indicates that during the course of the disease, either Type IV collagen is selectively removed from the lamina rara externa (but not from the lamina rara interna and densa), or, more likely, it is not being deposited in the lamina rara externa. We know that the mature GBM is the result of the fusion, at the level of the lamina densa, of two basement membranes: the epithelial and endothelial basement membranes.^{5,44} It would appear that the most likely explanation for the change in Type IV collagen distribution is that the visceral epithelial cells, affected by the immune-mediated glomerular injury, decrease their synthetic rate of Type IV collagen, the result being an almost complete absence of Type IV collagen in the lamina rara externa and in the spikes. If this is the case, it appears that the visceral epithelial cells respond to the injury, not only with a decreased secretion of Type IV collagen, but also with an increased secretion of laminin. The result of this increased secretion of laminin is the formation of spikes. Obviously we cannot rule out the redistribution of other basement membrane components, such as entactin, nidogen, or heparan sulfate proteoglycan. In addition, the possible relationship between changes in structural composition of the GBM and proteinuria was not addressed in the present study.

In nephritic mice Type IV collagen and laminin were observed within the rough endoplasmic reticulum of parietal epithelial cells (Figure 4). In the normal adult murine glomerulus, laminin and Type IV collagen have not been localized intracellularly,⁷⁻⁹ a fact that probably reflects the low synthetic rate of these molecules. In autoimmune glomerulonephritis in mice, the parietal epithelial cells must receive some signal that triggers an increased synthesis and secretion of laminin and Type IV collagen. The synthetic rate must be increased to such an extent that the antigens become detectable by immunohistochemistry. The fate of these basement membrane components is not clear: Bowman's capsule does not appear thickened; therefore, these antigens must be either lost in the glomerular filtrate or incorporated into the GBM.

In the present study, mice were immunized not only with whole glomeruli or isolated GBM but also with a cell-derived glomerular preparation. This was done to test the hypothesis that the pathogenic antigen-antibody interaction in murine autoimmune glomerulone-

phritis occurs at the level of the plasma membrane of the epithelial and/or endothelial cells, cells that produce the GBM. Our failure to induce significant disease in mice immunized with cellular fraction does not completely rule out this possibility, because basement membrane antigens expressed on the surface of glomerular capillary cells may have been lost during the preparative process.

It would be important to know, of all the basement membrane antigens present in human GBM, which one(s) elicits an antibody response, and which one(s) is responsible for the immunologic injury. No definitive answer is available, but it is clear that the ultrastructural localization of the IgG eluted from the diseased mouse kidneys is different from that of antibodies directed against laminin, heparan sulfate proteoglycan, and Type IV collagen. The eluted antibodies localized in normal mouse GBM in the lamina rara interna and to some extent in the lamina densa. Type IV collagen is present in the three laminae, but preferentially in the lamina densa.^{8,9} Laminin is also found in the three layers, but preferentially in the laminae rarae.⁷ Heparan sulfate proteoglycan is predominantly localized in discrete clusters in both laminae rarae.^{2,8} The localization of the eluted antibodies closely resembles that of entactin. This glycoprotein is found preferentially in the lamina rara interna and lamina densa.⁶ The localization of the eluted antibodies in tubular basement membrane is also similar to that of entactin.⁶ Obviously, similar ultrastructural localization is not definitive evidence, but it seems to rule out three components and suggests that entactin, or other GBM components with a localization similar to that of entactin, may play a crucial role in this murine model of anti-GBM antibody-mediated glomerulonephritis.

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