Glomerular Basement Membrane Anionic Charge Site Changes Early in Aminonucleoside Nephrosis

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Alterations of glomerular basement membrane (GBM) anionic (charge sites, CSs) in the development of proteinuria in a model of idiopathic nephrotic syndrome in man (puromycin aminonucleoside nephrotic syndrome [PAN] in the rat) were assessed quantitatively and sequentially early after disease induction. GBM CSs (known to consist mainly of heparan sulfate-rich proteoglycans) were stained *in vivo* and, in a separate group of animals by an *in vitro* method, with the cationic marker polyethyleneimine (PEI) and studied by electron microscopic examination. Four hours after administration of PAN, there was a significant decrease in GBM lamina rara externa CSs: 18 ± 0.7 *versus* 22.0 ± 2.2 per 1000 nm GBM in controls by PEI injection and 17.2 ± 2.7 *versus* 21.1 ± 1.6 per 1000 nm GBM in controls by PEI *in vitro* staining. This CS alteration coincided with changes in glomerular epithelial cell morphologic characteristics (increased cytoplasmic organelles and rough endoplasmic reticulum) and preceded the detection of foot process broadening (at 24 hours) and increased urinary albuminuria (suggested at 12–24 hours, statistically significant at 36–48 hours). These results suggest that GBM CS–heparan sulfate proteoglycan alterations consisting of either decreased number and/or less anionic charge occur early in PAN and support a role for glomerular epithelial cell maintenance of GBM CS for normal glomerular function. (Am J Pathol 1986, 125:393–401)

FIXED ANIONIC MACROMOLECULES (charge sites) have been demonstrated in the glomerular basement membrane (GBM) of several species.[1–4] These punctate sites appear as linear arrays in the lamina rara externa (LRE) and lamina rare interna (LRI), as revealed by a variety of cationic markers.[2,5] The sites consist predominantly of heparan sulfate proteoglycan[6] and resemble proteoglycan networks demonstrated in other basement membranes.[7,8]

The charge and physical-chemical characteristics of proteoglycan networks in the GBM appear to contribute to the permselectivity of the glomerular capillary wall.[9] Digestion of this network *in vivo* with heparinase and hyaluronidase increases ferritin and albumin penetration of the GBM.[10] Neutralization of GBM anionic sites with administration of polycations, such as protamine or polyhexedrine, causes proteinuria.[5] Alterations in GBM anionic sites occur in animal models of nephrotic syndrome[11,12] and immune complex nephritis[13] and in association with proteinuria in the congenital nephrotic syndrome[4] and diabetic nephropathy[14] in man.

The sequence of events leading to proteinuria in most renal diseases is poorly understood, but changes in the charge-selective function of the glomerular capillary wall occur in some proteinuric conditions.[15] Although early investigations focused on loss of glomerular epithelial cell anionic charge,[16] this alteration appears to be a late event and may be a secondary phenomenon.[17] Endothelial cell abnormalities may occur in some proteinuric conditions,[18] but studies by Farquhar,[9] Olson et al,[19] and others suggest that major changes of permeability occur in the GBM in most forms of proteinuria.

The aminonucleoside of puromycin (PAN) causes marked proteinuria, altered glomerular permeability, and nephrotic syndrome in the rat.[19,20] This experimental model runs a well-defined clinical and pathologic

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course[11,20] and appears to result from direct toxicity of the aminonucleoside for the glomerular epithelial cell.[21]

Several investigators have demonstrated decreased binding of cationic markers to the GBM in aminonucleoside nephrosis[11,12,19]; others have found no alterations of anionic charge in this model.[22] These studies, however, evaluated animals with well-established nephrotic syndrome, did not use quantitative morphologic techniques,[19] or used probes that may be insensitive to small changes in the GBM proteoglycan network.[22]

The present studies assess, quantitatively and sequentially, changes in GBM anionic sites and epithelial cell foot processes in PAN nephrotic syndrome. We chose the cationic hydrocarbon polyethyleneimine (PEI) for labeling GBM anionic sites *in vivo* and *in vitro*, because previous studies have used this compound to detect small changes in GBM anionic charge site density.[4,23] We were particularly interested in the GBM anionic sites and epithelial foot processes early after PAN administration before significant alteration in GBM function.

Materials and Methods

Male Sprague–Dawley rats weighing 160–200 g were kept in individual metabolic cages with free access to standard chow and water. After 3 days of acclimatization, baseline 24-hour urine collections were obtained. Test animals were then injected intraperitoneally with PAN (6-dimethylamino-9 [3′ amino-3′-deoxyrisbosyl] purine), 150 mg/kg body weight. Control animals received an identical volume of normal saline. Urine was collected at 12-hour intervals on all animals, and the cages and collection trays were rinsed with 3 ml distilled water at the end of each collection period.

Rat albuminuria was quantitated by radical immunodiffusion (RID), according to the technique of Mancini[24] as modified by Nevins et al.[25] Eight microliter aliquots of urine were assayed in duplicate by RID with five standards of rat albumin in concentrations from 11.3 to 180 mg/ml per plate. The timed urinary excretion of albumin was calculated for each animal and expressed as milligrams urinary albumin/12 hours during the collection period.

In Vivo PEI Studies

Control and test rats were studied at 4, 24, 48, 120, and 168 hours after injection of either normal saline or PAN (Table 1). Thirty minutes before PEI administration, the rats received diphenhydramine, 25 mg/kg intraperitoneally, followed by ether anesthesia and injection of PEI (1200 MW, Sigma) intravenously over 2 minutes in a dose of 20 μg/kg as a 0.5% solution in distilled water, pH 7.4. The animals were sacrificed 5 minutes after PEI injection. Cortical sections from the upper pole of the left kidney were cut into 1-mm cubes and fixed in 1% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4, 320 mosm/kg) for 1 hour, washed several times in buffer, and postfixed in 1% osmium tetroxide for 1 hour. The tissue was then rinsed, dehydrated through graded ethanol, and embedded in POLY BED 812 (Polysciences, Warrington, Pa).

In Vitro PEI Studies

Control and test animals were sacrificed at 0.5, 4, 24, 48, 72, and 120 hours after injection of either normal saline or PAN, and cortical sections were obtained from the upper pole of the left kidney. Samples were fixed in 1% glutaraldehyde and processed as above for glomerular epithelial cell foot process measurements. Other samples were fixed in 1% paraformaldehyde–lysine–sodium metaperiodate (PLP) for 2 hours, rinsed overnight in phosphate–sucrose buffer, freeze substituted in buffer with 20% glycerol, and stored at −20 C. The *in vitro* PLP specimens were all stained with PEI on the same day. Frozen samples were thawed in 8% glycerol–0.1 M Na cacodylate buffer for 2 hours

Table 1—*In Vivo* Technique: Lamina Rara Externa GBM Anionic Sites and Epithelial Foot Processes During Aminonucleoside Nephrosis

Time after PAN* (hours)	Animals	Glomeruli	LRE charge sites Per 1000 nm GBM	Foot process width (nm)
Control	3	10	22.0 ± 2.2†	245 ± 0.4†
4	2	8	18.0 ± 2.0‡	251 ± 9§
24	2	8	18.5 ± 1.2‡	277 ± 9‡
48	2	5	16.9 ± 1.3‡	264 ± 9‡
120	2	5	15.8 ± 1.1‡	285 ± 10‡
168	2	4	15.7 ± 1.0‡	407 ± 19‡

* Puromycin aminonucleoside.
† ± standard deviation.
‡ $P < 0.05$ *versus* control.
§ Not significant, $P > 0.05$.

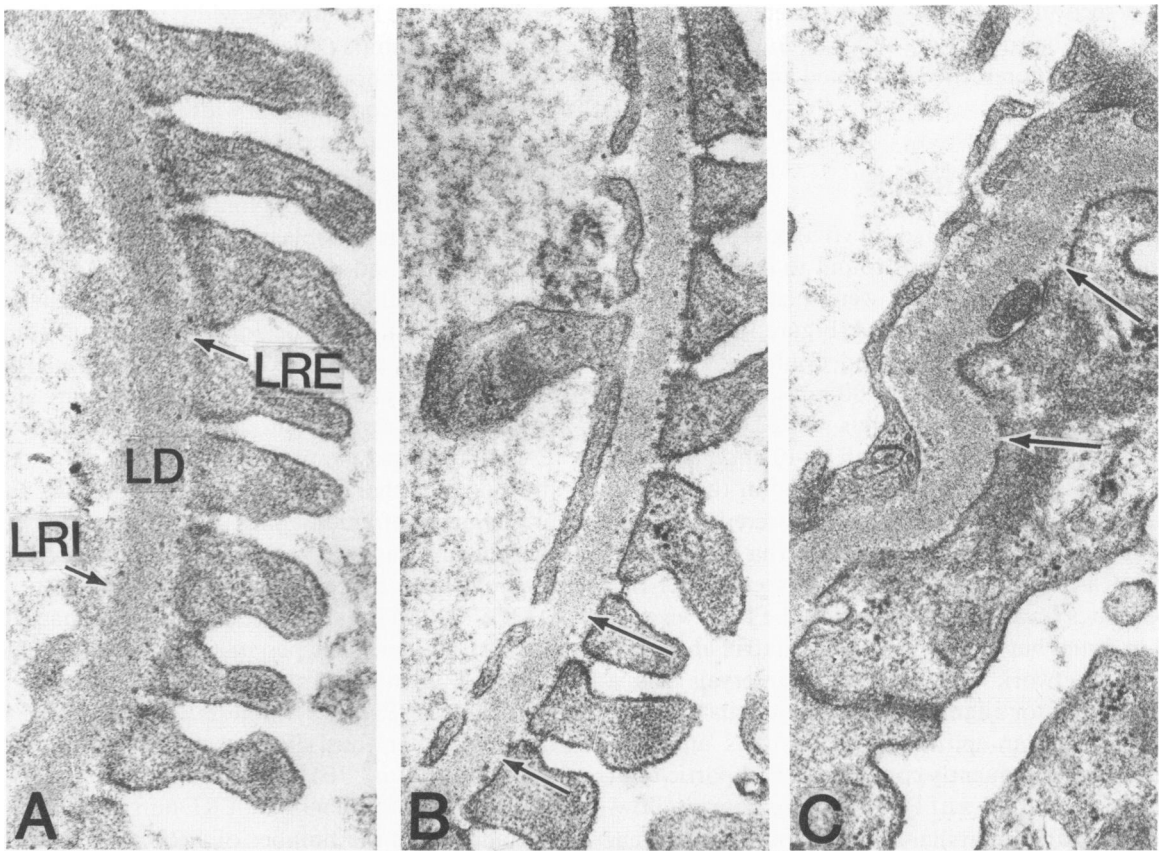


Figure 1—Electron micrographs of representative portions of rat glomerular capillary walls 5 minutes after injection of PEI. (×52,000) **A**—Control. Prior injection of normal saline. Labeled anionic sites are evident in this slightly tangential section as a staggered regular row of 20-nm dots in the LRE (average, 22 ± 2.2/1000 nm GBM length). There are a few labeled sites in the LRI and in the lamina densa. **B**—Comparable representative section obtained 4 hours after administration of PAN. The number of anionic sites in the LRE is reduced (average, 18.0 ± 2.0/1000 nm GBM length). Note the focal absence of labeled sites in several areas (*arrows*). **C**—At 168 hours after injection of PAN, the epithelial cell foot processes have disappeared, and relatively few normally labeled anionic sites remain within the LRE (average, 15.7/1000 nm GBM length). The sites that remain are smaller and less distinct than those visible in **A** and **B**.

and snap-frozen in liquid nitrogen, and 30 μ were sections cut. Sections were incubated in 1% PEI (1200 MW) in Na cacodylate buffer overnight at 4 C, washed twice in buffer, stained with 2% phosphotungstic acid/1% glutaraldehyde for 45 minutes, rinsed and dehydrated through graded ethanol, and embedded in POLY BED 812, as previously described.[4]

Morphometric Techniques

Half-micron sections were cut from glutaraldehyde fixed tissues and stained with toluidine blue. A minimum of three glomeruli per animal were selected; thin sections were obtained and stained with uranyl acetate and lead citrate. The sections were examined with a JOEL 100 CX electron microscope. At least 10 micrographs per glomerulus were recorded on 35-mm film at primary magnifications of 2380 and 6464 with the use of a systematic random sampling technique.[26] A carbon grating calibration grid was photographed on each roll of film and used to determine final magnifications of the micrographs, which ranged from 15,786 to 20,450 and 51,436 to 55,915.

The PEI-labeled anionic sites in the LRE of the GBM were measured and counted over a distance of 100–250 cm of GBM in the photomicrographs (representing 17 $\times$ 10^3 to 42 $\times$ 10^3 nm per glomerulus) and expressed as the mean number (± SD) of stained anionic sites per 1000-nm length of GBM. Obviously tangential sections were omitted. A minimum of 100 foot processes abutting peripheral GBM were measured per glomerulus with the use of a "Gunderson ruler," and the harmonic mean foot process width per animal was obtained.[27] Statistical analysis involved one-way analysis of variance, with group means compared by Tukey test. Significance was defined as $P < 0.05$.

Separate samples of renal tissue from PAN-injected animals and controls not injected with PEI were snap-frozen in liquid nitrogen for immunofluorescence studies. The tissue was processed by routine methods,[28]

and 4-μ sections from each animal were stained with fluorescein-labeled antisera to rat IgG, C_3, and albumin. Positive fluorescence was noted and graded from trace to 4+.

Results

In PEI 1200 MW injected (*in vivo*) control animals, a regular latticelike array of round to oval, 15-to 30-nm, electron-dense particles were located within the lamina rara externa of the GBM (Figure 1). Tangential sections demonstrated staggered double rows in the LRE. The number of sites per 100-nm-length GBM for the PEI-injected control animals was 22.0 ± 2.2 (Table 1). Variable numbers of small (5–10 nm diameter), less dense sites were randomly located in the lamina densa. Anionic sites of various sizes were irregularly distributed within the lamina rara interna of the GBM and much less frequent in number than seen in the LRE. Anionic sites similar to those seen in the LRE were located in many but not all mesangial matrix areas in a latticelike network with 60–120-nm intervals and a predisposition for alignment along mesangial cell surfaces. Glomerular epithelial cell surfaces and foot processes were frequently coated with PEI particles of varying sizes. Particles of PEI were also noted in Bowman's space, and in capillary lumina and focally on endothelial cell surfaces.

Anionic sites were also noted in the inner and outer portions of tubular basement membrane (TBM) in an irregular linear pattern at 60–120-nm intervals, with less dense, smaller particles in the remainder of the TBM. Peritubular capillary basal lamina anionic sites were distributed in staggered double rows at 40–60-nm intervals, while numerous electron-dense sites were noted in the interstitial matrix, with regular labeling of collagen fibrils at the 60-nm major banding intervals.

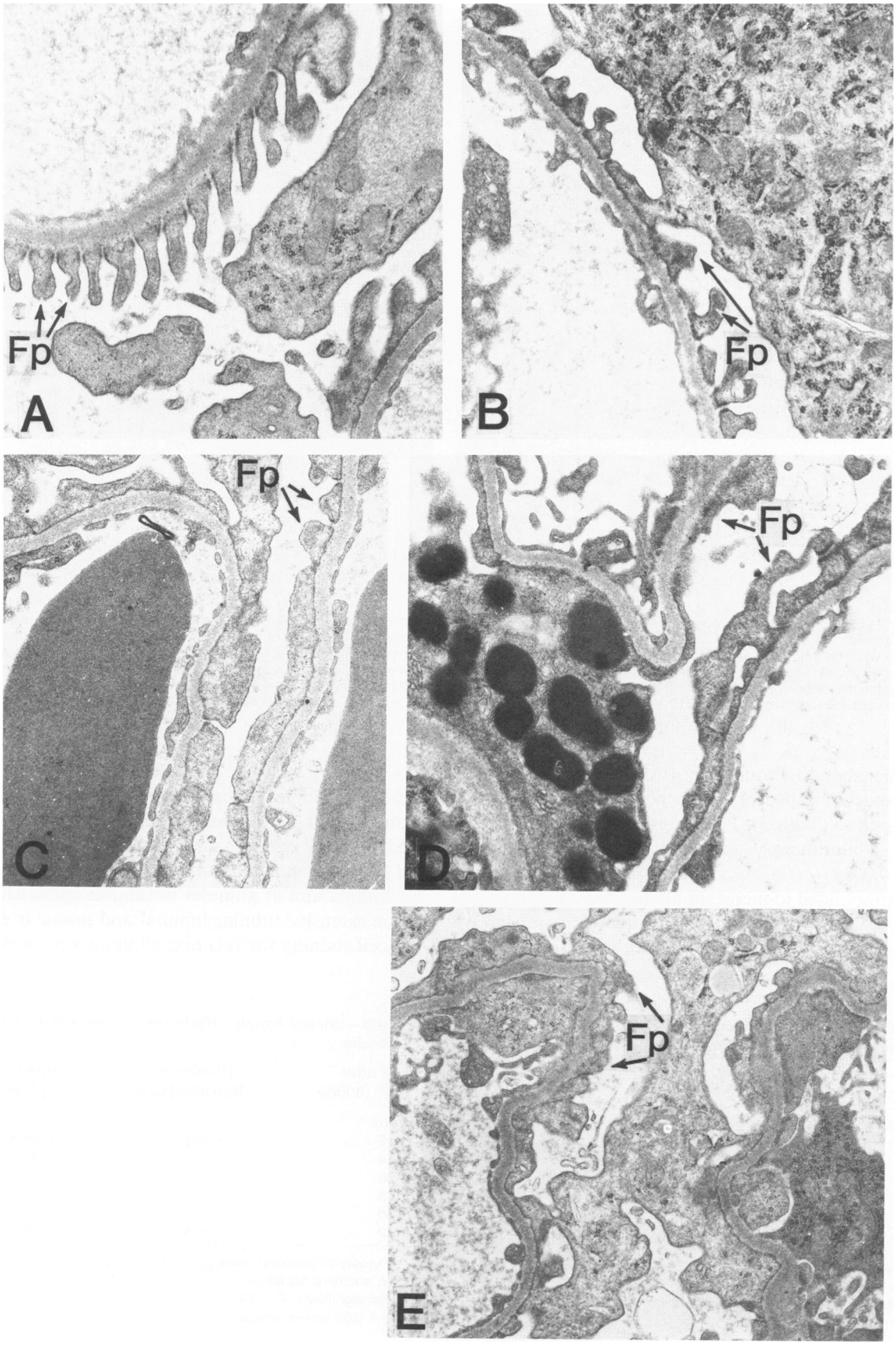
Four hours after administration of PAN, a significant decrease in stained LRE charge sites was observed (18 ± 0.7 per 1000 nm GBM length). In these animals, there was focal drop out of labeled LRE sites, and, in later time periods more extensive charge site loss and greater decreases in the LRE charge site number (Table 1) occurred. There did not appear to be any consistent change in the adjacent epithelial cell or foot processes related to the focal loss of LRE anionic sites.

Mean foot process width in control animals was 245 ± 0.4 nm. The foot process width was not statistically different at 4 hours (251 ± 9 nm) but was significantly increased at 24 hours. The overall trend over time was for increasing foot process width as described by the significant regression equation $y = 20.4 - 0.0867x + 0.00036x\ ^2\text{Time}$, $r^2 = 0.48$, $P < 0.02$ (Figure 2, Table 1). Increased numbers of glomerular epithelial cell organelles, rough endoplasmic reticulum (RER), Golgi vacuoles (G), and protein reabsorption droplets were noted at 4 hours, and more widespread epithelial cell cytoplasmic changes, including microvillous transformation, blebs, and further expansion of the RER and G, were noted at 24–48 hours. There were rare areas of epithelial cell detachment at 5 days. Throughout the PAN time course, glomerular endothelial cell and GBM morphologic characteristics remained normal, and the arrangement of anionic sites in the lamina densa, LRI mesangium, and other portions of the kidney were not different from that seen in control animals.

PEI 1200 MW *in vitro* control animals had 21.1 ± 1.6 LRE anionic sites per 1000 nm GBM arranged in a pattern identical to that revealed by the PEI injection method (Figure 3, Table 2). The sites were 10–20 nm in diameter, thus, slightly smaller than those noted with injection of PEI 1200 MW. At 30 minutes after PAN, there was no change in LRE number (21.6 ± 2.9), but at 4 hours the number of LRE anionic sites was significantly decreased to 17.3 ± 2.7, and this decrease was noted at all subsequent time periods. The sites were absent focally along the LRE; at later time periods, the drop out of sites became more pronounced (Figure 3). Although glomerular epithelial cells and foot processes were not well preserved with this fixative and their relationship to the LRE anionic sites was difficult to evaluate, obvious foot process broadening occurred 72 hours after PAN. The lamina densa and LRI anionic sites revealed by this technique were also slightly smaller and less consistent in density than noted after injection of PEI, but staining of glomerular epithelial cell surface foot processes and filtration slits with PEI was more consistent with the *in vitro* technique. The mesangial matrix, TBM, peritubular capillary, and interstitial anionic sites were labeled in patterns similar to those seen after injection of PEI. There were no differences in these sites or those in the lamina densa and LRI throughout the PAN disease course.

Urinary excretion of albumin began to increase dur-

Figure 2—Electron micrographs of representative glomerular capillary walls of saline- and PAN-injected rats photographed at a constant magnification for evaluation of epithelial cell foot process (Fp) width. (×16,000) **A**—Control. Saline-injected rat. Average Fp width, 245 ± 0.04 nm. **B**—Four hours after PAN injection. The epithelial cell also demonstrates an increased number of cytoplasmic organelles. Average Fp width, 251 ± 9 nm. **C**—Twenty-four hours after PAN injection. Average Fp width, 277 ± 9 nm. **D**—One-hundred twenty hours after PAN injection. Average Fp width, 285 ± 10 nm. Not the numerous electron-dense (lipoprotein) vacules in one epithelial cell. **E**—One-hundred sixty-eight hours (7 days) after PAN injection. Average Fp width, 407 ± 19 nm.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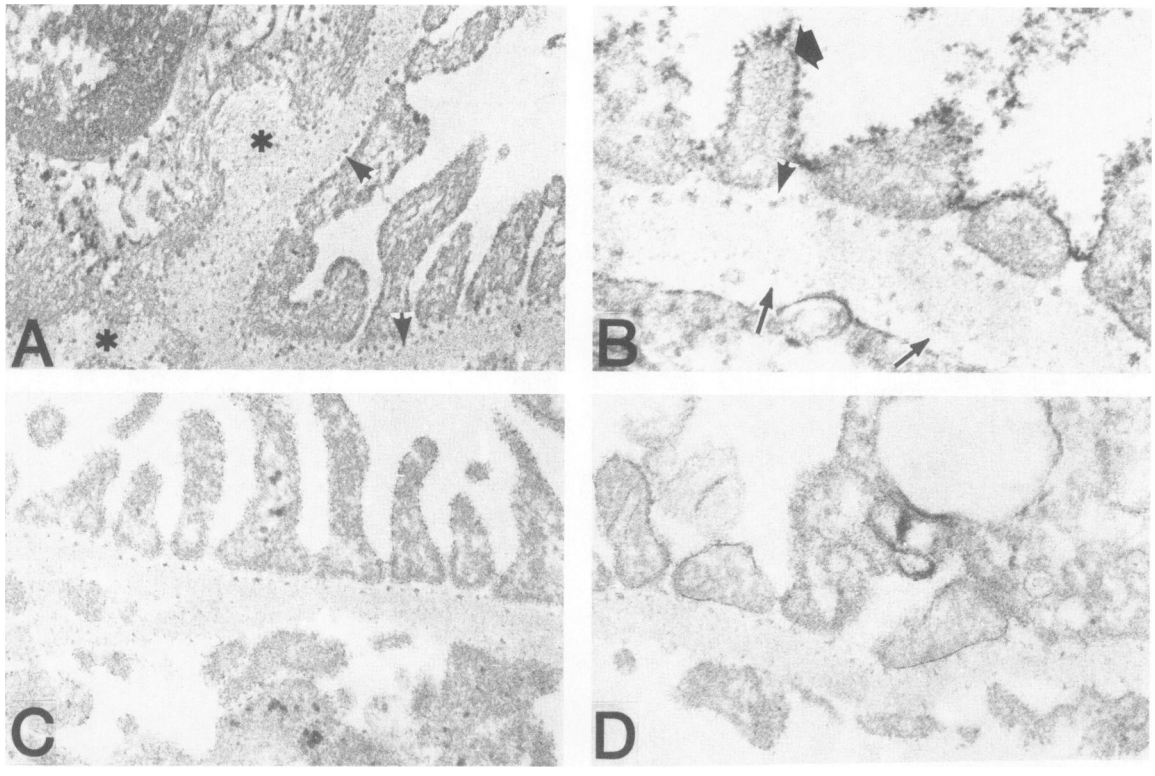

Figure 3—Electron micrographs of representative glomerular capillaries within sections labeled with PEI by the *in vitro* method. **A**—Control rat, Labeled anionic sites are distributed primarily within the LRE (*arrowheads*) with an average frequency of 21.2 ± 1.6/1000 nm GBM width. Labeled sites are also visible within the mesangial matrix (asterisks). (×50,000) **B**—Higher magnification of incubated section of normal rat kidney demonstrating the labeled anionic sites in the LRE (*arrowheads*), infrequent sites in the LRI (*small arrows*), and the prominent labeling of the epithelial cell coat (*broad arrows*) with this technique. (×110,000) **C**—Labeling of anionic sites in the LRE 24 hours after the injection of PAN. Average, 17.9 ± 2.4/1000 nm GBM length. **D**—Similar section obtained 72 hours after injection of PAN. Average, 15.5 ± 1.0/1000 nm GBM length.

ing the second 12 hours after PAN administration but did not reach statistical significance until the 26–48-hour time period (Table 3). The regression equation for urinary albuminuria was highly significant at $y = 0.21-0.003x + 0.00029\,x^2$ mg, $r^2 = 0.826$, $P < 0.0001$; it corresponded to increased albuminuria at each time period. In addition, individual animals consistently showed increased albuminuria in sequential collections.

Control animals had faint linear IgG immunofluorescent (IF) staining in many capillary loops but no evidence of C_3 or albumin deposition. Abnormal IF findings were just seen 48 hours after PAN, where focal (1+) IgG and albumin were detected in some tubular lumina and as granules in tubular epithelial cells. More extensive tubular luminal and granular epithelial cell staining for IgG and albumin were present in

Table 2—*In Vitro* Technique: Lamina Rara Externa GBM Anionic Sites During Aminonucleoside Nephrosis

Time after PAN* (hours)	Animals	Glomeruli	LRE charge sites per 1000 nm GMB
Control	3	6	21.1 ± 1.6†
0.5	4	9	21.6 ± 2.9‡
4	3	7	17.3 ± 2.7§
24	3	8	17.9 ± 2.4§
48	2	4	12.0 ± 2.6§
72	2	5	15.5 ± 1.0§
120	2	4	13.7 ± 2.5§

* Puromycin aminonucleoside.
† ± standard deviation.
‡ Not significant, $P > 0.05$.
§ $P < 0.05$ *versus* control.

Table 3—Urinary Albumin Excretion: During Aminonucleoside Nephrosis

Time after PAN* (hours)	Number of determinations	Urinary albumin mg/time period
Control	27	0.211 ± 0.083†
0–12	13	0.223 ± 0.077‡
12–24	9	0.264 ± 0.106‡
24–36	6	0.406 ± 0.117‡
36–48	4	0.886 ± 0.377§
48–60	7	1.074 ± 0.382§
60–72	8	1.456 ± 0.393§

* Puromycin aminonucleoside.
† ± standard deviation.
‡ Not significant, $P > 0.05$.
§ $P < 0.05$ *versus* control.

the animals studied after 120-hours. At no time was IF staining positive for mesangial or capillary C_3 or IgG.

Discussion

PAN-induced nephrotic syndrome in the rat has been extensively studied because of the similarity of the renal lesion to that seen in minimal-change nephrotic syndrome in man.[20] Studies performed five days after PAN administration had demonstrated altered charge-selective GBM function[29] and, later, both size and charge defects in glomerular permeability.[19] Nevins et al,[25] using morphometric techniques, reported the onset of foot process fusion 24 hours after PAN injection. Although Gang et al[30] demonstrated an increase in lanthanum binding to the GBM 7 days into the disorder and suggested an alteration in GBM organization, Nevins et al[25] and most other investigators[11,31,32] have described the GBM as normal by routine electron microsopic examination.

This study demonstrates changes in the array of LRE anionic sites in the GBM early in the course of PAN nephrotic syndrome. Focal loss of PEI-stained sites in the LRE was detected 4 hours after PAN administration, with more pronounced loss of stainable anionic sites as time progressed. Increased glomerular epithelial cell organelles were seen at 4 hours, although foot process widening was not detected until 24 hours. At that time, more extensive glomerular epithelial cell alterations, which included expansion of the rough endoplasmic reticulum and Golgi system and appearance of protein reabsorption droplets, were evident. The decrease in stainable LRE anionic sites was demonstrated before the increase in urinary albumin excretion was first suggested 12–24 hours after injection of PAN. The LRI and lamina densa anionic sites were not well stained with this technique but qualitatively appeared to be unchanged during the PAN disease course. The PAN appears to be a direct glomerular epithelial cell toxin, as recently demonstrated by Fishman and Karnovsky in a glomerular epitheial cell culture system.[21] Our study indicates that glomerular capillary wall structure and epithelial cell morphologic characteristics are altered within 4 hours of PAN exposure and that foot process fusion and widespread epithelial cell changes occur later. Studies in other disorders characterized by proteinuria have also noted early changes in charge sites after disease induction.[33,34]

Albuminuria in this model should be an excellent marker of altered glomerular permeability, because albumin is the major serum protein and is present in only small amounts in the urine of normal rats. The RID technique permits the detection of as little as 6 μg/ml albumin, and, because little proximal tubular reabsorption of albumin occurs in PAN nephrosis,[35] slight elevations in urinary albumin should reflect abnormal glomerular permeability. Although our method cannot exclude earlier mild changes in glomerular permeability, urinary albumin did not begin to increase until 12–24 hours after PAN, and broadening of epithelial foot processes did not become detectable until 24 hours into the disorder. There was no consistent relationship between the loss of LRE anionic sites and changes in adjacent foot processes or epithelial cell detachment at any time period.

The significant decrease in LRE sites was also noted when, in a separate group of experimental animals, PEI staining was performed *in vitro* after tissue fixation (Table 2). These techniques do not indicate whether the LRE anionic sites were absent or simply less anionic and therefore not able to interact electrostatically with the cationic probe. The enumeration of anionic charge sites does not necessarily assess net negative charge density, and the technique may overestimate the true change density in any particular region. In fact, in the PAN-injected animals, the stained sites in the LRE tended to be faint, smaller, and less distinct, which suggested an even greater decrease in charge density in the LRE of PAN-injected animals than that indicated by the decrease in LRE anionic site number.

The cationic tracer employed may influence the detection of anionic sites in injection and *in vitro* staining studies.[2,36] Pilla et al obtained different results in staining GBM charge sites in normal rats, based on the characteristics of the cationic tracer.[36] With PEI MW 40,000–60,000, these investigators noted the same number of LRE anionic sites (21 per 1000 nm GBM) as we did with PEI MW 1200. However, the larger PEI marker revealed approximately equal numbers of LRI anionic sites (20 per 1000 nm GBM), more than the number revealed in the LRI with the smaller PEI marker in our hands.

Caulfield and Farquhar noted a decrease in LRE anionic sites in PAN nephrosis with the cationic probes, lysozyme, alcian blue and ruthenium red.[11] Mynderse et al described a reduction in the staining of heparansulfate proteoglycan in the GBM in PAN rats by use of an immunohistochemical technique.[37] Kanwar and Jabubowsi, on the other hand, using tracers with greater charge density, (cationic ferritin [pI 7.2–7.4] and cytochrome C) could not detect a difference between PAN-injected and control animals.[22] This may have occurred because strongly charged probes still bind to LRE anionic sites that have lost some, but not all, of their negative charge, while less cationic probes can better detect subtle changes in anionic site charge.

The lattice-like network of anionic sites in GBM lamina rara was first demonstrated by Caulfield and Far-

quhar in 1976.[38] Studies that used a variety of cationic probes have confirmed the presence of this network in fixed renal tissue and the remarkable regularity of the spacing of these sites at 50–60-nm intervals in the fish, rat, dog, and man.[9] These anionic sites contain heparan-sulfate–rich proteoglycans and, in life, exist as complex hydrophilic macromolecules that occupy considerable domains.[9] Lamina rara anionic sites are involved in the structural organization of the GBM and also help define its permeability characteristics, because enzymatic removal[10] or polycation neutralization[5] of these sites induce increased permeability of anionic serum proteins. Whether altered permeability occurs as a result of disruption of complex GBM matrix relationships or, more specifically, through loss of intrinsic negative charges in the matrix is not yet known.[15]

Our data support the concept that alterations in the GBM proteoglycan network are involved in the early proteinuria of PAN nephrotic rats. GBM matrix components are synthesized and maintained by contiguous epithelial cells.[9] The striking alteration in LRE anionic sites noted early in PAN nephrotic syndrome raises the possibility of a direct effect of PAN on synthesis, transport, degradation, or neutralization of proteoglycans. Glomerular proteoglycan (PG) turnover is rapid, and an almost twofold increase in synthesis of glycosaminoglycan (GAG) by isolated glomeruli from PAN-injected rats as compared with controls has been demonstrated.[39] Thus, impaired glomerular epithelial cell synthesis of normal PG or GAG or transport of new proteoglycan moieties to maintain GBM structure may occur early in PAN. Further epithelial cell changes, such as broadening of foot processes and later detachment of the epithelium from the underlying GBM, may then represent a secondary phenomenon related to the persistent proteinuria.[5] Abnormal GBM permeability and "protein overload" of glomerular epithelial cells may also be responsible for the progression of the epithelial cell lesions seen in this disorder[31] and the eventual development of focal segmental glomerulosclerosis seen with chronic administration of PAN.[40]

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