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ORIGINAL ARTICLE

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Sequence of events in the glomerular capillary wall at the onset of proteinuria in passive Heymann nephritis

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Abstract Proteinuria in passive Heymann nephritis (PHN) results from complement-mediated glomerular injury, since complement depletion with cobra venom factor (CVF) prevents proteinuria. However, there are no comprehensive morphological studies identifying the sites of injury leading to onset of proteinuria. To address this issue, we attempted to locate sites of injury involved in the onset of proteinuria in PHN. PHN was induced in intact Munich-Wistar rats (PHN-rats, examined at days 3, 5, and 7) and in complement-depleted rats (CVF treated, PHN-CVF-rats, examined at days 3 and 5). The distribution of endogenous albumin in the glomerular basement membrane (GBM) was studied in in situ drip-fixed glomeruli using immunogold immunocytochemistry, and glomerular anionic sites were visualized by polyethyleneimine staining. In addition, the ultrastructural localization of an epitope recognized by a proteinuria-inducing monoclonal antibody (called 5-1-6) directed against the slit diaphragm was examined. Significant proteinuria was seen in intact PHN-rats, starting at day 5. The intensity of gold labeling for endogenous albumin was significantly increased at the outermost site of the GBM (GBM interfacing foot process and the filtration slit, designated area O) at day 3 in both PHN-rats and PHN-CVF-rats in comparison to untreated controls. At day 5, labeling for albumin in area O was decreased in PHN-rats, but not in PHN-CVF-rats, where it was then higher; in PHN-rats, some areas between epithelial cells and subepithelial deposits were almost free of albumin labeling at day 7. There was no evidence of epithelial cell detachment in any group at day 5, but on day 7 limited focal detachment was seen exclusively in PHN-rats. In proteinuric rats, amorphous material that stained for albumin could be seen in the urinary space, without any exocytosis of labeling by glomerular epithelial cells. A significant reduction of intensity of staining for anionic sites was seen in parallel in both groups, but only in the regions of the lamina rara externa adjacent to subepithelial deposits. This local loss of charge might contribute to enhanced permeability to albumin in both PHN- and PHN-CVF-rats. Changes in the appearance of the filtration slits and in the density and distribution of antigen recognised by monoclonal antibody 5-1-6 were similar in PHN- and PHN-CVF-rats at day 5. Complement depletion prevented neither the reduction in anionic sites of the GBM nor the changes in the slit diaphragm observed. These data suggest that albumin leakage between the epithelial cell and the GBM (area O) could occur in PHN-rats, perhaps as a result of epithelial foot-process changes. This may be the final link in the chain of events responsible for the onset of proteinuria in PHN.

Keywords Proteinuria · Heymann nephritis · Epithelial detachment · Anionic site · Slit diaphragm

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Introduction

Subepithelial electron-dense deposit formation, glomerular basement-membrane (GBM) thickening, foot-process retraction, and epithelial detachment have been recognized as the major morphological changes in passive Heymann nephritis (PHN) with established proteinuria. PHN is a widely used experimental model of human membranous nephropathy. Proteinuria in PHN is induced by anti-Fx1A antibody, which binds to the glomerular epithelial cell (GEC) and has been generally believed to result from GEC injury perpetrated by the complement 5b-9 membrane attack complex [9, 10]. Decomplementa-

tion of rats with cobra venom factor (CVF) inhibits the occurrence of proteinuria without exerting any obvious effect on the formation of subepithelial immune deposits [7, 9, 28]. In relation to the epithelial detachment in PHN, evidence has emerged that anti-Fx1A antibody recognizes the β1-integrin matrix receptor on cultured rat GECs and, thus, could inhibit adhesion to several extracellular matrix components bearing β1-integrin [1].

Recently, Neale et al. [25] reported that, in PHN with established proteinuria, GECs express cytochrome b558 and this enzyme complex generates reactive oxygen species, which are found even within the GBM where they could modify type-IV collagen [25]. This latter pathway was also suppressed by complement depletion with CVF [24]. In addition, treatment of rats with oxygen-radical scavengers efficiently reduced proteinuria, while the formation of immune deposits was not obviously affected [30]. Furthermore, McMillan et al. [22] reported that induction of GEC injury in PHN leads to a rapid increase in matrix metalloproteinase-9 synthesis in the time interval associated with maximal proteinuria. These data, considered in association with the known reduction of anionic sites in the GBM [3], suggest that qualitative GBM changes will exist in PHN.

The ultrafiltration unit of the glomerulus consists of the endothelial fenestrae, the GBM, and epithelial-foot processes with intervening slit diaphragms. Damage to any of these structures may induce an abnormal loss of plasma protein into the urinary space [18]. However, there is no satisfactory evidence pinpointing those structures of the glomerular capillary wall primarily involved during the onset of proteinuria in PHN. To address this issue, we examined the in vivo distribution of endogenous albumin in the glomerular capillary wall, as a parameter of glomerular permselectivity for macromolecules, by immunocytochemical ultrastructural techniques [11]. Changes in glomerular anionic sites were visualized using polyethyleneimine (PEI) staining [29]. In addition, to evaluate the changes in the slit diaphragm, we studied the ultrastructural localization of an epitope which is recognized by a proteinuria-inducing monoclonal antibody (MoAb) 5-1-6, directed against the slit diaphragm [13, 26]. These studies were performed in both intact and complement-depleted rats.

Materials and methods

Antibodies

Anti-Fx1A IgG was prepared in a sheep, and the globulin fraction was obtained by ammonium-sulfate precipitation, yielding an IgG concentration of 60 mg/ml. Details of the production of anti-Fx1A have been given elsewhere [15]. The following antibodies were used: affinity-purified rabbit anti-rat albumin (Nordic Immunological Laboratories, Kempten, Germany), rabbit anti-mouse IgG1 (Cappel Laboratories, Cochraneville, Penn., USA), fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep IgG (Behringwerke, Marburg, Germany), FITC-conjugated rabbit anti-rat C3 (Cappel Laboratories), FITC-conjugated mouse anti-rat IgG (Dianova Immunotech, Hamburg, Germany), FITC-conjugated rabbit anti-rat albumin (Nordic), FITC-conjugated donkey anti-mouse IgG (preabsorbed with rat and sheep serum) (Dianova), donkey anti-rabbit IgG conjugated with 12-nm gold particles (Dianova), and goat anti-rabbit IgG conjugated with 10-nm gold particles (Ultra Biosols, Liverpool, UK). MoAb 5-1-6 (mouse IgG1 subclass) was prepared according to previously described methods [26], and the crude culture supernatant, containing fetal calf serum, was used for immunohistochemical study. As a control, a non-relevant monoclonal antibody RVG1 (mouse IgG1 subclass-matched) was used.

Induction of PHN

PHN was induced in intact male Munich-Wistar rats (120–150 g, Harlan Winkelmann, Borchen, Germany or Charles River Japan, Atsugi, Japan) by i.v. injection of 30 mg of anti-Fx1A IgG in 0.5 ml in phosphate-buffered saline (PBS), pH 7.4 (PHN-rats, examined at days 3, 5, and 7) and in complement depleted rats (CVF treated, PHN-CVF-rats, examined at days 3 and 5). To ensure complement depletion, 15 µg of CVF (a gift of Dr. Bitter-Suermann [8]) were injected i.p. at 18 h and 12 h before induction of PHN and thereafter once at day 3. As in our previous study [12], the serum C3 levels, measured by single radial immunodiffusion [21], could be maintained below 3% of a standard of normal, pooled rat serum till day 5 in CVF-treated rats. Sixteen-hour urinary protein excretion, before sacrifice of rats, was measured with the biuret method [31]. The groups studied were as follows: for study of the location of endogenous albumin and glomerular anionic sites, PHN-rats were examined at days 3 (n=3), 5 (n=4), and 7 (n=2); PHN-CVF-rats were examined at days 3 (n=3) and 5 (n=4); and normal rats (n=4) served as controls; for the study of ultrastructural location of antigen recognized by MoAb 5-1-6, PHN-rats at day 5 (n=4), PHN-CVF-rats at day 5 (n=3), and normal rats (n=4) as controls were used.

Tissue processing

The rats were anesthetized with sodium pentobarbital (50 mg/kg body weight) and positioned on an operating table heated at 37°C. The ventral aspect of the left kidney was exposed through a middle incision of the abdomen, and the renal capsule was gently stripped from the surface. This exposed region was fixed in situ for 60 min by dripping freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) onto the tissue [11]. The fixed cortical tissues were sliced, cut into 1 mm cubes, immersed in the same fixative for 120 min at 4°C, and then processed for immunoelectron microscopy to visualize the localization of endogenous albumin. After removal of the left kidneys, portions of renal tissues were snap frozen in N-hexan and stored at –70°C for immunofluorescence studies. Then, the nephrectomized rats were injected i.v. with 0.2 ml of a 0.5% PEI solution (MW 40 000–60 000, 33% aqueous solution, Polyscience, Eppelbeim, Germany) in 0.9% NaCl, pH 7.4; after 15 min the cortex of the right kidney was cut into small pieces and processed for electron microscopy to visualize glomerular anionic sites.

Ultrastructural distribution of endogenous albumin in glomeruli

Paraformaldehyde-fixed (4%) cortical tissues were dehydrated in a graded ethanol series and embedded in Unicryl (British BioCell, UK) at –30°C according to the company's instructions. The superficial glomeruli were identified on semithin sections, as previously reported [11]. Then, ultrathin sections having superficial glomeruli were incubated with rabbit anti-rat albumin antiserum (diluted 1:2000 in PBS) and, subsequently, with donkey anti-rabbit IgG antiserum conjugated with 12-nm gold (Dianova), diluted 1:40 in 0.02 M Tris-HCl buffer (pH 7.0) containing 1% bovine serum albumin. For histological controls, the first antibody was omitted or replaced by normal rabbit serum. Sections were stained with uranyl acetate, then examined in a Zeiss EM 10A electron microscope.

Detection of glomerular anionic sites

Cubes (1 mm) of cortical tissue obtained after injection of PEI solution were then fixed by immersion in 0.1% glutaraldehyde and 2% phosphotungstic acid mixture, washed in 0.2 M cacodylate buffer, post-fixed with 1% osmic acid, and embedded in Epon. The ultrathin sections were stained with uranyl acetate and lead citrate and examined in the electron microscope.

Immunofluorescence microscopy

Cryostat sections (4 µm) were stained directly with FITC-conjugated anti-sheep IgG, anti-rat C3, anti-rat IgG or anti-rat albumin, respectively, and indirectly stained with MoAb 5-1-6 (spent culture supernatant) using FITC-conjugated anti-mouse IgG (Dianova) as the second antibody.

Localization of antigen recognized by MoAb 5-1-6

In an attempt to detect differences in the slit diaphragm between PHN-rats and PHN-CVF-rats at day 5, the distribution of an antigen associated with the slit diaphragm (recognized by MoAb 5-1-6) was examined in additional groups of rats. Following the surgical procedure outlined above, a 27-gauge needle was inserted into the aorta below the junction of the left renal artery. Shortly after perfusion was started, the aorta was clamped above the renal vessels and the inferior vena cava was severed. PBS containing protease inhibitors (5 mM sodium tetrathionate, 5 mM benzamidine, and 5 mM EDTA) was perfused for 3 min at 120 mm Hg. Renal glomeruli were isolated from the kidney by sequential sieving, fixed with 4% paraformaldehyde for 2 h at room temperature, incubated with MoAb 5-1-6 (spent culture supernatant, diluted 1:50 in 10% normal goat serum), or MoAb RVG1 as an IgG subclass-matched control overnight at 4°C, and then fixed again with 4% paraformaldehyde for 30 min at room temperature. The glomeruli were dehydrated and embedded in Unicryl at –30°C. Ultrathin sections were preincubated with 10% normal goat serum in PBS, then incubated with rabbit anti-mouse IgG1 (absorbed with rat and sheep serum, diluted 1:200 in PBS) and subsequently with goat anti-rabbit IgG conjugated with 10-nm gold particles. Sections were stained with uranyl acetate and examined with an JEM-1220 electron microscope (JEOL, Tokyo, Japan).

Morphometric analysis

In the cases of immuno-gold-labeling procedures and PEI staining, a minimum of ten electron micrographs of the GBM were taken for each procedure in at least three glomeruli per rat. The photographs were printed at a final magnification of ×30 000. Morphometry was performed using a computerized image analyzer (MOP-Videoplan, Carl Zeiss, Germany). In order to obtain a semiquantitative estimate of the density of albumin molecules at the outermost site (epithelial side) of the GBM, the number of gold particles at this site (GBM interfacing foot process and the filtration slit, designated area O) per 10 µm GBM was determined. To assess the density of anionic sites in the lamina rare externa (LRE) of the GBM, PEI stainable sites in the LRE were expressed as the number of electron-dense deposits per µm GBM. For evaluating MoAb 5-1-6, which recognizes antigen at the site of the filtration slit, the number of gold particles and the number of filtration slits per µm GBM were counted, and the data was expressed as the density of gold particles per filtration slit.

Statistical analysis

Data are expressed as mean±SD. Differences between data sets were analyzed by performing a one-way analysis of variance followed by a Scheffe test. A level of $P<0.05$ was accepted as being statistically significant.

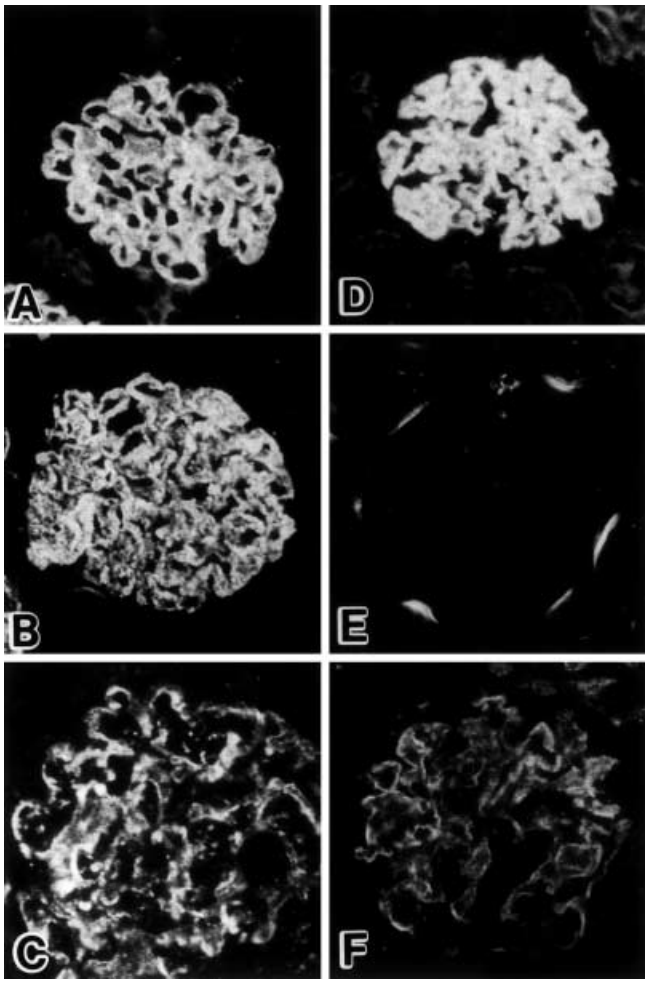

Fig. 1 Immunofluorescent staining of immune reactants in glomeruli of left kidneys of passive-Heymann-nephritis (PHN) -rats (**A–C**) and PHN-cobra-venom-factor (CVF) -rats at day 5 (**D–F**). **A** and **D** show sheep IgG, **B** and **E** show rat C3, and **C** and **F** show rat IgG

Results

Proteinuria

Significant proteinuria was noted only in PHN-rats, starting at day 5 (PHN-rats at day 5: 51.9±7.9 mg/day, at day 7: 110 mg/day), whereas abnormal urinary protein excretion (more than 10 mg/day of urinary protein excretion) was not detected in normal rats, PHN-rats at day 3, and PHN-CVF-rats at days 3 and 5.

Immunofluorescence findings

All rats injected with anti-Fx1A IgG showed dense, granular immune deposits of sheep IgG along the capillary wall after three days, the intensity of staining was similar in PHN- and PHN-CVF-rats (Fig. 1A, D). Staining for rat C3 was positive in PHN-rats after three days (Fig. 1B), whereas it was negative in PHN-CVF-rats

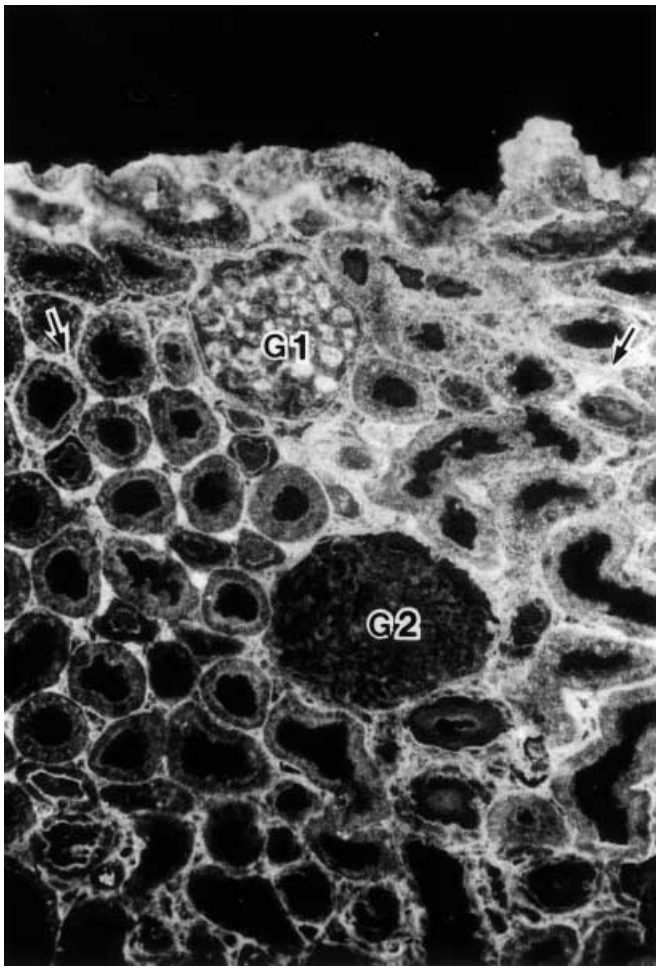

Fig. 2 Immunofluorescent staining for rat albumin in the left kidney of a normal Munich Wistar rat after in situ drip-fixation. Note that, in the superficial cortical area, staining for rat albumin is seen in the glomerular capillary lumen (*G1*), as well as in the peritubular capillaries (*arrows*), but not in the glomerular capillary lumen (*G2*) in the deeper cortical area

(Fig. 1E). Staining for rat IgG was positive after five days in both groups, but staining intensity was stronger in PHN-rats (Fig. 1C) than in PHN-CVF-rats (Fig. 1F). Glomerular staining for rat albumin was equivocal in all rats. Occasional protein droplets (rat albumin) in the tubular epithelial cells were seen only in proteinuric rats.

In the superficial cortical area of in situ drip-fixed kidneys, staining for rat albumin was clearly seen in the glomerular capillary lumen, as well as in peritubular capillarys, in all rats, including normal rats (Fig. 2), indicating that endogenous albumin molecules were homogeneously fixed by in situ drip-fixation in superficial glomeruli. In contrast, glomeruli in deeper cortical areas showed a lack of such a staining pattern in the glomerular capillary lumens (Fig. 2).

Staining for MoAb 5-1-6 was in a linear to fine granular pattern along the capillary wall, the intensity of staining was similar in control rats and in both PHN groups at all time points (Fig. 3A–C), except for PHN-rats at day 7, which then showed a much more granular pattern (Fig. 3D).

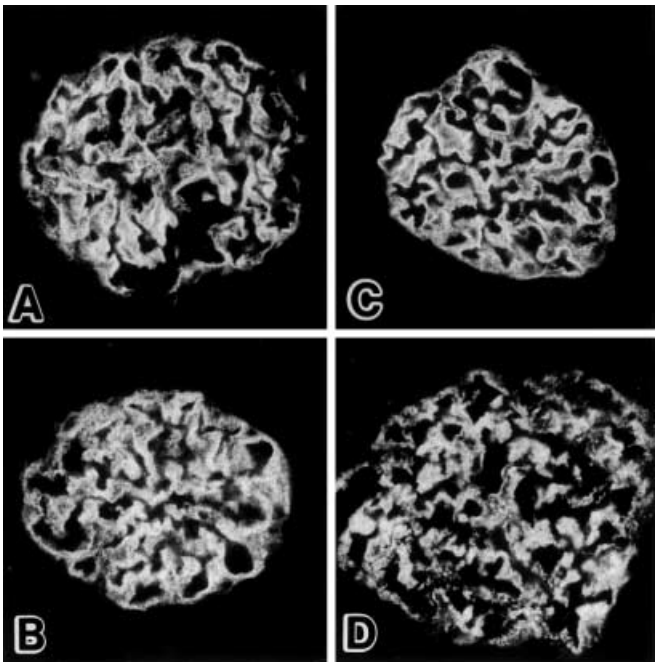

Fig. 3 Immunofluorescent staining for MoAb 5-1-6 in glomeruli of a normal rat (**A**), a passive-Heymann-nephritis (PHN) -rat at day 5 (**B**), a PHN-cobra-venom-factor (CVF) -rat at day 5 (**C**), and a PHN-rat at day 7 (**D**)

Glomerular endogenous albumin distribution (ultrastructure)

In normal control rats, as reported elsewhere [11], gold particles indicating the presence of albumin were distributed homogeneously over most of the capillary lumen, they were not seen on the apical membrane of the podocytes (Fig. 4A). Gold particles revealing albumin were also found over the GBM; the density of gold labeling was highest on the inner part of the GBM. The labeling was sparse in the subepithelial, subslit membrane and subendothelial regions (Fig. 4A). The distribution of gold particles in the GBM in PHN-rats and in PHN-CVF-rats was similar at day 3 (Fig. 4B, C). The density of gold labeling was significantly increased at the outermost site of the GBM (area O) at day 3 in PHN-rats and PHN-CVF-rats (Fig. 4B, C) when compared with control rats (Fig. 4A, Table 1). By day 5 in PHN-rats, the density of labeling in the subepithelial deposits had increased and gold labeling was also found in the GECs (Fig. 5A), however, the labeling density in area O decreased by days 5 and 7 (Fig. 5A, C; Table 1). At day 7 in PHN-rats, some discrete areas between epithelial cells and subepithelial deposits were almost free of labeling (Fig. 5C), and labeling was sometimes found in the vacuoles of the GECs, without any exocytosis of vacuoles with labeling (Fig. 5D). In proteinuric rats (PHN-rats at days 5 and 7), amorphous material with gold labeling could be seen in the urinary space (Fig. 5A, C). On the other hand, in PHN-CVF-rats, gold labeling in area O increased from day 3 to day 5 (Figs. 4C and 5B), where it was then

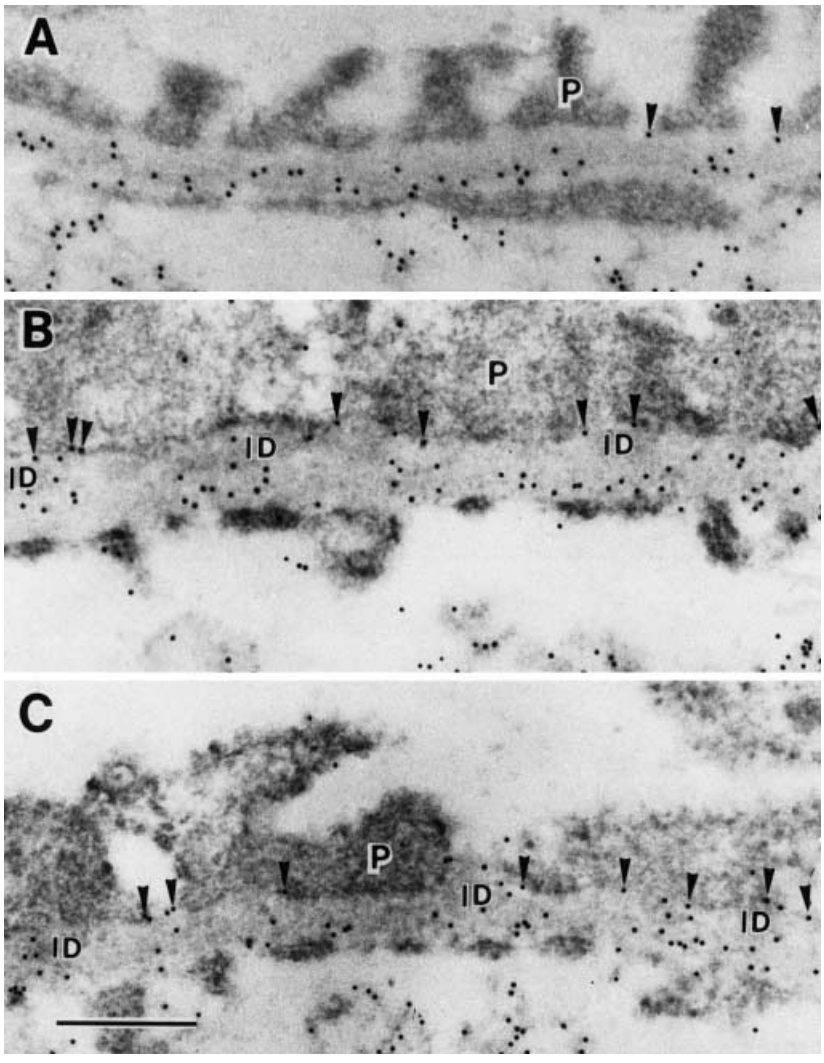

Fig. 4A–C Immunoelectron-microscopic findings on the distribution of rat endogenous albumin in the glomerular capillary wall. In a normal Munich Wistar rat (**A**), gold particles, indicating albumin, were found over the glomerular basement membrane (GBM); the density of gold labeling was highest at the inner part of the GBM. The labeling was sparse in the subepithelial, subslit membrane, and the subendothelial region, and was not seen at all on the apical membranes of the podocytes. At day 3, in both a passive-Heymann-nephritis (PHN) -rat (**B**) and a PHN-cobra-venom-factor (CVF) -rat (**C**), gold labeling for albumin was increased at the outermost site of the GBM (GBM interfacing foot process and the filtration slit) in comparison to a control rat (**A**). *Arrow heads* Gold particles indicating rat albumin, *P* podocyte, *ID* immune deposit. *Bar* 0.3 µm

Table 1 Density of albumin at the outermost site of the glomerular basement membrane (GBM) (GBM interfacing foot process and the filtration slit). *area O* Site of GBM interfacing foot process and the filtration slit. PHN Passive Heymann nephritis, PHN-CVF passive Heymann nephritis-cobra venom factor

Group	*n*	Length measured (µm)	No. of gold particles in area O / 10 µm GBM
Control	4	791.4	0.47±0.11
PHN			
Day 3	3	583.5	2.24±0.14[a]
Day 5	4	640.3	1.93±0.16[a,b]
Day 7	2	306.9	1.65
PHN-CVF			
Day 3	3	462.3	2.23±0.11[a,c]
Day 5	4	594.6	2.64±0.24[a,d,e,f]

[a] $P<0.001$ vs. control
[b] $P<0.05$ vs. PHN-rats at day 3
[c] $P<0.05$ vs. PHN-rats at day 5
[d] $P<0.01$ vs. PHN-rats at day 3
[e] $P<0.001$ vs. PHN-rats at day 5
[f] $P<0.01$ vs. PHN-CVF-rats at day 3

higher than in PHN-rats at day 5 (Fig. 5A; Table 1). In PHN-CVF-rats, labeling was sometimes found in the GECs (Fig. 5B), indicating that the quantity of albumin which could reach area O had increased without leakage into the urinary space in detectable quantities. There was no evidence of epithelial detachment of podocytes, defined as the spatial separation of the basal podocyte-cell membrane from the lamina rara externa of the GBM, before day 5 in both groups. In proteinuric rats (PHN-rats at days 5 and 7), increased labeling in lysozomal bodies in the proximal tubular epithelial cells was noted (Fig. 5E). Histochemical control sections showed little labeling over any renal structures.

Glomerular anionic sites labeled by PEI

The results of the quantitation of PEI aggregates in the LRE of the GBM are shown in Table 2. In the glomeruli of both pre-proteinuric PHN-rats and non-proteinuric PHN-CVF-rats at day 3, there was a significant decrease in PEI aggregates in the LRE compared with normal controls. This de-

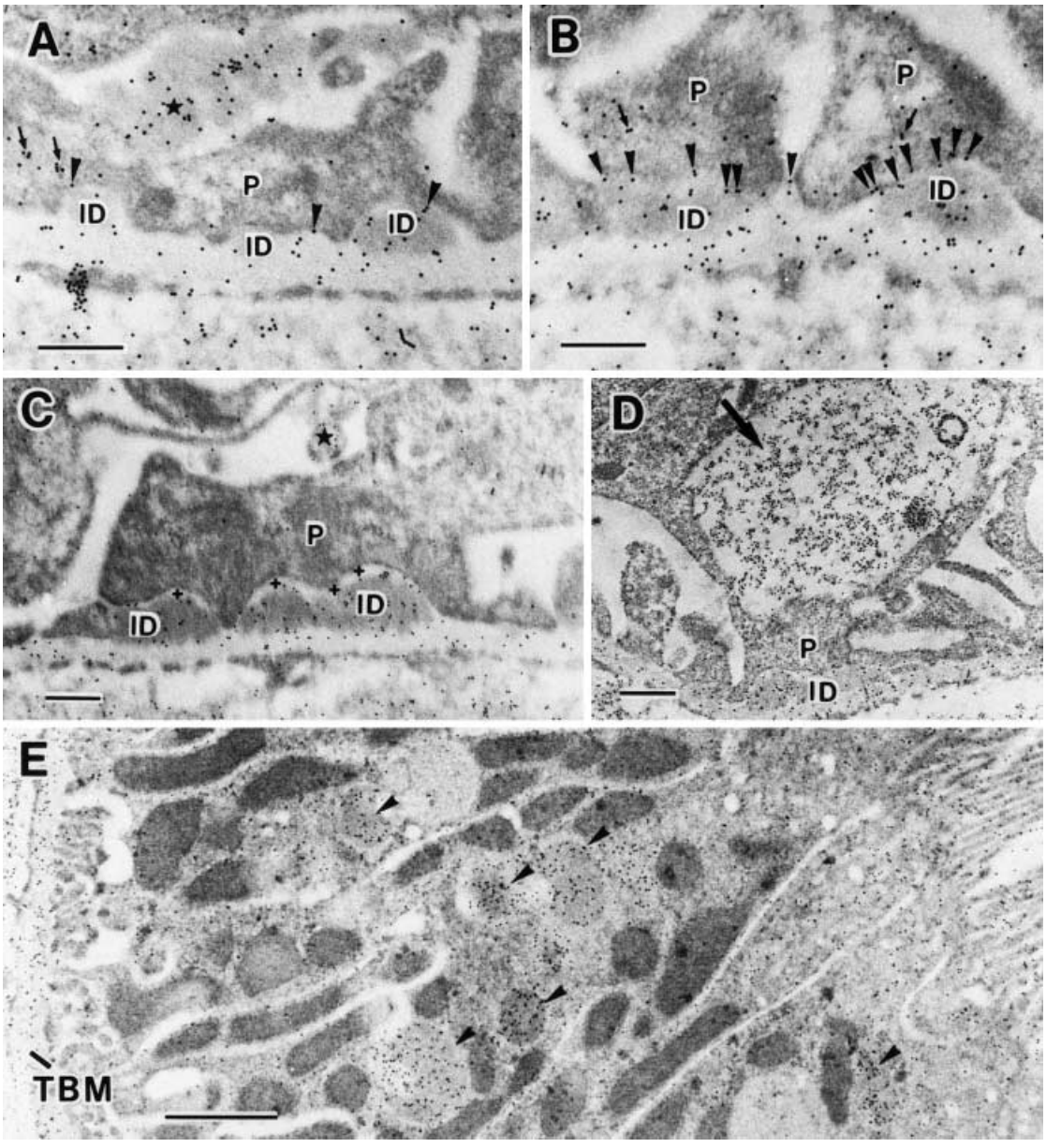

Fig. 5A–E Immunoelectron-microscopic findings of rat endogenous albumin in the glomerular capillary walls. At day 5 in a passive-Heymann-nephritis (PHN) -rat (**A**), the density of gold labeling indicating rat albumin was increased in the subepithelial immune deposits (*ID*), and gold labeling was also found in the visceral glomerular epithelial cells (*arrows*); however, the labeling density at the outermost site of the glomerular basement membrane (GBM) was decreased by days 5 (**A**, *arrow heads*) and 7 (**C**). Areas between the epithelial cell and subepithelial deposit were almost free of labeling (+) in the PHN-rat at day 7 (**C**), and labeling was found in the vacuole of the visceral glomerular epithelial cell (**D**, *arrow*). In proteinuric rats (PHN-rats at days 5 and 7), amorphous material with gold labeling could be seen in the urinary space (**A**, **C**, *asterisks*). On the other hand, in the PHN-CVF-rat, gold labeling at the outermost site of the GBM was increased from day 3 (Fig. 4C) to day 5 (**B**, *arrow heads*), where it was then higher than in the PHN-rat at day 5 (**A**). Labeling was found in the glomerular epithelial cells (**B**, *arrows*). In the PHN-rat at day 7 (**E**), increased labeling in lysozomal bodies (*arrow-heads*) in the proximal tubular epithelial cells were noted. *P* Podocyte, *TBM* tubular basement membrane. *Bars* (**A–D**) 0.3 µm, (**E**) 1.0 µm

crease in the number of PEI aggregates became very marked at day 5, this was especially prominent in the areas where subepithelial immune deposits were located (Fig. 6). In areas of the LRE that were remote from IC deposits, no differences in respect of the number of PEI aggregates compared with normal control rats could be seen (Fig. 6A, B).

Ultrastructural localization of filtration slit related antigen

PHN-rats, at day 5, revealed partial effacement of the foot processes of the GEC over areas containing large subepithelial deposits. Foot-process fusion was also seen in PHN-CVF-rats at day 5, but was less extensive and the subepithelial deposits were smaller than in PHN-rats. As we previously reported [13] in normal rats, gold particles, representing the sites of binding of the MoAb 5-1-6 recognizing antigen, were mainly localized in the

Table 2 Density of polyethyleneimine particles in the lamina rara externa of the glomerular basement membrane (GBM). *LRE* Lamina rare externa, *ID* immune deposit, PHN passive Heymann nephritis, PHN-CVF passive Heymann nephritis-cobra venom factor

Group	*n*	Length measured (μm)	No. of particles per 10 μm GBM in LRE			ID area / total (%)
			ID area[a]	ID free[b]	Total	
Control	4	657.8		19.89±0.38	19.89±0.38	0.0
PHN						
Day 3	3	356.2	13.09±0.34	19.50±0.11	18.55±0.09[g]	16.2
Day 5	4	374.8	12.38±0.34[c]	19.58±0.09	16.98±0.18[c,h]	35.4
Day 7	2	160.4	10.08	20.08	15.4	46.9
PHN-CVF						
Day 3	3	366.7	13.08±0.12[d]	19.58±0.08	18.57±0.05[d,g]	15.7
Day 5	4	348.0	12.48±0.14[e,f]	19.77±0.47	16.80±0.26[c,f,h]	34.4

[a] Area where the subepithelial immune deposits were located
[b] Area remote from immune deposits
[c] $P<0.001$ vs. PHN-rats at day 3
[d] $P<0.001$ vs. PHN-rats at day 5
[e] $P<0.001$ vs. PHN-rats at day 3
[f] $P<0.001$ vs. PHN-CVF-rats at day 3
[g] $P<0.005$ vs. control
[h] $P<0.001$ vs. control

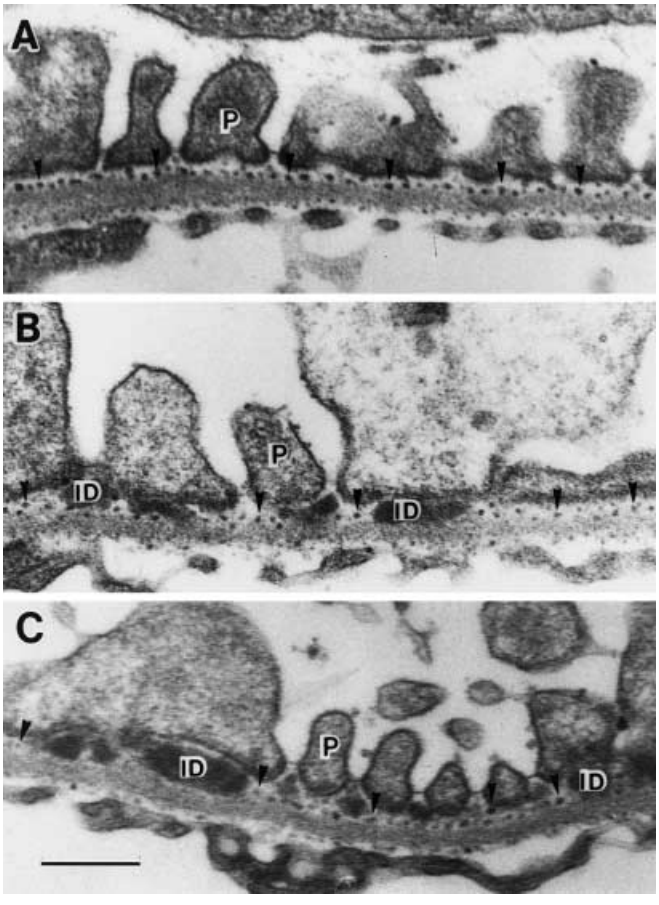

Fig. 6 Electron-microscopic findings of polyethyleneimine (PEI) aggregates in the glomerular basement membrane (GBM). In glomeruli of proteinuric a passive-Heymann-nephritis (PHN) -rat at day 5 (**B**), there was a significant decrease in PEI aggregates in the lamina rare externa (LRE) compared with a normal control rat (**A**). This was prominent in the areas where the subepithelial immune deposits were located. In the LRE remote from IC deposits, no difference between control (**A**) and experimental rats (**B**) could be seen regarding the number of PEI aggregates. PEI counts in the PHN-cobra-venom-factor (CVF) -rat at day 5 (**C**) were comparable to those in the PHN-rat (**B**). *P* Podocyte, *ID* immune deposits, *arrow heads* PEI aggregates. *Bar* 0.3 μm

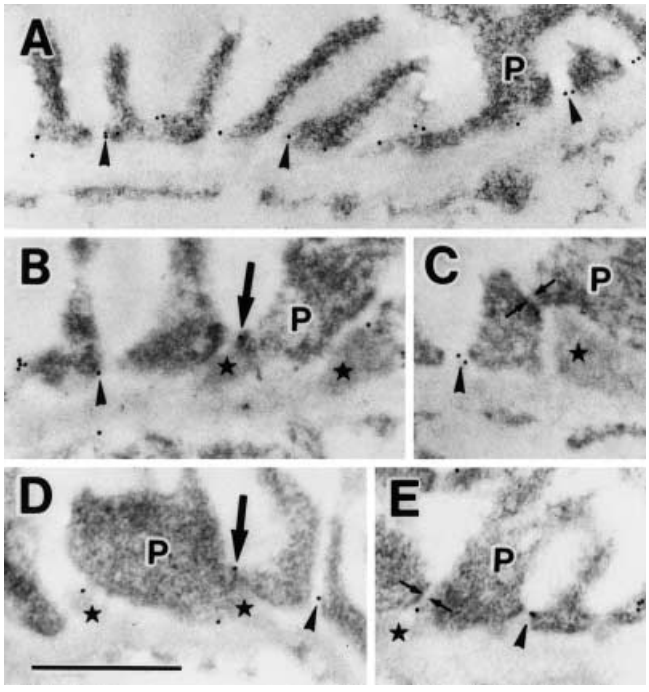

Fig. 7 Localization of antigen recognized by MoAb 5-1-6 by indirect immunogold electron microscopy using isolated glomeruli and a postembedding method in a normal rat (**A**), passive-Heymann-nephritis (PHN) -rats at day 5 (**B, C**), and PHN-cobra-venom-factor (CVF) -rats at day 5 (**D, E**). In normal rats, the gold particles (*arrowheads*), which indicate the binding sites of MoAb 5-1-6, were mainly localized in the filtration slit (**A**). In both PHN-rats and PHN-CVF-rats at day 5, gold particles (*arrowheads*) (**B–E**) were also located in the filtration slits and could be seen to be associated with dislocated slit diaphragms (*arrows*) (**B, D**). The occluding-type junctions are not labeled (*double arrows*) (**C, E**). *P* Podocyte, *asterisk* immune deposit. *Bar* 1.0 μm

filtration slit (Fig. 7A). Also, in both PHN groups at day 5, a similar distribution of gold particles was found (Fig. 7B–E), gold particles could often be seen associated with the dislocated slit diaphragms (Fig. 7B, D). The occluding-type junctions were not labeled (Fig. 7C, E).

Table 3 Morphometrical analysis of gold particles for antigen recognized by MoAb 5-1-6. GBM Glomerular basement membrane. PHN passive Heymann nephritis, PHN-CVF passive Heymann nephritis-cobra venom factor

Groups	*n*	Length measured (µm)	No. of gold particles / µm GBM	No. of filtration slits / µm GBM	Density of gold particles / filtration slit
Control	4	520.2	5.30±0.83	3.19±0.22	1.66±0.21
PHN day 5	4	635.1	2.54±0.49*	1.65±0.19**	1.54±0.22
PHN-CVF day 5	3	478.0	2.89±0.81*	1.82±0.24*	1.57±0.26

*P<0.01 vs. control, **P<0.001 vs. control

Histochemical controls using MoAb RVG1 showed little labeling over glomeruli. The number of gold particles and the number of filtration slits per µm GBM were significantly reduced in both groups at day 5, compared with normal rats, but the density of gold particles in the filtration slits remained the same in all groups (Table 3).

Discussion

In this study, the onset of significant proteinuria was seen at day 5 in PHN-rats, whereas decomplementation of rats inhibited the onset of proteinuria, with accompanying negative glomerular staining for rat C3. These results are in line with those detailed in previous reports [9, 10, 15]. It appears that the early phase of proteinuria follows a chain of events, including subepithelial deposit formation, loss of fixed anionic charges, and changes in the GEC podocytes and the slit diaphragm, leading to leakage of protein between GEC and GBM in discrete areas.

The distribution of endogenous albumin in in situ drip-fixed normal rat glomeruli showed a concentration gradient in the GBM, peaking at the inner (endothelial) side, as shown previously [11]. This method of fixation is considered to be more suitable for the type of study performed, as it minimizes losses of albumin during tissue processing. When glomerular permeability for albumin is enhanced, the distribution of albumin in the GBM would be expected to shift towards the outer (epithelial) side of the GBM. This expected alteration in the distribution of endogenous albumin could in fact be demonstrated in a previous study in proteinuric rats [14]. Therefore, we believe that it is possible to detect and interpret subtle changes of permeability in the glomerular filter by studying alterations in the localization of endogenous albumin using the present technique. Naturally, results obtained with the method of in situ drip-fixation must be interpreted with some caution, because artifacts could arise via perturbations of glomerular capillary hemodynamics and/or re-distribution of soluble endogenous albumin in the process of fixation. However, drip-fixation is the only method currently available which will minimize artifacts known to be caused by immersion fixation as well as perfusion fixation [11].

The first original observation in the current study was that the density of albumin in the outermost region of the GBM was significantly increased in both PHN-rats and PHN-CVF-rats at day 3 (both non-proteinuric) compared with control rats. This indicates that the permeability of the GBM itself was already enhanced in these rats and implies that subepithelial deposit formation per se, with or without complement activation, can perturb the integrity of the glomerular permselective filter in the GBM. The observed reduction in the glomerular anionic sites in the LRE in PHN, with or without complement depletion, supports this suggestion, as proposed in an earlier report [4]. However, as seen in PHN-CVF-rats, the reduction in glomerular anionic sites in itself was not sufficient to lead to significant proteinuria.

Enhanced permeability of the GBM, together with damage to the filtration barrier located beyond the GBM at the slit diaphragm and/or at the foot processes of the GEC, appears to be necessary for proteinuria to develop. One of the main regulatory factors governing permeability of the GBM is believed to be the fixed anionic charges [5, 17, 20]; however, with the technique used here, these charges do not appear to be different in proteinuric PHN-rats and non-proteinuric PHN-CVF-rats.

The second new observation is that, in PHN-CVF-rats, accumulation of albumin at area O (interfacing foot process + filtration slit) increased from day 3 to day 5, but, in contrast, in PHN-rats it decreased during this period. These findings indicate that changes in the part of the filtration barrier located beyond the GBM (the slit diaphragm and/or foot process of the GEC) may be the final, essential step in induction of proteinuria in PHN-rats. At the onset of proteinuria, no evidence of glomerular epithelial cell detachment (defined as the spatial separation of the basal podocyte cell membrane from the lamina rara externa of the GBM) was found after an extensive survey by electron microscopy, both by us (data not shown) and others [4]. However, in PHN-rats at day 7, we observed local detachment, producing spaces between the GECs and the subepithelial deposits, where almost no albumin accumulation could be seen. In proteinuric rats, amorphous material with labeling could be seen in the urinary space without any exocytosis of labeling by glomerular epithelial cells, suggesting that an intra-epithelial route of albumin passage was unlikely. These findings suggested that albumin that has penetrated to the outermost site of the GBM might leak into the urinary space between the GEC and the GBM in PHN-rats at day 5 (even when no clear morphological changes are seen) and/or that the permeability of the slit diaphragm might be enhanced.

An epitope on an antigen of the slit diaphragm, which is recognized by MoAb 5-1-6, has been shown to regulate glomerular permeability in rats [13, 14, 26] and al-

terations in the quantity and/or distribution of this antigen could be expected to be associated with proteinuria. In the current study, the intensity of immunofluorescent staining for MoAb 5-1-6 at day 5 was similar in normal control rats and both experimental groups, indicating that there was no extensive change in the distribution of this particular antigen in the slit diaphragm in PHN-rats. However, the immunofluorescent antibody technique is not sensitive enough to detect subtle changes, thus we compared the density of gold labeling at the electron-microscopic level. Dislocation of the filtration slit and a reduction of the quantity of antigen recognized by MoAb 5-1-6 were found; in addition, the density of the antigen per µm GBM in both PHN groups at day 5 was significantly reduced compared with controls. However, these changes were similar in both PHN- and PHN-CVF-rats at day 5. These findings suggest that alterations in the slit diaphragm really do exist, as reflected by the changes in the binding of MoAb 5-1-6, but that even when these changes are involved in onset of proteinuria in PHN, they are not the final event. The terminal change necessary for causing proteinuria may be a loosening of the adhesion of GEC to GBM.

Epithelial detachment has been reported to change the hydraulic conductivity and cause exaggerated perturbations in the permselectivity of the GBM itself [16, 19, 32, 33]. The mechanism of epithelial detachment in PHN is unclear. The events following the formation of the complement 5b-9 membrane attack complex on the GEC in PHN [9, 10] may contribute to epithelial detachment by modifying the molecules involving cell adhesion to the GEC and/or in the GBM. Adler et al. reported that anti-Fx1A antibody recognizes the β1-integrin matrix receptor on cultured rat GECs [1] and inhibits their adhesion to the GBM via a process that is initiated by integrin cross-linking, rather than through simple interference with cell adhesion per se [2]. It is also known that some of the GEC antigens implicated in the classic complement-mediated model of Heymann nephritis, namely GP330 and dipeptidyl peptidase IV (GP108), show matrix binding properties [6, 23, 27]. It has also been shown that not only epithelial cell membranes, but even the GBM itself can be modified by reactive oxygen species in PHN [24, 25]. Treatment of rats with PHN with oxygen-radical scavengers efficiently reduced proteinuria [30]. Furthermore, it was reported that a rapid increase in matrix metalloproteinase-9 synthesis occurred at a time point associated with maximal proteinuria in PHN [22], suggesting that a direct link exists between GEC proteolytic activity and loss of glomerular permselectivity. These complement-mediated events might also affect GBM constituents, including fibronectin, collagen, and laminin, which are involved in binding to the GEC. All of the above-mentioned mechanisms might contribute to loss of adhesion of GEC to the GBM, allowing, first, protein leakage and, later, concrete detachment of glomerular epithelial cells.

In summary, subepithelial immune deposit formation may perturb the filtration barrier in the GBM (probably reflected by the reduction in glomerular anionic sites), but this alteration of the GBM is not the sole event leading to proteinuria in PHN. Local, small-scale epithelial-cell detachment from the GBM, even if it is a functional disorder without any gross morphological changes, may be a primary pathogenetic event initiating overt proteinuria. However, these data do not exclude the possibility that other unrecognized changes in the capillary wall and/or slit diaphragm may contribute to enhanced protein excretion.

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