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Reactive oxygen species and neutrophil respiratory burst cytochrome b_{558} are produced by kidney glomerular cells in passive Heymann nephritis

(glomerular damage/autoimmune disease/renal pathology)

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ABSTRACT **Reactive oxygen species (ROS) have been implicated in the production of glomerular damage in passive Heymann nephritis (PHN), an experimental form of membranous nephropathy with neutrophil-independent proteinuria. Immunohistochemistry with monoclonal antibodies specific for cytochrome b_{558} (a major component of the oxidoreductase complex of the respiratory burst in stimulated neutrophilic granulocytes) showed that this enzyme is localized within visceral glomerular epithelial cells (GECs) in a dense, granular pattern in rats with PHN and proteinuria. By immunoelectron-microscopy, the cytochrome was found in membrane vesicles within the GEC and also extracellularly on the GEC membranes facing the glomerular basement membrane (GBM). By immunoblotting, cytochrome b_{558} was detected in highest concentration in lysates of isolated glomeruli from proteinuric rats. By contrast, only traces were found in normal glomeruli by immunohistochemistry. Depletion of complement abolished the expression of the cytochrome. Using an ultrastructural cerium–H_2O_2 histochemistry technique, the functional activity of the glomerular ROS-generating system was demonstrated exclusively in proteinuric PHN, where H_2O_2 was found in highest concentration within the GBM. These results provide evidence that in rats with PHN and proteinuria, the GECs express and externalize respiratory-burst enzymes that generate ROS in a manner similar to neutrophilic granulocytes, which could then lead to glomerular damage.**

Passive Heymann nephritis (PHN) is an experimental model of human membranous nephropathy that is induced in rats by injection of antibodies directed against crude fractions of kidney cortex (Fx1A) (1). There is evidence that the subepithelial immune deposits in PHN are formed *in situ* from immune complexes of the membrane glycoprotein (gp) complex gp330/44 kDa, which is present in glomerular visceral epithelial cells (GEC) and from circulating antibody (2–4). The causes of glomerular damage and of the resulting proteinuria are less well understood. Reactive oxygen species (ROS) have recently been implicated in the mediation of proteinuria in PHN (5–7), and in other experimental models of renal disease (8–16). ROS are known to be generated in large amounts by activated neutrophilic granulocytes and macrophages by a NADPH oxidoreductase enzyme complex that is responsible for the respiratory burst (17). We wanted to investigate whether or not there is a possible intrinsic glomerular source of ROS in PHN by the identification of this enzyme complex within glomeruli and to localize the distribution of ROS in the glomeruli of proteinuric PHN rats.

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MATERIALS AND METHODS

Antibodies. The monoclonal antibody mAb 48 with specificity for the cytochrome b_{558} high molecular weight B subunit (M_r, ≈100 kDa) has been prepared and characterized (18). Anti-Fx1A IgG with identical properties to that described originally for induction of PHN (1) was prepared in a sheep in Wellington, New Zealand (19, 20), and the globulin fraction was obtained by double precipitation with 50% saturated ammonium sulfate, yielding an IgG concentration of 23 mg/ml. Goat anti-rabbit IgG conjugated to 10-nm gold particles (Auroprobe) was obtained from Amersham; affinity-purified rabbit anti-mouse IgG, from Dako; fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse $F(ab')_2$, from Jaxell-Accurate (Westbury, NY); and mAb mouse anti-FITC IgG, from Sigma. Absorption of these IgGs with the respective IgGs from other species prevented reactivity by indirect immunofluorescence on cryostat sections of rat kidneys 7 days after induction of PHN, excluding cross reactivity with both sheep (heterologous) and rat (autologous) deposited IgGs.

Induction of Passive Heymann Nephritis. Male Sprague–Dawley rats (250–300 g) were injected i.v. with 11.5 mg of anti-Fx1A IgG in 0.5 ml of phosphate-buffered saline (PBS; 150 mM NaCl/20 mM phosphate buffer, pH 7.4) and were sacrificed after 3 or 7 days ("3-day PHN" or "7-day PHN" rats). Some 3-day PHN rats were depleted of complement by injection of Cobra venom factor (*Naja naja kaouthia*, Sigma) for 3 days (21). Controls consisted of rats injected i.v. with 600 μg of affinity-purified rabbit anti-gp330 IgG (22) or of rats injected with PBS. Proteinuria was determined by a Coomassie blue assay (Bio-Rad). The use of experimental animals in this study was authorized by the Austrian Ministry of Science.

Immunohistochemistry. Rat kidneys were fixed by perfusion with freshly prepared (from paraformaldehyde) 4% formaldehyde/7% sucrose in PBS (pH 7.4), and tissue blocks were further fixed for 6 hr at 20°C. Cryostat sections (4 μm) were incubated with mAb 48 (8 μg/ml), a bridging affinity-purified $F(ab')_2$ rabbit anti-mouse antibody (preabsorbed against immobilized rat and goat IgG) at a dilution of 1:50, and FITC-labeled $F(ab')_2$ goat anti-rabbit antibody (diluted 1:100, absorbed with rat serum, and shown to have no reactivity with rat IgG). For marked amplification of the signal, 1-μm frozen sections were cut on a Reichert Ultracut UMO 2

Abbreviations: DMTU, dimethylthiourea; GBM, glomerular basement membrane; GEC, glomerular epithelial cell; PHN, passive Heymann nephritis; ROS, reactive oxygen species; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate.

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ultramicrotome equipped with an F4 cryoattachment, quenched with 0.1% egg albumin in PBS followed by incubation in mAb 48 (8 μg/ml) for 45 min, incubated in PBS with 0.1% egg albumin and then in sequence (*i*) in $F(ab')_2$ sheep anti-mouse IgG, FITC-labeled (preabsorbed with rat serum) diluted 1:50; (*ii*) in mouse mAb anti-FITC IgG diluted 1:100; and (*iii*) again in FITC-labeled sheep anti-mouse IgG diluted 1:50. Sections were mounted in *p*-phenylenediamine/glycerol (pH 8.0) and were examined with a Leitz fluorescence microscope.

Ultrathin frozen sections were cut from a 7-day PHN rat kidney perfusion-fixed with 4% formaldehyde/7% sucrose in PBS, quenched in 1% bovine serum albumin in PBS, and incubated in mAb 48 (8 μg/ml) followed by affinity-purified rabbit $F(ab')_2$ anti-mouse IgG (preabsorbed with rat and goat IgG), and 10-nm gold–goat anti-rabbit IgG conjugate (1:10). As positive controls some sections were incubated with mAb anti-C5b-9 IgG (23); as negative controls the primary antibody was omitted. The labeled sections were processed as described by Tokuyasu (24) and were examined in a Zeiss EM9S electron microscope.

Immunoblotting. Glomeruli of controls injected with PBS and of rats 3 and 7 days after injection of anti-Fx1A IgG were isolated by graded sieving and solubilized in reducing SDS sample buffer, and the protein concentration was determined (25). For a comparative quantitative estimate, precisely 50 μg of protein was loaded into each slot. After electrophoresis in 5–15% polyacrylamide gradient/SDS gels, the proteins were transferred onto nitrocellulose and stained in 0.5% ponceau S in 6% trichloroacetic acid. Strips were incubated in mAb 48 (8 μg of IgG per ml) in PBS containing 5% dried milk and 0.1% Tween 20 at 20°C for 4 hr, followed by detection with alkaline phosphatase-labeled anti-mouse IgG and nitrotetrazolium blue (Promega).

Histochemical Localization of H_2O_2. In this study we applied the method of Briggs *et al.* (26), which was developed for the ultrastructural localization of H_2O_2 in stimulated neutrophilic granulocytes. The left kidneys were perfused at 37°C at a rate of 2.5 ml/min by retrograde aortic perfusion with 4 ml of each of the following solutions: (*i*) PBS; (*ii*) 20 mM Tris maleate buffer (pH 7.2) containing 7% sucrose (TMS); (*iii*) TMS containing 1 mM aminotriazole (Aldrich); (*iv*) TMS containing 10 mM aminotriazole and 1 mM cerium chloride heptahydrate (Sigma), passed twice through a 0.22-μm Millipore filter before adding 2.56 mM β-nicotinamide adenine dinucleotide (β-NADPH, Sigma, Grade 11); and (*v*) TMS followed by 10 ml of 2.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.2) as fixative. Pieces of kidney were fixed further for 2 hr at 20°C followed by treatment with cacodylate-buffered 2% osmium tetroxide and were embedded in Epon. In some experiments, dimethylthiourea (DMTU) (150 μg/ml) (27, 28) was perfused immediately before cerium exposure. The animal groups studied were as follows: Normal rats (n = 7), 3-day PHN rats (n = 5), 7-day PHN rats (n = 8), DMTU-pretreated 7-day PHN rats (n = 3), and anti-gp 330 IgG-treated rats (controls; n = 2).

RESULTS

Passive Heymann Nephritis. Rats injected with sheep anti-Fx1A IgG showed dense granular immune deposits in all capillary loops (Fig. 1*A*), and a similar pattern was observed in rats injected with purified anti-gp330 IgG (22). Only rats of the anti-Fx1A 7-day group were proteinuric (average 72.4 ± 12.0 mg/day), whereas <10 mg of urinary protein excretion per day was measured in rats injected with purified anti-gp330 IgG 7 days before sacrifice, in rats injected with sheep anti-Fx1A IgG 3 days before sacrifice, and also in controls injected with PBS alone.

Cytochrome b_{558} in Glomeruli. By indirect immunofluorescence mAb 48 (specific for the large ≈100-kDa subunit of cytochrome b_{558}) showed a diffusely distributed, punctate, cytoplasmic staining most evident in the GECs but also in endothelial and mesangial cells in glomeruli of 7-day PHN rats (Fig. 1*C*). The discrete granular nature of the reactivity was most apparent in the 1-μm thick sections (Fig. 1*D*). The intensity of the labeling was weaker on day 3 of PHN (Fig. 2*A*). The pattern of distribution was distinct from that of the IgG in the glomerular immune deposits (Fig. 1*A*). In controls injected with affinity-purified anti-gp330 IgG or with PBS (Fig. 1*B*), the cytochrome was not detected in glomeruli when the same level of enhancement of the fluorescent signal was used as for Fig. 1*C*—that is, with the bridging antibody alone (Fig. 1*B*). However, when the sensitivity of the immunofluorescence was increased by additional enhancement, a faint

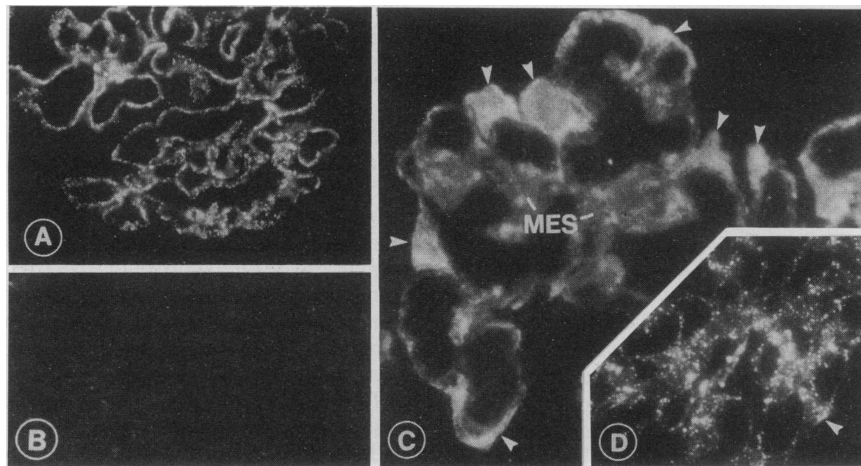

FIG. 1. Localization of sheep IgG (*A*) and of cytochrome b_{558} (*B–D*) by immunofluorescence on frozen sections. (*A*) Localization of sheep IgG in a glomerulus, 7 days after injection of anti-Fx1A IgG, in a fine granular pattern characteristic for PHN. (*B*) Localization of the B subunit of cytochrome b_{558} with mAb 48 in a normal rat glomerulus (1-μm-thick section). At this level of sensitivity of the indirect immunofluorescence technique (the same as used in *C* and *D*), the enzyme is visualized only in traces. (*C*) Localization of cytochrome b_{558} in a 4-μm cryostat section (to emphasize its overall distribution) of a glomerulus of a 7-day proteinuric PHN rat. There is an intense labeling for cytochrome b_{558} associated with the GECs (arrowheads) and a weaker signal also in the mesangial area (MES). (*D*) In a 1-μm section (to emphasize high resolution) of the same tissue as in *C*, the cytochrome b_{558} is found in a discrete punctate pattern in GECs (arrowhead) as well as in the mesangium. (*A* and *B*, ×600; *C*, ×950; *D*, ×800.)

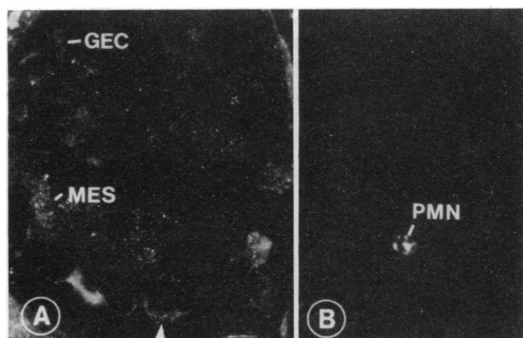

FIG. 2. Localization of cytochrome b_{558} in a glomerulus of a rat 3 days (an optimal time point for the depletion of complement) after injection of anti-Fx1A IgG (*A*) and a rat depleted of complement during this period of time by treatment with cobra venom factor (*B*), demonstrating a granular pattern of distribution in the GECs of the untreated animal in a relatively weak intensity, as typically found in PHN 3 days after injection of anti-Fx1A IgG (*A*), but its complete absence in the decomplemented rat (*B*). Note the bright staining of a trapped neutrophilic granulocyte (PMN). (×550.)

granular distribution could be seen in mesangial cells and also in GECs (not shown).

In the glomeruli of rats depleted of complement, the cytochrome was not detected by immunofluorescence (Fig. 2*B*) or by immunoblotting (not shown). By contrast, it was highly concentrated within the cytoplasm of a rare neutrophilic granulocyte captured within a glomerular capillary loop (Fig. 2*B*).

By immuno-electron-microscopy on ultrathin frozen sections of glomeruli of proteinuric 7-day PHN rats injected with anti-Fx1A IgG, binding of mAb 48 was confined to the membranes of small vesicles within the GEC cytoplasm (Fig. 3 *A* and *C*). There was no labeling of mitochondria. On the GEC surface membranes, gold particles were found along the "soles" of foot processes and sometimes also in extracellular clusters close to immune deposits (Fig. 3 *B* and *D–F*). Labeling was also observed in a few cytoplasmic membrane vesicles in endothelial and in mesangial cells (not shown).

Biochemical Detection of Cytochrome b_{558}. By immunoblotting with mAb 48, a single band with an apparent M_r of ≈100 kDa was labeled in SDS lysates of 7-day PHN rat glomeruli; a weak signal was obtained in 3-day PHN preparations and none in PBS-injected controls (Fig. 4). The amount and the pattern of proteins transferred onto the nitrocellulose membranes from the different glomerular lysates was identical.

Histochemical Detection of H_2O_2. The granular, electron-dense reaction product of cerium perhydroxide was found in ≈75% of the capillary loops exclusively in the glomeruli of proteinuric 7-day PHN rats. Invariably the precipitate was distributed diffusely throughout the GBM (Fig. 5*A*). The reaction product was also observed within GECs in lysosome-like vesicles (Fig. 5 *B* and *C*) and occasionally also in endothelial cells. At no time was the reaction product evident in mitochondria. Controls injected with PBS or anti-gp330 IgG as well as nonproteinuric 3-day PHN rats did not show any reaction product. When the kidneys of proteinuric 7-day PHN rats were preperfused with DMTU, no cerium–H_2O_2 reaction product was deposited.

DISCUSSION

Previous *in vivo* interventional studies with ROS scavengers have established a major role for ROS in the mediation of proteinuria in PHN (5–7). In the current study, the mechanisms of the *in situ* production and the location of ROS in glomeruli of proteinuric PHN rats were investigated. We have localized by immunocytochemistry and detected by immunoblotting in isolated rat glomeruli cytochrome b_{558}, an essential component of the NADPH-oxidoreductase enzymatic complex that mediates the respiratory burst in neutrophilic granulocytes (18, 26). Expression was greatly amplified in proteinuric PHN rats when compared with nonproteinuric controls and was dependent on complement. An ultrastructural cerium histochemistry technique showed the location of H_2O_2 within proteinuric glomerular capillary walls, notably within the GBMs and GECs, and its absence in nonproteinuric controls. These results indicate that—in coincidence with the development of proteinuria in PHN—intrinsic glomerular cells, in particular GECs, produce and liberate ROS.

The group of compounds of the ROS (superoxide anion, H_2O_2, hydroxyl radical) that result from the stepwise activation of the respiratory burst have been extensively studied in professional phagocytes such as neutrophils and are known to be potent mediators of cell injury in a variety of pathologic

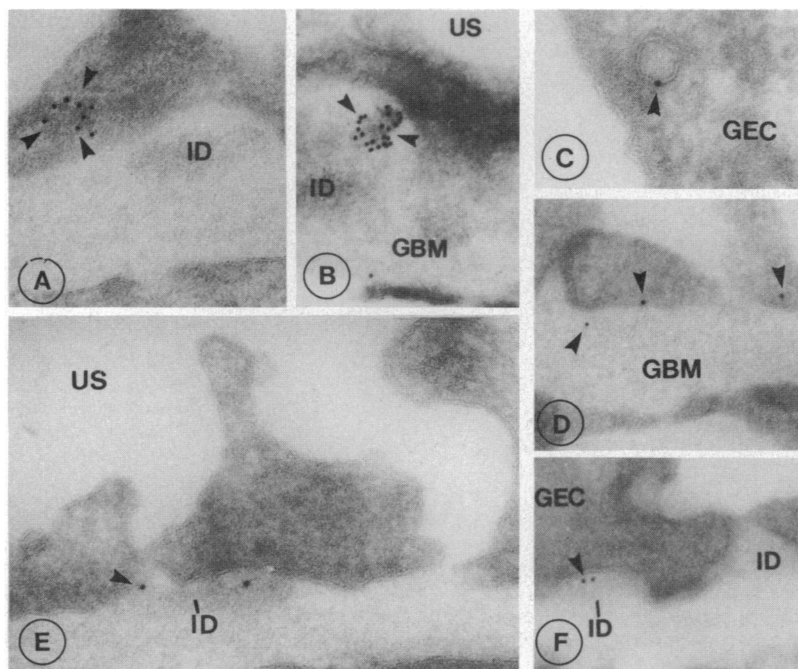

FIG. 3. Localization of cytochrome b_{558} by immunogold electron microscopy on ultrathin frozen sections of glomeruli of proteinuric rats 7 days after injection of anti-Fx1A IgG. (*A*) Gold particles outline a vesicle (arrowheads) in the GEC's cytoplasm, corresponding to the granular distribution of this enzyme seen by immunofluorescence. (*B*) Extracellularly located cytochrome b_{558} is indicated by a cluster of gold grains adjacent to an immune complex deposit. (*C*) Another example of gold particles localized in an intracellular vesicle in a GEC. (*D*) Extracellular localization of cytochrome b_{558} along the cell membrane of a foot process, indicated by gold particles (arrowheads). (*E* and *F*) Association of cytochrome b_{558} with the membrane at the "soles" of the GECs in proximity of immune complex deposits (ID). US, urinary space; GBM, glomerular basement membrane; E, endothelial cell. (*A*, *B*, *D*, and *F*, ×32,000; *C*, ×60,000; *E*, ×40,000.)

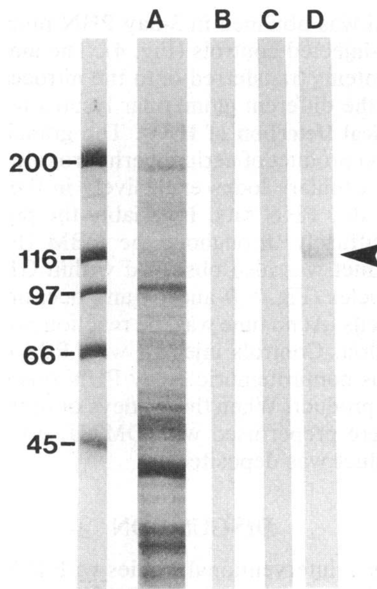

FIG. 4. Immunoblots with monoclonal anti-cytochrome b_{558} IgG (mAb 48) on nitrocellulose transfers of lysates of isolated glomeruli from normal rats (lane B) and from rats injected with anti-Fx1A IgG for 3 days (lane C) or 7 days (lane D) before sacrifice. All lanes were loaded with precisely the same amount of protein and overlayed with mAb 48 under identical conditions to permit a quantitative comparative estimate. Lanes: A, Coomassie blue-stained proteins of a glomerular lysate of a normal rat (similar protein patterns were obtained from rats with PHN; not shown); B, C, and D, immunoblots of normal, 3-day, and 7-day PHN glomerular transfers, respectively, revealing a single dense band at ≈100 kDa in the 7-day PHN transfer (arrowhead), a barely detectable band in 3-day PHN (lane C), and no detectable band in the glomeruli of normal rats (lane B). The leftmost lane shows molecular weight markers (200, 116, 97, 66, and 45 kDa).

processes—for example, ischemia, reperfusion injury, respiratory distress syndrome, neoplasia, etc. (27–30). Oxygen metabolites have been implicated previously in several experimental models of renal disease, including aminonucleoside nephrosis, anti-GBM antibody-initiated nephritis, concanavalin A-nephropathy, neutrophil-dependent glomerular injury induced by the infusion of phorbol myristic acid or cobra venom factor, etc. (5–16). In PHN it was observed that hydroxyl radical scavengers such as DMTU, which also inhibits H_2O_2 (31, 32), and the iron chelator deferoxamine effectively reduced proteinuria (6). These findings confirm a substantial role for ROS in the glomerular damage and the associated proteinuria in PHN, although the precise species primarily responsible is not established. However, there is evidence that H_2O_2 can be directly nephrotoxic, as intraarterial administration in rats produces transient massive proteinuria associated with impairment of molecular size selectivity (33).

The respiratory burst in activated neutrophilic granulocytes is followed by generation of ROS by a cyanide-insensitive nonmitochondrial multicomponent NADPH-oxidase enzyme complex (27–30) of which cytochrome b_{558} is a membrane-bound heterodimeric component (18). This enzyme complex is functionally dormant in resting phagocytes (34, 35) and appears restricted to the membranes of intracytoplasmic granules. However, when activated by phagocytosable particles or a variety of soluble factors (chemotactic peptides, complement components, lectins, fatty acids), the enzyme inserts into the neutrophil's plasmalemma by exocytosis of granules and then generates ROS on the cell surface (26, 34, 35). GECs resemble neutrophils in this respect because cytochrome b_{558} is associated with cytoplasmic membrane vesicles in GECs, which could be subsequently transported and exposed to the GEC's basal cell surface facing the GBM, presumably by exocytosis or secretion.

Recent investigations have indicated that the NADPH oxidoreductase, and in particular the cytochrome b_{558} component, is not restricted in its expression to neutrophils and macrophages. Human mesangial cells (36) and fibroblasts (37) express the cytochrome. Moreover, human mesangial cells in culture liberate ROS independently of phagocytosis when stimulated by cytokines (38). Our studies now provide evidence that rat GECs also express the cytochrome *in vivo* and that this is markedly amplified in proteinuric PHN in conjunction with the production of injurious ROS.

The results of the histochemical experiments provide direct evidence that H_2O_2 is produced in glomeruli of proteinuric PHN rats. This indicates that not only cytochrome b_{558} but also the entire NADPH oxidoreductase enzyme complex is fully functional. The precipitates of H_2O_2 perhydroxide are readily detectable in the GBMs and in the GECs of proteinuric PHN rats but not of normal rats, suggesting that the GBM could be exposed to ROS *in vivo*, resulting in proteinuria. However, the determination of the precise site of production of H_2O_2 is limited by the resolution of the histochemical

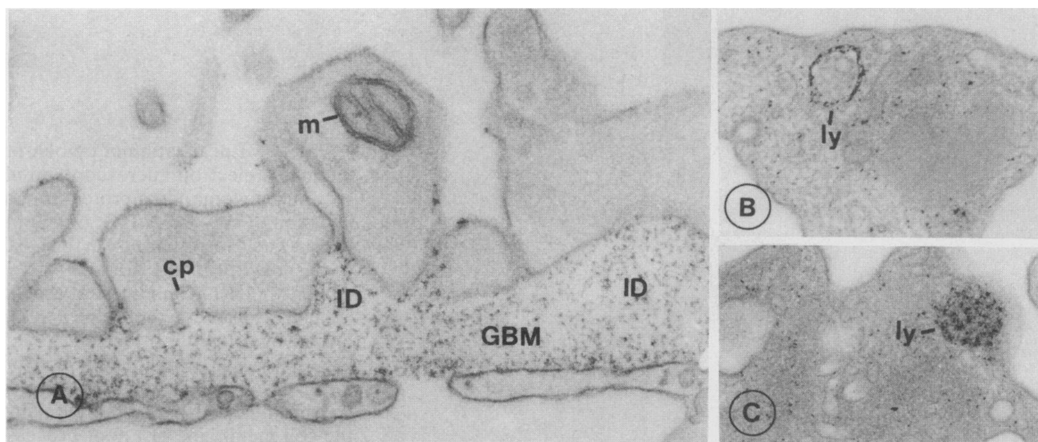

FIG. 5. Localization of H_2O_2 by a histochemical cerium-precipitation method applied to isolated and perfused kidneys of proteinuric rats 7 days after injection of anti-Fx1A IgG. (*A*) Granular, electron-dense reaction product of cerium perhydrate is seen prominently within the entire GBM, including the immune deposits (ID). Mitochondria (m) are not labeled. Occasionally, the reaction product is observed on the external surface of GECs facing the urinary space. (*B* and *C*) Dense reaction product is seen within the lumen of lysosome-like vesicles (ly) in the GECs. cp, coated pit. (*A*, ×35,000; *B* and *C*, ×25,000.)

technique used mainly because H_2O_2 is known to diffuse rapidly across cell membranes (39), and the cerium-reaction product could be redistributed within the GBM during the perfusion procedure.

Complement activation and formation of the C5b-9 membrane-attack complex in immune deposits in PHN were found to correlate with proteinuria, which usually develops 5–6 days after the injection of anti-Fx1A IgG (14). Complement activation causes sublytic damage to GECs (40), which could also trigger the induction of cytochrome b_{558} and the subsequent production of ROS. There is suggestive evidence that subsequent Ca influx and protein kinase C may limit the extent of GEC damage, at least *in vitro* (41).

Recent findings have suggested a role for macrophages in the development of proteinuria in PHN (42). The number of macrophages per glomerulus increased from ≈5 to 24 in proteinuric rats, and the onset of proteinuria was delayed when macrophages were depleted. This raises the possibility that the cytochrome b_{558} observed in intrinsic glomerular cells could be derived from infiltrating macrophages after shedding of the transmembrane enzyme and endocytic uptake by glomerular cells. However, this seems unlikely, as we have found that depletion of macrophages with cyclophosphamide did not decrease the amount of cytochrome b_{558} expressed in the glomeruli of proteinuric PHN rats (unpublished observations).

While this study provides evidence that intrinsic glomerular cells can generate ROS by molecular mechanisms that resemble those found in neutrophilic granulocytes, it remains to be determined which component(s) of the capillary wall become chemically modified to the extent that the permselectivity of the filtation barrier is destroyed, resulting in proteinuria.

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