Immunoenzymatic study of the protein pathway through the glomerular barrier in rat glomerulonephritides

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Immunoenzymatic study of the protein pathway through the glomerular barrier in rat glomerulonephritides. Circulating anti-horseradish peroxidase (HRP) IgG antibodies were used in the rat to study the glomerular leakage of proteins in glomerulonephritis (GN) induced by aminonucleoside (AN) and in glomerulonephritis induced by mercuric chloride to produce anti-glomerular basement membrane (GBM) antibodies. In ANGN, autologous albumin and fibrinogen were also detected by immunoperoxidase techniques. In both types of GN, the proteins studied were observed in the glomerular urinary space and proximal tubular cells. No channels were visible in the lamina densa. No accumulation of proteins was seen under the epithelial slits that were not closed. In ANGN, accumulation of proteins was observed in the subepithelial space where the podocytes act as a barrier (closed slits, subepithelial blind pockets, areas covered by broad sheets of cytoplasm), but no accumulation was seen in the lamina rara externa under normal or enlarged slits and areas of large epithelial cytoplasm detachment. Statistical analysis showed that in ANGN, at the time of maximal proteinuria, the number of "micropinocytotic" vesicles in the GBM-embedded part of podocytes was not increased as compared with controls. Such vesicles were not labeled. We conclude that in both types of GN, the permeability of the GBM is diffusely increased and that the plasma proteins pass into the urinary space via an extracellular pathway.

Etude immunoenzymatique du passage de protéines à travers la barrière glomérulaire au cours de glomérulonéphrites du rat. La détection d'IgG circulantes anti-peroxydase (PO) a été employée chez le rat pour étudier la fuite glomérulaire des protéines dans la glomérulonéphrite (GN) par aminonucléoside (AN) et la GN par anticorps anti-membrane basale glomérulaire (MBG) induite par le chlorure de mercure. Dans la GNAN, l'albumine et le fibrinogène autologues ont aussi été détectés par les techniques d'immunoperoxydase. Dans les deux GN, les protéines étudiées ont été mises en évidence dans l'espace urinaire glomérulaire et dans les cellules tubulaires proximales. Il n'a pas été vu d'accumulation de protéines sous les fentes épithéliales, ni de passage privilégié de celles-ci à travers certains segments de la lamina densa. Dans la GNAN, une accumulation de protéines a été observée sous les podocytes là où ceux-ci jouaient un rôle de barrière (jonctions étroites, poches aveugles sous épithéliales, zones de "fusion" des pédicelles). En revanche, aucune accumulation n'a été vue dans la lamina rara externa là où existaient des fentes épithéliales normales ou élargies, ou bien des zones de détachement épithélial. L'analyse statistique a montré que le

nombre des vésicules de "micropinocytose" à l'insertion des podocytes sur la MBG n'était pas supérieur dans la GNAN à celui observé chez les rats contrôles. Ces vésicules étaient négatives. Ces faits montrent que la perméabilité de la MBG est augmentée de façon diffuse dans les deux GN et que les protéines plasmatiques passent dans l'espace urinaire par voie extracellulaire.

Mechanisms responsible for proteinuria in human and experimental glomerulonephritis (GN) are still much debated. The glomerular lesions produced by aminonucleoside (AN) are well known [1–11], but the leakage of proteins from glomeruli is interpreted diversely, depending on the type of intoxication, the type of fixation, and the tracer used [5, 7, 9–15] (Table 1). Protein leakage has been studied also ultrastructurally in GN induced by antiglomerular basement membrane (GBM) antibodies [16–18].

The detection of circulating autologous or heterologous antihorseradish peroxidase (HRP) IgG antibodies is a more reliable technique for the study of the glomerular filtration barrier in normal rats than are the classical immunoenzymatic techniques using conjugates [19, 20]. Indeed, circulating anti-HRP antibodies can be detected by incubating the sections with HRP, as HRP diffuses uniformly into the tissues because of its low mol wt (40,000 daltons). Detection of protein by the classical immunoenzymatic techniques requires incubation of the

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Table 1. Data on glomerular filtration barrier in aminonucleoside glomerulonephritis[a]

Reference no.	Tracer and mol wt *daltons*	Fixation	AN injections	Level of the main barrier	Permeability of the GBM	Protein pathway
[5]	Ferritin 480,000	Injection in situ	Multiple	GBM	Diffusely increased	In major part through the podocytes by pinocytotic process
[9]	Ferritin 480,000	Immersion	Single	GBM	Focally increased	Sites of externally bare GBM
[7]	HRP 40,000	Immersion	Multiple			Remnant slits; epithelial vacuolar pathway
[13]	Catalase 240,000	Immersion	Multiple	GBM, slit diaphragms	Increased	Epithelial vacuolar pathway
[10]	Dextrans 62,000, 125,000	Injection in situ	Multiple	GBM	Diffusely increased	Remnant slits
[14]	Lanthanum	Immersion	Multiple		Diffusely or focally increased among the protein	
[15]	IgG 160,000 Alb 69,000 (detected by immunoperoxidase)	Drop in situ	Single	Endothelial fenestrae level	Focally increased	Sites of externally bare GBM
Present study	Antiperoxidase IgG	Drop in situ	Single	Lamina densa	Diffusely increased	Extracellular pathway

[a] Abbreviations used are GBM, glomerular basement membrane; HRP, horseradish peroxidase; Alb, albumin.

sections with HRP-labeled antibodies (conjugates), which diffuse heterogeneously because of their high molecular weight.

Methods

We used 15 Munich-Wistar (MW) male rats, each weighing 180 to 250 g, and eight Brown Norway (BN) male rats, each weighing 150 to 200 g, purchased from the Centre de Sélection et d'Elevage d'Animaux de Laboratoire (Centre National de la Recherche Scientifique, Orléans, La Source, France). We also used 6 PVG/c male rats, each weighing 200 g, obtained from the Laboratory Animals Center, Medical Research Council (Carshalton Surrey, England).

Proteins studied

Anti-HRP IgG antibodies. Two kinds of anti-HRP IgG antibodies were used: heterologous anti-HRP antibodies and autologous anti-HRP antibodies. The first of these was obtained from a sheep immunized with HRP as previously described [19, 20]. The antiserum was tested with HRP by Ouchterlony's technique and by immunoelectrophoretic analysis using a rabbit antiwhole normal sheep serum antiserum. Precipitation lines were stained after incubation with HRP. It was thus shown that the anti-HRP antibodies were IgG antibodies. The

antiserum contained 4 mg/ml sheep anti-HRP IgG as determined by quantitative immunoadsorption [21]. The autologous anti-HRP antibodies were detected in MW rats immunized with HRP as previously described [19]. These antibodies were also shown to be IgG antibodies. The concentration of specific autologous anti-HRP IgG antibodies was not determined. Detection of both autologous and heterologous antibodies was performed to determine whether IgG circulating for a limited or prolonged lapse of time could influence their pathway into the urinary space.

Autologous albumin and fibrinogen. We used classical immunoenzymatic techniques [19] to detect autologous rat albumin and rat fibrinogen. Antirat albumin and antirat fibrinogen antisera were raised in sheep. The procedures used for obtaining antisera, for the purification of antibodies, and for labeling by HRP have been described elsewhere [19]. For control experiments, we purified and labeled with HRP normal sheep IgG.

Experimental procedures

Glomerulonephritis induced by aminonucleoside. We injected each of 11 MW rats and 6 PVG/c rats with a single i.v. dose of puromycin aminonucleoside (6-dimethylamino-9[3'-amino-3' deoxyribosyl] purine) (Sigma Chemical Company, Saint

Table 2. Statistical analysis of the number of micropinocytotic vesicles in normal rats and in rats with aminonucleoside glomerulonephritis at the time of maximal proteinuria[a]

	GBM length μm	No. of "micropinocytotic" vesicles
Normal rats ($N = 40$)	12.72 ± 8.12	1.18 ± 1.24
Proteinuric rats ($N = 40$)	9.86 ± 6.24	1.78 ± 1.98
P	< 0.05	< 0.001

[a] Values are the means $\pm$ SD.

Louis, Missouri), 15 mg/100 g of body wt, as a 2% solution in saline. We looked for heterologous anti-HRP antibodies 45 min after the i.v. injection of sheep anti-HRP antiserum, 1 ml/100 g body wt, in 4 MW rats. We looked for autologous anti-HRP IgG antibodies in 2 MW rats immunized with HRP. In 5 MW rats and in the 6 PVG/c rats, we looked for autologous albumin and fibrinogen. Rats were sacrificed on days 2, 4, 7, 8, and 21 after the AN injection. Twenty-four-hour urinary protein excretion was measured on the day before the sacrifice.

Anti-GBM antibodies induced by mercuric chloride. We injected i.v. 6 BN rats three times a week with mercuric chloride at 0.10 mg/100 g body wt [22, 23]. Urinary protein excretion was measured three times a week. Rats were sacrificed on days 14 or 23 after the beginning of the mercuric chloride injections. Rats were sacrificed either 5 min, 45 min, or 24 hours after the injection of anti-HRP antiserum.

Controls. We injected s.c. 4 normal MW rats and 2 BN rats with water only to use as controls. We looked for anti-HRP antibodies in 2 normal MW rats and the 2 control BN rats, 45 min after the i.v. injection of anti-HRP antiserum. We looked for autologous albumin and fibrinogen in 2 normal MW rats.

Immunohistochemical and ultrastructural procedures

Ultrastructural detection of proteins. The fixation procedure depended on the strain studied. We processed the MW rat kidneys by in situ fixation with a 2% glutaraldehyde solution according to Ryan, Hein, and Karnovsky [15]. For PVG/c and BN rats, which do not have superficial glomeruli, we used immersion fixation with Karnovsky's mixture [24]. Both fixation procedures are reliable [19]. To detect anti-HRP antibodies, we incubated kidney sections with HRP alone, as previously described [25]. HRP activity was then revealed [26]. To detect autologous rat albumin and fibrinogen, we incubated kidney sections with specific HRP-la-

beled conjugates [19], and HRP activity was then revealed. Finally, we processed the sections for electron microscopy, as described elsewhere [19]. The sections were examined without counterstaining.

Serial kidney sections were performed on at least three glomeruli on each rat with autologous or heterologous anti-HRP IgG antibodies sacrificed on days 7, 8, or 21 after AN injection.

Morphometric analysis. A morphometric analysis was performed to compare the number of "micropinocytotic" vesicles located in the part of the podocytes adjacent to the GBM in the 4 MW rats sacrificed at the time of maximal proteinuria on days 7 through 8 after AN injection with the number present in the 4 MW control rats. The only vesicles referred to as "micropinocytotic" were those that appeared closed on the section plane and that were located less than one vesicular diameter from the GBM-embedded cell coat of podocytes. To obtain a significant number of vesicles and a significant length of GBM for a given surface of micrograph, we used a final magnification of $\times 31,000$. Forty micrographs in each group of ANGN and control rats were randomly sampled after the lengths of the GBM samples of each group were checked for a normal distribution. The precision achieved with the digitizer (Hewlett Packard, Model 9864 A) was of the order of 2%. The H_o hypothesis for the difference between homologous columns (Table 2) was tested by Student's t test.

Immunofluorescence microscopy. At sacrifice, kidney cryostat sections from BN rats were studied by an immunofluorescence technique using a fluoresceinated sheep antirat IgG antiserum to test for linear staining along the glomerular capillary wall.

Controls. Kidney sections from normal rats were studied by electron microscopy after incubation with HRP-labeled normal sheep IgG, HRP, or 1,4-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide. Normal MW kidney rat sections were also studied after incubation with sheep anti-HRP antiserum and further processed for detection of HRP activity.

Results

Control rats. Proteinuria was insignificant (<15 mg per 24 hours). Results obtained with anti-HRP antiserum, antirat albumin, and antirat fibrinogen conjugates after in situ or immersion fixation of kidney tissue from MW and BN rats were similar to those previously described in normal MW rats [19, 20].
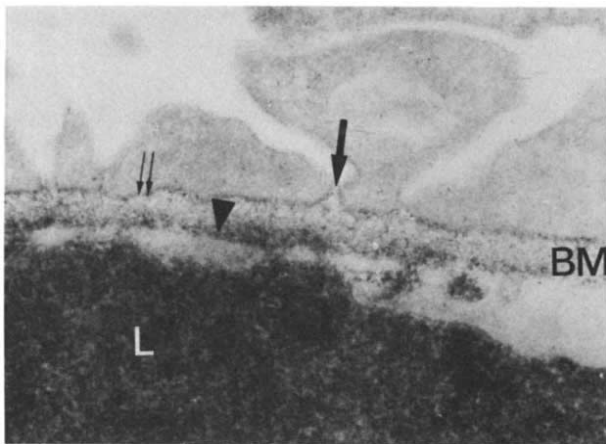
Fig. 1. *Munich-Wistar rat sacrificed 2 days after aminonucleo-side injection, 45 min after injection of antiperoxidase anti-serum: Superficial glomerulus fixed in situ.* The plasma is homogeneously labeled in the lumen (L). In the basement membrane (BM), the lamina rara interna *(arrow head)* is more intensely labeled than are the lamina densa and the lamina rara externa. The embedded cell coat of pedicels *(arrows)* is stained. The free cell coat of pedicels is unlabeled. No accumulation of reaction product is seen under the slit diaphragms *(arrow)* (×34,000)

Glomerulonephritis induced by aminonucleoside

(1) Detection of anti-HRP IgG antibodies after in situ fixation of the kidney:

(a) Rat sacrificed 2 days after AN injection and 45 min after injection of anti-HRP antiserum. Proteinuria was insignificant (13 mg per 24 hours). The ultrastructural appearance and the staining pattern were identical to those of normal rats (Fig. 1). The lamina rara interna (LRI) appeared much more intensely stained than did the lamina densa (LD). The embedded cell coat of pedicels continuous with the lamina rara externa (LRE) was weakly stained. No accumulation of reaction product was seen under the slit diaphragms. No staining was seen in the urinary space and on the free cell coat of pedicels.

(b) Rats sacrificed 7 to 8 days after AN injection and 45 min after the injection of anti-HRP anti-serum and rats immunized with HRP and sacrificed 7 to 8 days after AN injection. The glomerular changes in rats immunized with HRP and in those injected with anti-HRP antiserum were identical. At the time of sacrifice, proteinuria was 390 to 1,135 mg per 24 hours. The well-known [1–11] ultrastructural lesions of podocytes were observed. Broad continuous sheets of epithelial cytoplasm covered the outer aspect of the GBM. The contours of the podocytes were highly irregular with many sinuses and recesses. The spaces were usually bordered by one or more cells. The number of epithelial slits was reduced. The remaining slits appeared normal, enlarged, or closed. In numerous areas, there was irregular enlargement of the LRE. In other areas, subepithelial blind pockets or focal loss of the epithelial covering were observed. The endothelial cells were often swollen with a decreased number of fenestrae.

The staining patterns observed with autologous anti-HRP IgG and heterologous anti-HRP IgG were identical and therefore will be described together. In the glomerular loops, the LRI was generally more intensely stained than was the lamina densa LD. When the irregularly enlarged LRE was covered by broad sheets of epithelial cytoplasm without slits, it was stained as or more intensely than the LD, and the staining of the LRI was often of the same intensity as that of the LD (Fig. 2, a and b). In contrast, when the LRE was covered with pedicels separated by normal or enlarged slits, it was more sparsely stained than the LD (Fig. 3), and reaction product was seen along the free cell coat of pedicels and in the urinary space. The small subepithelial pockets contained a material that was often as dense and homogeneous as plasma (Fig. 4), but when the subepithelial pockets were the result of extensive detachment of the podocyte, they contained stained material that was usually scattered (Fig. 5). Accumulation of the reaction product was seen under the closed slits (Fig. 6). The LD was homogeneously stained and its staining was not enhanced in the sites underlying the epithelial slits, the pockets containing positive material, or the areas of bare GBM. The numerous and apparently intracytoplasmic epithelial "vacuoles" contained stained material. Serial sections clearly demonstrated that most of these "vacuoles" were in fact recesses of irregularly shaped extracellular spaces (Fig. 7) or subepithelial blind pockets (Fig. 8) opening onto the GBM. No extrusion of stained material from the podocytes into the urinary space was observed. Morphometric analysis revealed no significant differences between the mean lengths of GBM or the mean number of "micropinocytic" vesicles located in the GBM embedded part of podocytes, between proteinuric and control rats (Table 2). Such vesicles were not stained. In contrast, on the other side of the GBM, numerous stained "micropinocytotic" vesicles were seen in swollen endothelial cells (Fig. 2 b).

The GBM and the podocytes that cover the mesangial areas had a similar appearance to those that cover the loops. When the GBM was covered
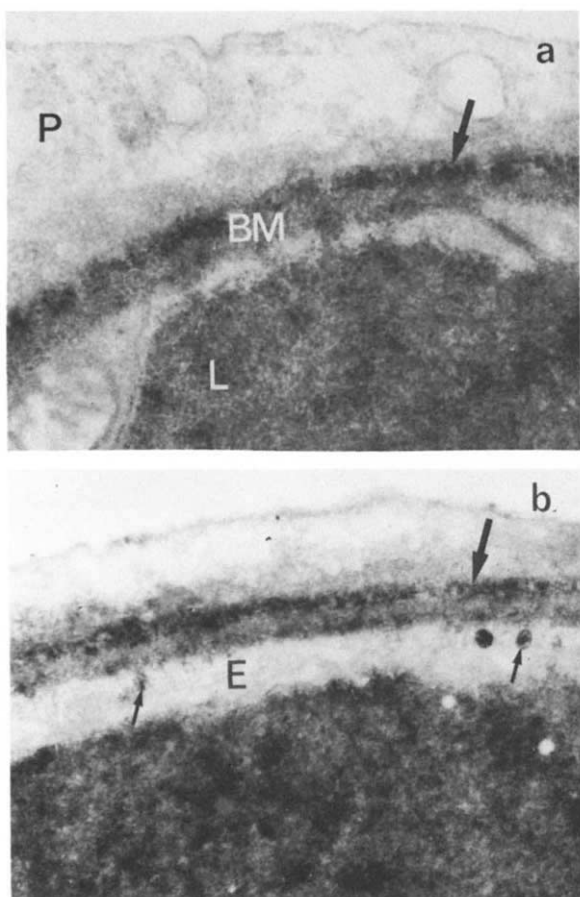
Fig. 2. a *Munich-Wistar rat immunized with peroxidase, sacrificed 8 days after aminonucleoside injection.* **b** *Munich-Wistar rat sacrificed 8 days after aminonucleoside injection, 45 min after the injection of antiperoxidase antiserum: Superficial glomeruli fixed in situ.* In both cases, the staining pattern is identical. The plasma is homogeneously labeled in the lumen (L). The basement membrane (BM) is completely covered by a broad sheet of epithelial cytoplasm (P) without positive "micropinocytotic" vesicles. Accumulation of reaction product *(thick arrow)* is seen in the widened lamina rara externa. The "micropinocytotic" vesicles observed in the swollen endothelial cell (E) are stained *(thin arrow).* (× 34,000)

Fig. 3. *Same rat shown in Fig. 2a: Superficial glomerulus.* In this area, the lamina rara externa, located under remnant pedicels (P) and remnant slits is very sparsely labeled *(arrow head).* No accumulation of reaction product is observed under the slit diaphragms *(arrow).* No channels are visible in the basement membrane (BM). The lamina rara interna *(arrow heads)* is more intensely labeled than is the lamina densa. No channels are visible in the lamina densa. Reaction product is seen along the entire cell coat of pedicels and free in the urinary space (U). L denotes lumen. (× 34,000)

by a broad sheet of epithelial cytoplasm, the LRE was intensely stained (Fig. 9). In contrast, when epithelial detachment and remaining enlarged slits were present, the LRE was sparsely stained (Fig. 10). The mesangial matrix was always more markedly stained than the overlying LD. In numerous proximal convoluted tubules (PCT), lysosomes were positive (Fig. 11). Moreover, stained lysosomes were seen in certain cells of several distal tubules.

(c) Rat sacrificed 21 days after AN injection and 45 min after the injection of anti-HRP antiserum. Proteinuria was 385 mg per 24 hours at the time of
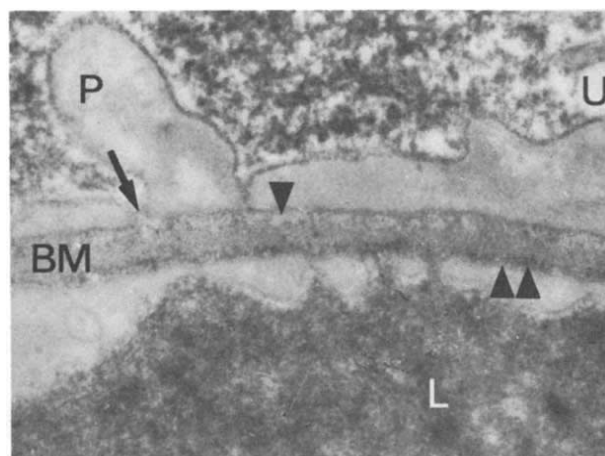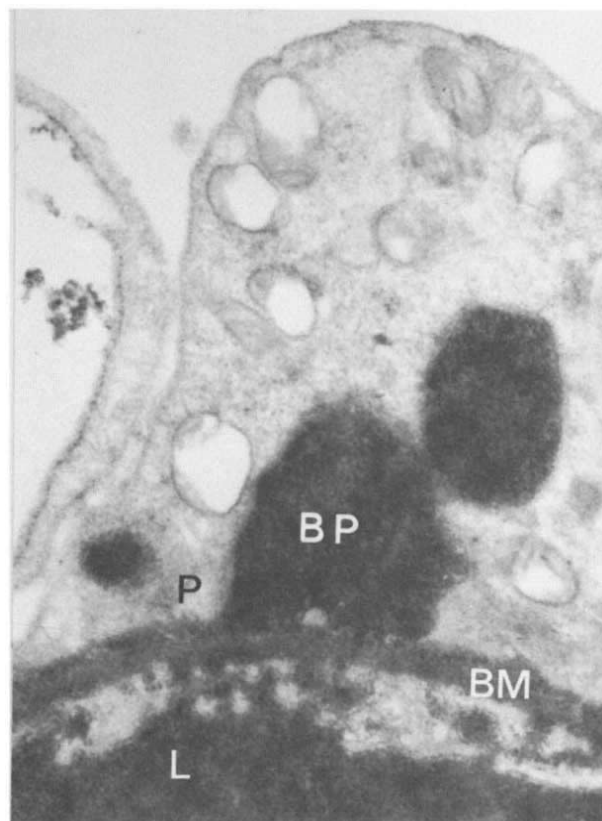

Fig. 4. *Same rat shown in Fig. 2 a: Superficial glomerulus.* The basement membrane (BM) is covered by a broad sheet of epithelial cytoplasm (P). The small subepithelial blind pocket (BP) is filled with homogeneous reaction product. L denotes lumen. (× 25,000)
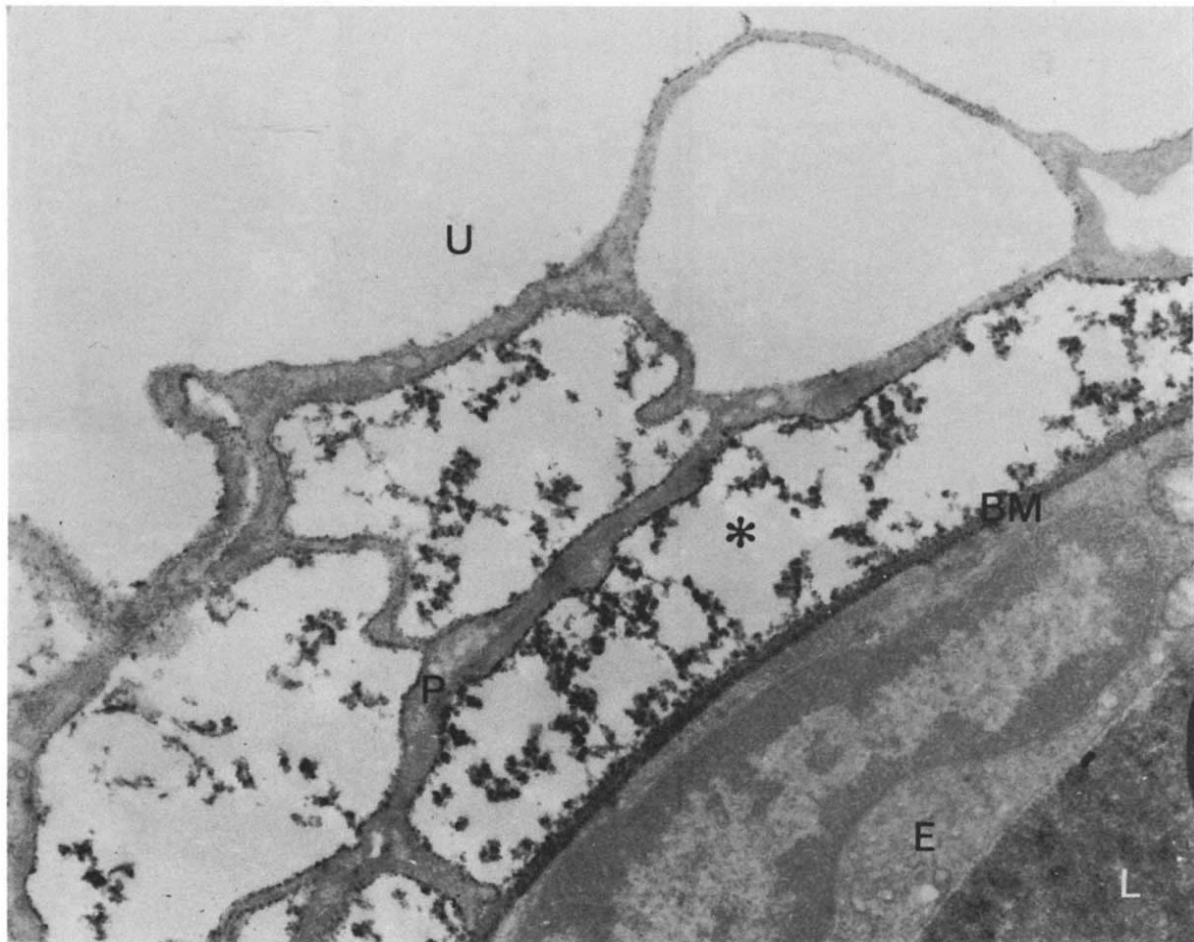
Fig. 5. *Same rat shown in Fig. 2 a.* The basement membrane (BM) decreased in thickness is not covered by a podocyte (P). In the area of extensive epithelial detachment *(asterisk),* note the scattered positive material. E denotes endothelial cell; L, lumen. (×25,000)
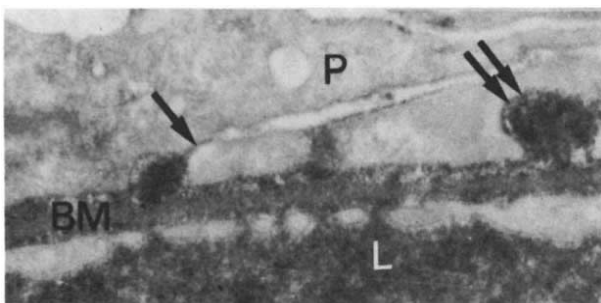

Fig. 6. *Same rat shown in Fig. 2 a.* Note the accumulation of reaction product under closed junction *(arrow)* and in blind pocket *(arrows).* P denotes podocyte; BM, basement membrane; L, lumen. (×34,000)

sacrifice. The ultrastructural glomerular lesions were more focal than they were in rats sacrificed on day 7. In numerous areas, the pedicels were differentiated. The LRI was more heavily stained than the LD. The staining of the irregularly widened

LRE was more dispersed than that of the LD, particularly where pedicels and slits were present. The embedded cell coat of the pedicels was faintly stained. Reaction product was detected between some pedicels and in the urinary space. The staining of the LD was not increased in areas adjacent to the slits containing positive material. Positive staining was also observed in the endocytotic apparatus and the lysosomes of some PCT.

(2) *Detection of autologous albumin and fibrinogen after in situ fixation of the kidney in rats sacrificed on day 7 after AN injection.* Proteinuria was 507 to 598 mg per 24 hours at the time of sacrifice. The well-developed ultrastructural changes of ANGN were observed. The penetration of conjugates was somewhat heterogeneous, but when the penetration of conjugates was considered adequate, the distribution pattern of albumin and fibrinogen in glomeruli was identical to that observed with anti-HRP antiserum in rats sacrificed 7
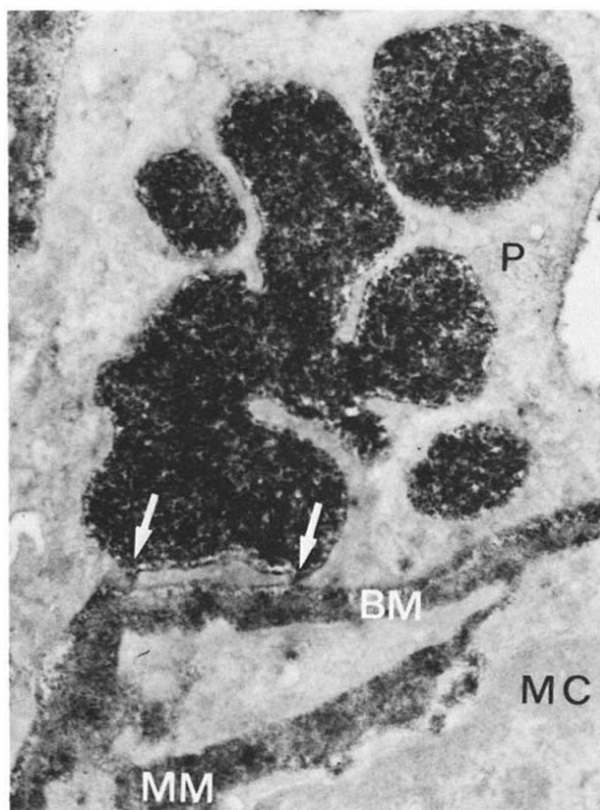
Fig. 7. *Munich-Wistar rat sacrificed 8 days after aminonucleo-side injection, 45 min after the injection of antiperoxidase antiserum: Superficial glomerulus fixed in situ.* In the podocyte (P), an irregularly shaped extracellular space is open to the basement membrane (BM) at the site of remnant slits *(arrow)*. MC denotes mesangial cell; MM, mesangial matrix. (× 20,000)

PCT were collapsed and contained stained material. The brush borders were heterogeneously stained.

Glomerulonephritis induced by mercuric chloride

No proteinuria. One rat treated with mercuric chloride had no proteinuria (13 mg per 24 hours) at the time of sacrifice on day 14. Linear fixation of the fluoresceinated antirat IgG antiserum was observed along the capillary walls of all glomeruli. The kidney specimen was taken 45 min after anti-HRP antiserum injection. Ultrastructural lesions were identical to those previously described [27] with an afflux of monocytes, which were seen in close contact with the inner aspect of the GBM in capillary lumina. But no differences were observed in the staining pattern when compared with control rats, with no reaction product seen in the urinary space.

Proteinuria. Five rats treated with mercuric chloride had proteinuria. Proteinuria was 57 to 262 mg/24 hours at the time of sacrifice. In all 5 rats, linear fixation of the fluoresceinated antirat IgG antiserum was seen along the glomerular capillary walls of all glomeruli. The staining pattern observed by electron microscopy was identical regardless of whether the rats were sacrificed on days 14 or 23 or whether the anti-HRP antiserum was injected 5 min, 45 min, or 24 hours before the biopsy was performed. Numerous endothelial cells were swollen and detached from the GBM. The pedicels were swollen, with a decreased number of slits. The remaining slits appeared normal. In the irregularly widened subendothelial space, the reaction product showed the same homogeneity and the same density as it did in the capillary lumen. A sharp drop in the intensity of the labeling was seen on the inner side of the LD (Fig. 14). The staining pattern of the LD, LRE, and embedded cell coat of the pedicels in the loops and in the mesangium were all similar to those of control rats. Some clumps of reaction product as positive as the plasma were seen in the urinary space between and beyond the slits whether or not the endothelial cells were detached from the GBM. Labeling of the free cell coat of the pedicels was observed where free reaction product was found in the urinary space. No accumulation of the reaction product was seen under the slit diaphragms. No increase in staining intensity of the LD was observed where monocytes were in contact with the GBM (Fig. 15) or in the sites under the slits containing positive material. No channels were visible in the LD. Positive "micropinocytotic" vesicles in pedicels and positive vacuoles inside
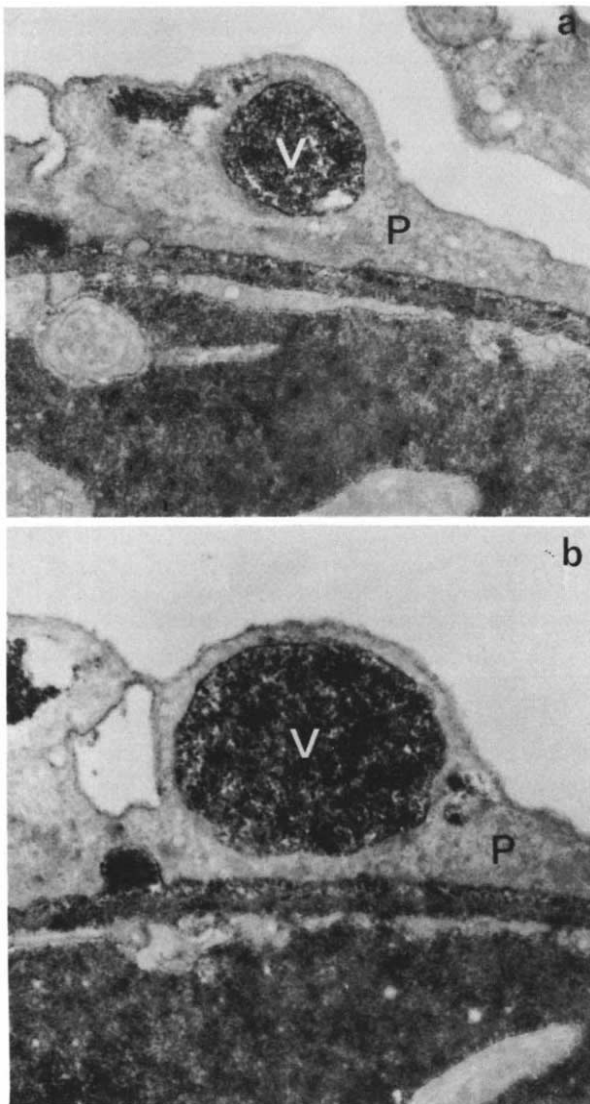
to 8 days after AN injection (Fig. 12). But with albumin, the LRI and the LD were heavily stained so that it was often difficult to distinguish between the two laminae. Albumin and fibrinogen were detected on brush borders and in the endocytotic apparatus of numerous PCT, but not in lysosomes (Fig. 13).

(3) Detection of autologous albumin and fibrinogen after immersion fixation of the kidney in rats sacrificed on days 4, 7, or 21 after AN injection. Proteinuria was 278 to 396 mg per 24 hours in rats biopsied on day 4, 507 to 598 mg per 24 hours in rats biopsied on day 7, and 506 to 713 mg per 24 hours in rats sacrificed on day 21 after AN injection. The glomerular ultrastructural lesions were more severe and diffuse on day 7 than day 4 or 21. Albumin and fibrinogen had a similar distribution, identical to that observed after in situ fixation. The lumina, however, were often empty, and in these instances, the LRI was poorly stained. All the lumina of the

Fig. 8. *Same rat shown in Fig. 7: Superficial glomerulus (serial sections).* The subepithelial blind pocket open *(arrow)* to the basement membrane (BM) in section c appears to be an intraepithelial vacuole in sections a and b. P denotes podocyte. ($\times$ 20,000)

podocytes or mesangial cells were seen in exceptional instances. At the periphery of the podocytes, however, some positive "micropinocytotic" vesicles were observed in contact with clumps of free reaction product in the urinary space. Staining was observed in the endocytotic vesicles of some PCT in the specimens taken 5 min, 45 min, or 24 hours after injection of the anti-HRP antiserum. Staining was also seen in the lysosomes of some PCT in the specimens taken 45 min or 24 hours after the injection. Such tubular staining was seen whether
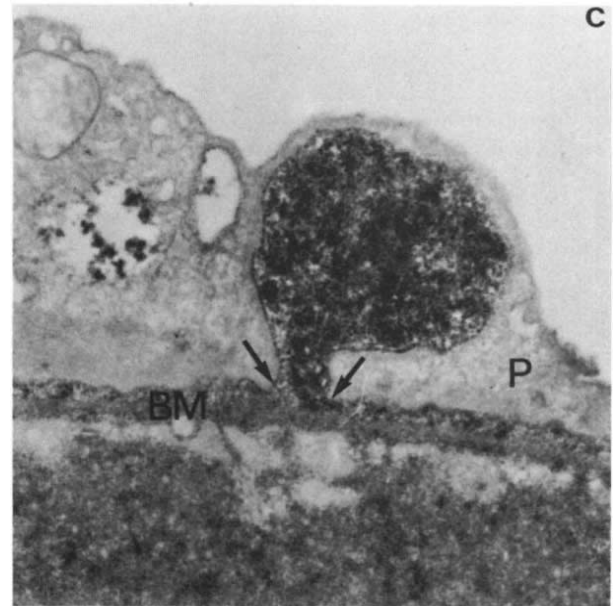
the classical tubular lesions induced by mercuric chloride were present or not.

Histochemical controls

After incubation with DAB and hydrogen peroxide alone, no HRP activity was seen except for the well-known endogenous activity of red blood cells, polymorphonuclear cells, and occasional microbodies and mitochondrial cristae. Similar results were observed when sections were incubated with *(1)* HRP, *(2)* anti-HRP antiserum, or *(3)* HRP-labeled normal sheep IgG.

Discussion

The present immunoelectromicroscopy study of glomerular permeability in rats with GN induced by aminonucleoside or mercuric chloride was concerned with the detection of serum proteins, as these are more physiologic markers than enzymatic or particulate tracers are. The most reliable results were obtained with anti-HRP IgG antibodies, which were detected by incubating the renal cortex with HRP, a low molecular-weight marker. With this technique, it has been shown in normal rats [19, 20] that circulating anti-HRP IgG are homogeneously detected throughout the renal cortex, even intracellularly.

Glomerulonephritis induced by aminonucleoside

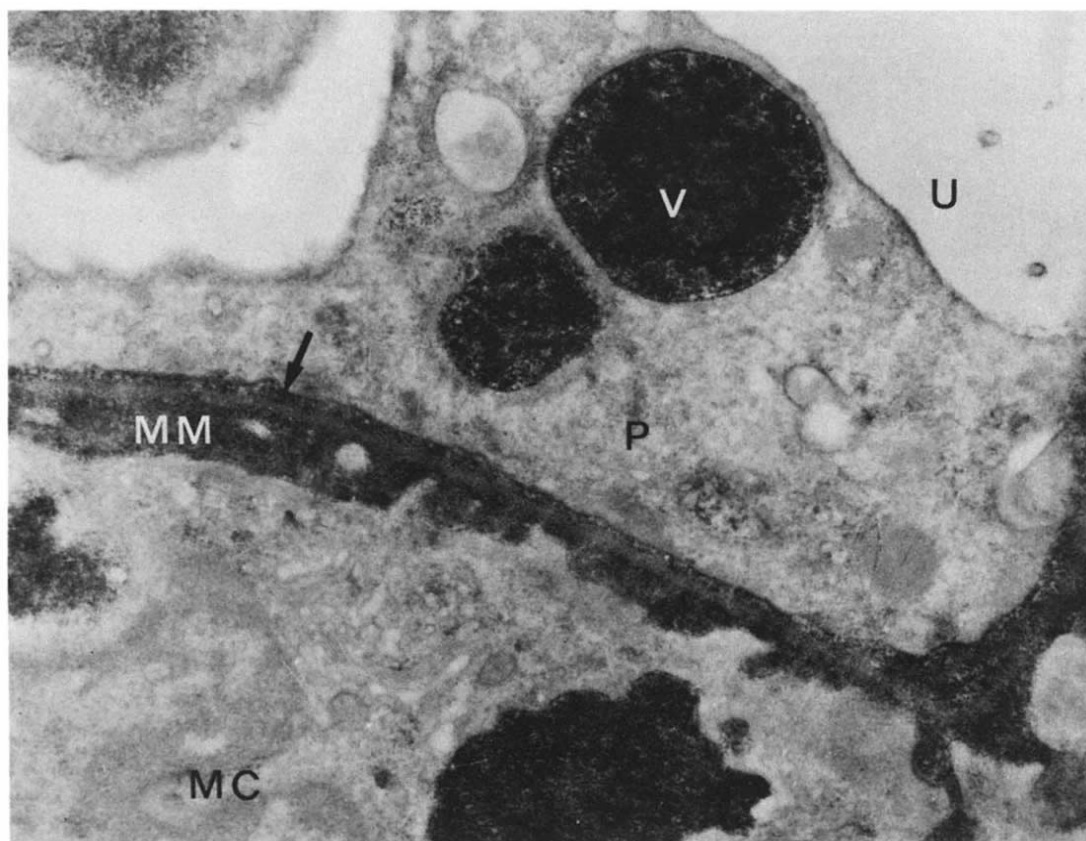Although the natural history of the ultrastructural glomerular lesions has been described extensively

Fig. 9. *Munich-Wistar rat sacrificed 8 days after aminonucleoside injection, 45 min after injection of antiperoxidase antiserum: Mesangial area of a superficial glomerulus fixed in situ.* The lamina rara externa is completely covered by a broad sheet of epithelial cytoplasm (P) and is intensely labeled *(arrow)*. The mesangial matrix (MM) is more intensely stained than is the overlying lamina densa. MC denotes mesangial cell; V, structure appearing as an intracytoplasmic vacuole; U, urinary space. (×20,000)

[1–11], the ultrastructural and biochemical basis for increased glomerular permeability is still much debated. The interpretation of various authors are summarized in Table 1. The diversity of the protocols used explains, in part, the various conclusions reached.

In the present work, the most informative results concerning the pathway of protein leakage were obtained by the detection of circulating anti-HRP IgG antibodies. The pattern of labeling was identical with autologous and heterologous IgG, that is, in two instances where IgG antibodies circulate for a long or a short period of time. In rats sacrificed on days 7, 8, or 21 after AN injection, the anti-HRP IgG passed into the urine, for the reaction product was observed in the glomerular urinary space, in the endocytotic apparatus, and in the lysosomes of the PCT.

Several arguments support the concept that the passage of the studied proteins into the urinary space is essentially extracellular. *(1) Pattern of*

GBM staining. The LD was homogeneous and no channels were observed. The staining of the LRE was quite different depending on the epithelial covering. An accumulation of the reaction product was observed under the closed slits, in the subepithelial blind pockets, and in areas where the LRE was completely covered with broad sheets of epithelial cytoplasm. On the contrary, no accumulation of reaction product was seen under normal or enlarged slits, broad epithelial detachment, and sections of bare GBM. Free reaction product was detected in the urinary space facing these areas. *(2) Morphometric analysis.* There was no significant difference in the number of "micropinocytotic" vesicles in the GBM-embedded part of podocytes for a given GBM length between heavily proteinuric and normal control rats. *(3) Serial sections.* Vacuoles that appeared to be intracytoplasmic on section planes proved to be part of the extracellular compartment on serial sections and were not the morphologic expression of a micropinocytotic mechanism.
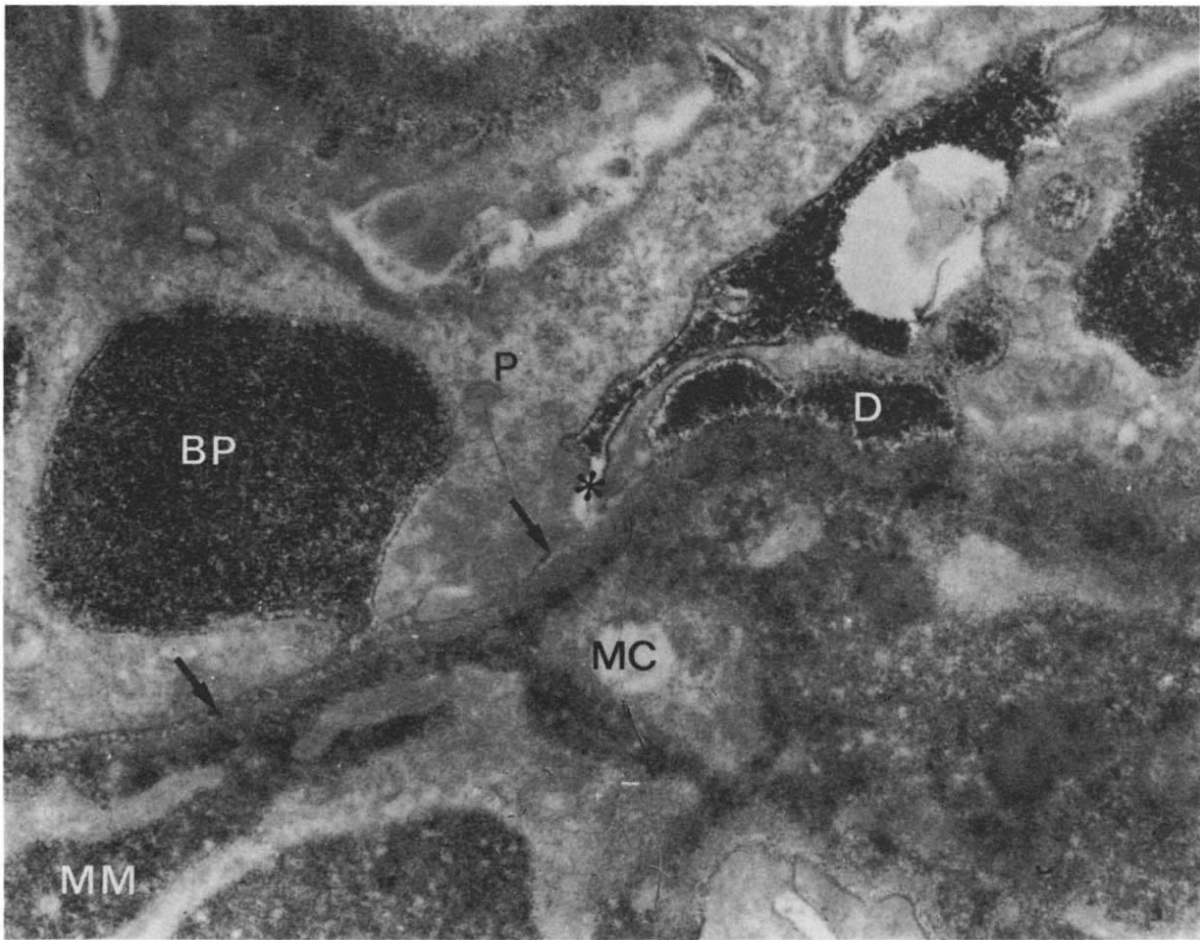
Fig. 10. *Same specimen shown in Fig. 9: Mesangial area of a superficial glomerulus.* Overlying the basement membrane, the following lesions are seen: epithelial detachment (D), enlarged slit *(asterisk)*, and blind pocket (BP), all containing positive material. The lamina rara externa is sparsely labeled *(arrow)*. The mesangial matrix (MM) is more intensely labeled than is the overlying lamina densa. P denotes podocyte; MC, mesangial cell. (×20,000)
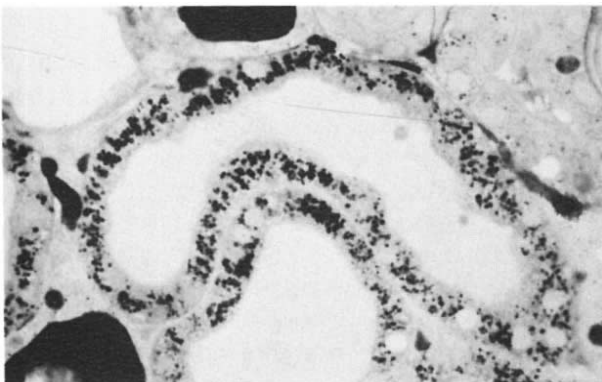

Fig. 11. *Munich-Wistar rat immunized with peroxidase, sacrificed 8 days after aminonucleoside injection: In situ fixation.* Numerous positive lysosomes are seen in the proximal tubular cells. (×500)

These findings suggest that the permeability of the LD is diffusely increased and that the studied proteins cross the LD to pass into the urinary space where slits and sites of bare GBM are present. In contrast, there was accumulation of the studied proteins in the subepithelial space in areas where the podocytes act as a barrier, that is, under the closed slits, in the blind pockets, in the LRE where it is completely covered with broad sheets of epithelial cytoplasm. An identical staining pattern in both the loops and the mesangial areas suggests that the latter are also involved in protein leakage.

Farquhar and Palade [5] and Caulfield and Farquhar [10], using ferritin and dextrans, also observed a diffuse increase in the permeability of the
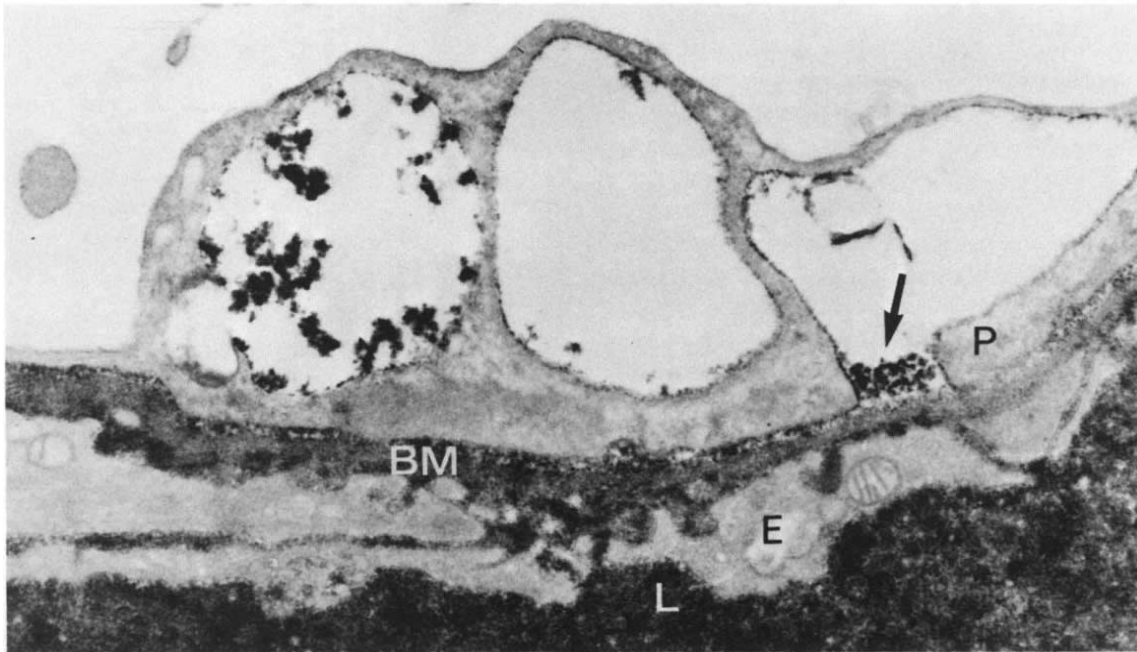
Fig. 12. *Munich-Wistar rat sacrificed 7 days after aminonucleoside injection: Detection of autologous in fibrinogen in superficial glomerulus fixed in situ.* Reaction product is observed in the area of epithelial detachment *(arrow).* P denotes podocyte; BM, basement membrane; E, swollen endothelium; L, lumen. (×20,000)

Fig. 13. *Same rat shown in Fig. 12: Detection of autologous fibrinogen in proximal convoluted tubule.* The brush border and some apical vesicles are labeled. (×20,000)

GBM, but Ryan and Karnovsky [9] found a focal increase with ferritin. Using the immunoperoxidase technique, the same authors also described a focal increase in GBM permeability [15]. Some of these discrepancies are probably related to the immunoenzymatic techniques, as we have previously

pointed out [19] (type of conjugates used, procedure of incubation with conjugates, and heterogeneous penetration of conjugates). Nevertheless, in the present study, when the penetration of conjugate was considered to be satisfactory, the glomerular and tubular distribution of the autologous proteins was similar to that of the anti-HRP IgG antibodies. These data demonstrate that, in ANGN, the glomerular pathway of albumin and fibrinogen is identical to that of anti-HRP IgG antibodies.

Particles such as ferritin [5] or dextrans [10] penetrate into GBM-embedded podocytes by "micropinocytosis." No positive "micropinocytotic" vesicles were seen in the GBM-embedded part of podocytes either with antialbumin and antifibrinogen conjugates, or with anti-HRP IgG revealed by incubation with HRP. Several hypotheses might explain the discrepancies seen between the pattern observed with particulate tracers and our present results: *(1)* In experiments using particulate tracers, GN was induced by repeated injections of AN [5, 10], whereas in the present study a single massive dose of AN was used. It is possible that the behavior of podocytes in these two experimental procedures is different. *(2)* The "micropinocytotic" vesicles in the GBM-embedded part of podocytes are unlabeled because conjugates or HRP have no

Fig. 14. *Brown-Norway rat treated with mercuric chloride sacrificed 24 hours after injection of antiperoxidase antiserum.* Proteinuria was 262 mg per 24 hours at the time of sacrifice. In the widened subendothelial space (SE), the reaction product is the same density as that in the capillary lumen (L). A sharp drop in the intensity of labeling is observed on the inner side of the lamina densa *(arrow)*. No accumulation of reaction product is seen in the lamina rara externa and under the slit diaphragms. Numerous epithelial slits persist, and reaction product is seen in the urinary space (U). E denotes endothelial cell; P, podocyte. ($\times$20,000)

access to intracellular structures. Indeed, negative results are difficult to interpret in immunohistochemistry. Such an hypothesis is feasible when conjugates are used to detect albumin and fibrinogen. But, when anti-HRP antibodies are revealed after incubation with free HRP, HRP easily penetrates into the cells, as has been shown in various circumstances [20, 25]. Furthermore, in the present study, numerous positive "micropinocytotic" vesicles were seen on the other side of the GBM in swollen endothelial cells. *(3)* The serum proteins could behave differently to particles such as ferritin and dextrans. Indeed, in the present study, at the time of maximal proteinuria, the number of "micropinocytotic" vesicles in the GBM-embedded part of podocytes was not statistically different from that
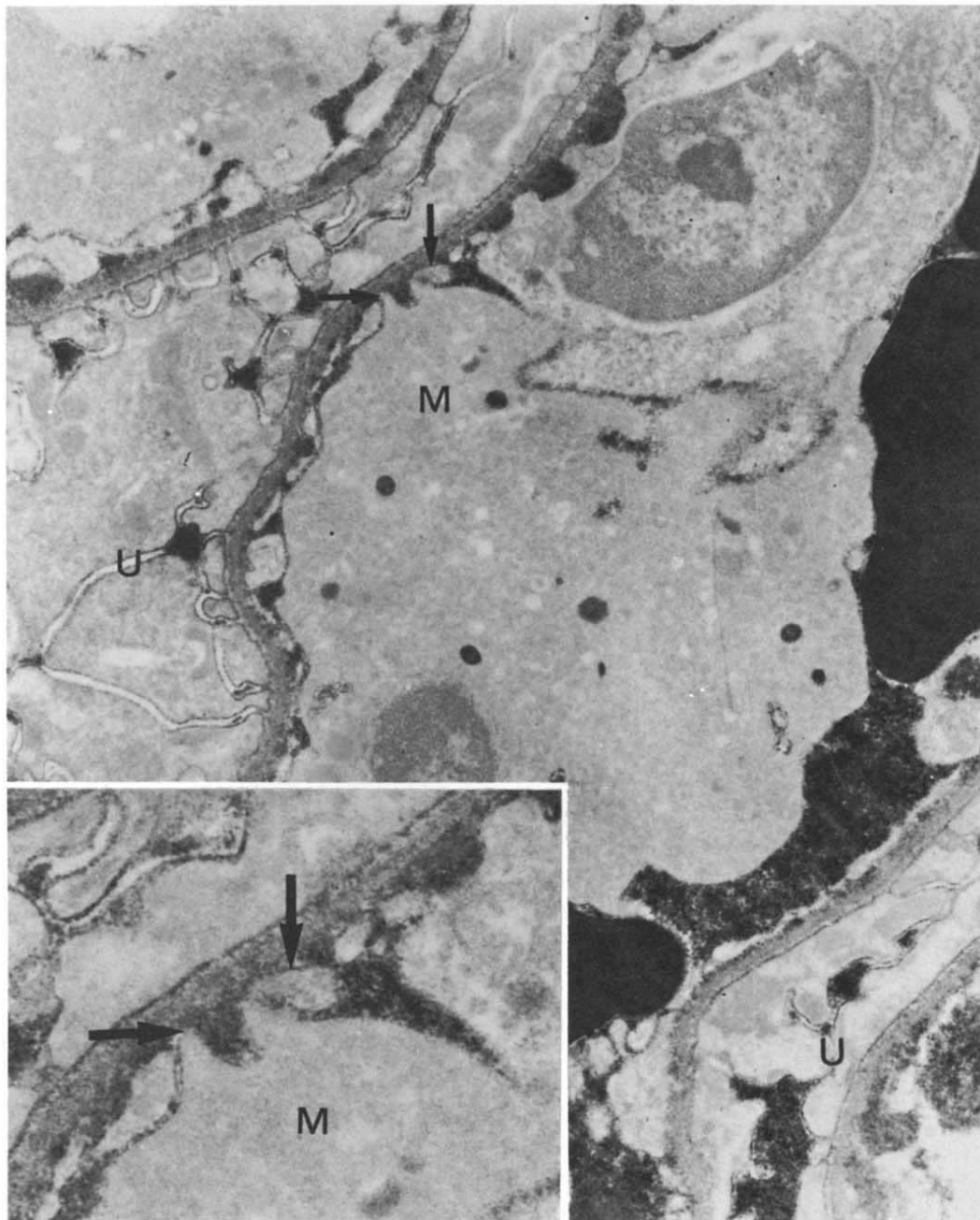
Fig. 15. *Same specimen shown in Fig. 14: Monocyte (M) in capillary lumen.* Note the contact of the monocyte with the inner aspect of the basement membrane between the two *arrows*. No channel of reaction product is seen at this site. Reaction product is seen in the urinary space (U). ($\times$17,000; insert, $\times$35,000)

found in control rats. The subepithelial accumulation of reaction product in the areas where the podocytes act as a barrier, as well as the absence of accumulation of reaction product in the LRE where an extracellular pathway exists between the GBM and urinary space, suggests that transepithelial protein leakage is a minor phenomenon if it does exist

at all. *(4)* The aim of this work was to study the protein pathway through the glomerular barrier. For this reason, serial sections and morphometric statistical analysis of micropinocytotic vesicles were done at the time of maximal proteinuria. It is possible that the endocytotic process described by Caulfield, Reid, and Farquhar in podocytes [11]

only occurs transiently at an early stage of the disease induced by a single injection of AN.

The role of electric charge was not evaluated in this study, but the staining pattern of proteins having very different IP, such as albumin, fibrinogen and IgG, was the same.

Glomerulonephritis induced by mercuric chloride

Because nephropathy from mercuric chloride can only be induced in BN rats [22], which lack superficial glomeruli, in situ fixation of the kidneys has not been used. Staining in the slits and in the PCT was only observed in rats with proteinuria and not in the control rats or in the rat treated with mercuric chloride, which had anti-GBM antibodies on its glomeruli but which had no proteinuria. This demonstrates that staining in the urinary space and in the PCT is not due to a diffusion process occurring during biopsy or fixation. Several points should be commented on. A sharp drop in the intensity of labeling was seen on the inner side of the LD. This was particularly striking in areas where the subendothelium was widened by the detachment of the endothelial cell. The staining in these areas was as intense as it was in the capillary lumen. The widened subendothelium is therefore permeable to plasma proteins, and the inner side of the LD thus acts as an important barrier. The LD was uniformly stained even in places where there was endothelial detachment or contact with monocytes. This suggests that there are no channels or areas across which the anti-HRP antibodies can selectively pass, as opposed to previous reports using ferritin and albumin systems [18]. Based on our data, there is no evidence to support the existence of a second barrier at the level of the slits or of the slit diaphragms, as no accumulation or reaction product was seen under the slit diaphragm or in the LRE. The staining in the spaces between the pedicels only observed in proteinuric animals, together with the lack of intracellular staining in the podocytes and the lack of positive "micropinocytotic" vesicles on the embedded cell coat of the pedicels, suggests that serum proteins that have penetrated into the LD pass freely into the urinary space across the slits. We found no evidence supporting a cellular pathway. The basement membrane that covers the mesangial area behaves in the same way as does that of the loops, which suggests that the mesangium is involved in protein leakage. Tubular reabsorption of filtered anti-HRP antibodies occurred even in tubular cells with lesions induced by mercuric chloride. This demonstrates that mercuric chloride, which is known to alter the tubules, does not impair the pattern of endocytosis or the lysosomal uptake of anti-HRP antibodies in the PCT.

Conclusion. In both types of GN with different physiopathologic mechanisms, proteinuria occurred because of a diffuse increase in the permeability of the LD. In both diseases, the proteins were transferred into the urinary space via an extracellular pathway, and no barrier was visible in the remaining normal or enlarged slits. The closed slits, the large expanses of podocytes, and the subepithelial blind pockets observed in ANGN appeared to prevent the leakage into the urinary space of the proteins that had crossed the GBM.

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