New Mechanism for Glomerular Injury

Myeloperoxidase-Hydrogen Peroxide-Halide System

Richard J. Johnson, William G. Couser, Emil Y. Chi, Stephen Adler, and Seymour J. Klebanoff
Divisions of Infectious Diseases and Nephrology, Department of Medicine and Department of Pathology, University of Washington, Seattle, Washington 98195

Abstract

Reactive oxygen species, particularly hydrogen peroxide (H_2O_2), participate in neutrophil-mediated glomerulonephritis. However, the mechanism of H_2O_2 neptrotoxicity is unknown. Myeloperoxidase (MPO), a neutrophil cationic enzyme that localizes in glomeruli, can react with H_2O_2 and halides to form highly reactive products. We tested the hypothesis that the MPO-H_2O_2-halide system may induce glomerular injury by infusing MPO followed by H_2O_2 in a chloride-containing solution into the renal artery of rats. Controls received MPO or H_2O_2 alone. MPO-H_2O_2-perfused rats developed significant proteinuria, endothelial cell swelling, and epithelial cell foot process effacement, whereas control kidneys were normal. In the presence of free ^{125}I, MPO-H_2O_2-perfused rats incorporated large amounts of ^{125}I, localized to the glomerular basement membrane and mesangium by autoradiography, into glomeruli. Glomerular iodination was greatly decreased or absent in controls. The MPO-H_2O_2-halide system causes glomerular injury and may be important in neutrophil-mediated glomerulonephritis.

Introduction

There is considerable evidence suggesting that neutrophils mediate glomerular injury in certain experimental models of nephritis (reviewed in reference 1). The evidence for neutrophil involvement is best documented in complement-dependent models of anti-glomerular basement membrane (anti-GBM)[1] nephritis in the rabbit and rat. In these models, neutrophils can be seen infiltrating the glomerulus within 3–4 h of antibody administration (2, 3). Proteinuria correlates with the number of neutrophils observed within the glomerulus and can be decreased or prevented by depletion of neutrophils with anti-neutrophil serum or cytotoxic agents (2, 4). The intravenous infusion of neutrophils to a leukocyte-depleted animal after administration of anti-GBM antibody can partially reconstitute injury and proteinuria (5). Neutrophils have also been reported to be important in complement-independent models such as the accelerated autologous anti-GBM nephritis in rabbits (6).

Although early studies had implicated the release of proteolytic enzymes in neutrophil-mediated glomerular injury (7–10), recent studies have emphasized the role of inflammatory cell-derived reactive oxygen species (ROS) (11–13). Neutrophils respond to stimulation with a burst of oxygen consumption, and the ROS produced during the respiratory burst include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$), and possibly singlet oxygen ($^1O_2$). The toxicity of H_2O_2 is greatly increased by reaction with the peroxidase of neutrophils (myeloperoxidase [MPO]) and a halide (chloride, bromide, iodide) (14, 15) with the halide undergoing oxidation to a toxic species such as the hypohalous acid or halogen. Evidence that ROS are important mediators of glomerular injury derives largely from the effects of ROS scavengers on the injury produced by circulating inflammatory cells. Superoxide dismutase (SOD) is a scavenger of O_2^-; catalase degrades H_2O_2, and a number of reagents (e.g., mannitol, ethanol, dimethyl sulfoxide [DMSO]) can scavenger $OH^\cdot$. Although one early study suggested a protective effect of SOD on glomerulonephritis induced by infusion of preformed immune complexes in mice (16, 17), most evidence now favors H_2O_2 as the most important ROS in neutrophil-dependent glomerular disease. Thus infusion of phorbol myristate acetate (PMA) to activate neutrophils, cobra venom factor (CVF) to activate complement, or anti-rat GBM antibody into the renal artery of rats results in significant proteinuria associated with a mild glomerular neutrophil infiltrate (11–13). In each of these studies, neutrophil depletion or pretreatment with catalase markedly reduced glomerular damage whereas SOD was ineffective, thereby implicating H_2O_2 as the major mediator of these neutrophil-dependent forms of glomerular injury (11–13). The dependence on H_2O_2 did not appear to be due to $OH^\cdot$ formation because treatment with the $OH^\cdot$ scavenger DMSO was also nonprotective (12, 13).

We reasoned that the H_2O_2-mediated glomerular injury may involve the MPO-H_2O_2-halide system. This hypothesis seemed particularly attractive in view of the highly cationic nature of MPO (isoelectric point > 10 [18]) and recent studies that demonstrate glomerular localization of neutrophil and platelet-derived cationic proteins in glomerulonephritis (19, 20). The evidence presented here demonstrates that very small quantities of MPO localize, presumably on a charge basis, in the glomerular capillary wall without causing demonstrable injury, but that subsequent exposure to nontoxic concentrations of H_2O_2 result in severe glomerular damage associated with halogenation of glomerular basement membrane.

These studies were presented in part at the 18th Annual Meeting of the American Society of Nephrology in New Orleans, LA, December 1985, and appeared in abstract form (1986. *Kidney Int.* 29:278).

Address reprint requests to Richard J. Johnson, Department of Medicine, Mail Stop RM-11, University of Washington, Seattle, WA 98195.

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1. *Abbreviations used in this paper:* CETAB, cetyltrimethylammonium bromide; CVF, cobra venom factor; DAB, diaminobenzidine; EM, electron microscopy; GBM, glomerular basement membrane; HD, half distance; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; $^1O_2$, singlet oxygen; O_2^-, superoxide anion; $OH^\cdot$, hydroxyl radical; PMA, phorbol myristate acetate; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid.

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Methods

Special reagents. MPO was prepared from canine pyometria by the method of Agner (21) to the end of step 6. The preparation was dialyzed against water and stored at −20°C until use. MPO activity was determined immediately before each experiment by the guaiacol assay (22). 1 U of enzyme is the amount that oxidizes 1 μmol of guaiacol per min. In most experiments MPO was diluted with phosphate buffered saline (PBS), pH 7.0, to a concentration of 18 U/ml, with 0.5 ml (108 μg MPO) used in each experiment. H_2O_2 (30%), obtained from American Scientific and Chemical Co., Seattle, WA, was assayed immediately before use by its absorption at 230 nm, using an extinction coefficient of 81 M^{-1} cm^{-1}. Na ^{125}I, sp act 17.4 Ci/mg NaI, was obtained from New England Nuclear Corp., Boston, MA.

Renal artery perfusion. Sprague-Dawley rats (Tyler Labs, Bellevue, WA) weighing 240–310 g were anesthetized with intraperitoneally administered chloral hydrate (0.1 ml of 3.6% solution per 10 g body weight). The left renal artery was isolated, the renal blood flow was interrupted, and a 30-gauge needle was carefully introduced into the lumen with the aid of a dissecting microscope. The kidney was initially perfused with 0.5 ml of PBS to displace the blood. The kidney was then perfused with either (*a*) 1.0–2.0 ml of PBS, (*b*) 0.5 ml of PBS followed by 2.0 ml of 10^{-3} M H_2O_2 in PBS, (*c*) 0.5 ml of MPO (9 U, 108 μg) in PBS followed by 2.0 ml of PBS, or (*d*) 0.5 ml of MPO (9 U) followed by 2.0 ml of 10^{-3} M H_2O_2 in PBS. All perfusions were performed with a constant infusion pump (Sage Instruments Div., Orion Research Inc., Cambridge, MA) at a rate of 0.5 ml/min. Following the perfusion the needle was removed, gentle pressure and gelfoam (Upjohn Co., Kalamazoo, MI) were applied to the puncture site, and the kidney was allowed to reperfuse. Total ischemia time was always < 10 min. Only rats in which the kidney appeared to reperfuse normally were studied. The rats were placed under a heat lamp for 2 h and then housed in metabolic cages with water ad libitum for the remaining 22 h of the day. Urine was collected and analyzed for protein content using the sulfosalicylic acid method (23).

In vivo iodination. To test the hypothesis that MPO-H_2O_2 induced glomerular damage is associated with halogenation of glomerular tissues, Sprague-Dawley rats were perfused as described above except that 16 nmol NaI containing 0.25 μCi ^{125}I was added to the last perfusion (H_2O_2 or PBS) in each animal. Following the iodide perfusion an additional 9 ml of PBS was infused at 0.5 ml/min and the kidney was removed. Glomeruli were immediately isolated according to standard methods using differential sieving techniques (24). The total cpm in a washed glomerular pellet was determined in a Prias scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL) and the total number of glomeruli was determined by visual counting with a Fuchs-Rosenthal hemocytometer. Assuming 38,000 glomeruli in each rat kidney (25), we expressed iodination as cpm/total glomeruli with appropriate correction for background. The glomerular pellet was solubilized with 1 M NaOH at 56°C for 1 h, neutralized with an equal volume of 1 N HCl, and the percent of cpm present in a 10% trichloroacetic acid (TCA) precipitate was determined.

Quantitation of MPO retained in glomeruli. Separate experiments were performed to determine the amount of MPO that bound to glomeruli following perfusion. In four rats the left kidney was removed immediately after perfusion with MPO (9 U, 108 μg) and 2 ml of 10^{-3} M H_2O_2. The glomeruli from each kidney were isolated and counted as previously described and the MPO was extracted with the cationic detergent cetyltrimethylammonium bromide (CETAB) (26). Briefly, the glomeruli were suspended in 0.5 ml of 0.3% CETAB in 0.01 M phosphate buffer, pH 7.0, and disrupted with a sonicator (Branson Sonic Power Co., Danbury, CT). The MPO was extracted by incubation at 4°C for 1 h. Following centrifugation, the supernatant was assayed for MPO, and activity expressed as the amount of MPO present in 38,000 glomeruli.

Histology and tissue processing. Rats were perfused initially with PBS to displace the blood from the left kidney and then received either PBS, H_2O_2, MPO, or H_2O_2 and MPO as described above. Kidneys were fixed in vivo by perfusion with 2 ml of 2% glutaraldehyde (37°C) before removal. Tissue for routine light microscopy was fixed further in 2% glutaraldehyde for 30 min, washed in 0.1 M sodium cacodylate buffer, pH 7.4, dehydrated in graded ethanols, and embedded in glycomethylmethacrylate (Polysciences, Inc., Warrington, PA) (27). 3-micron sections were stained with periodic acid–Schiff reagent. Specimens for routine electron microscopy (EM) were immersed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4–6 h. They were then rinsed in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide for 2 h, washed with distilled water, and block stained in 0.5% uranyl acetate. The tissue was then dehydrated in graded ethanol, cleared in propylene oxide, and embedded in epoxy resin. 1 micron and ultrathin sections were cut on a LKB-Ultramicrotome (LKB Produkter, Stockholm, Sweden) using a diamond knife (Diatome, Bienne, Switzerland). Thin sections were stained on the grid with uranyl acetate and lead citrate and examined in a JEOL-100 B electron microscope (Japanese Electronics, Tokyo, Japan).

Tissue was also processed for localization of MPO using a modification of the method of Graham and Karnovsky (28). Following glutaraldehyde perfusion, the biopsy tissue was further fixed for 30 min with 2% glutaraldehyde, then washed twice with 0.1 M sodium cacodylate buffer. Tissue sections of ~ 40 microns were prepared with a McIlwain tissue chopper (Mickie Laboratory and Engineering Co., Gomshall, England) and then incubated for 30 min in 0.1 M cacodylate buffer containing 0.01 percent H_2O_2 and 10 mg/ml diaminobenzidine (DAB) (Scientific Chemical, Huntington Beach, CA). Tissues were further processed for light and electron microscopy as previously described, except that for EM the specimens were not stained either in the block or on the grid with uranyl acetate or lead.

EM autoradiography. EM autoradiography was performed to better localize the site of iodination, following perfusion with MPO, H_2O_2, and iodide. The experimental design was similar to the in vivo iodination protocol described above except that a larger amount of Na ^{125}I was added to the last perfusion (55.3 μCi). Following the iodide perfusion, the kidney was further perfused with PBS (9.0 ml) followed by in vivo fixation with 3 ml of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer. The kidney was then removed and prepared for EM as described above. Ultrathin sections were placed on nickel grids, stained with lead citrate and uranyl acetate, carbon coated, and then coated with a monolayer of Ilford-L4 emulsion using the wire loop method (29). Grids were exposed for 9 d, beginning 6 d after surgery. They were then developed with Microdol-X (Eastman Kodak Co., Rochester, NY) at room temperature for 3 min and fixed for 1.5 min in Rapid Fixer (Eastman Kodak Co.).

The distribution of 538 silver grains located within 0.2 microns of the peripheral glomerular capillary wall (excluding mesangial areas) was analyzed. Silver grain position was measured as the shortest distance from the center of the silver grain to the center of the basement membrane. The half distance (HD) (i.e., distance within which 50% of the developed grains were located) as defined by Salpeter (30) was determined, and the density of grains relative to the basement membrane was compared to the theoretical distribution of grains around a radioactive solid band source (30).

Statistical analysis. All values are expressed as the mean±SEM and were analyzed for statistical differences by Student's *t* test with significance defined as $P < 0.05$.

Results

Effect of MPO-H_2O_2-Cl infusion on protein excretion. Fig. 1 demonstrates the effect of the perfusion of either MPO or H_2O_2 alone or in combination on protein excretion. In initial experiments in which 1–2 ml of PBS was infused in the left renal artery of rats, the 24-h baseline protein excretion was 10.0±1.6 mg (n = 9). When the kidney was perfused with either 0.5 ml of MPO (9.0 U) followed by 2.0 ml of PBS or with 0.5 ml PBS followed by 2.0 ml of 10^{-3} M H_2O_2 in PBS, 24-h protein excretion was 8.4 mg and 9.2 mg respectively, values which were not significantly different from the PBS control. However, when per-

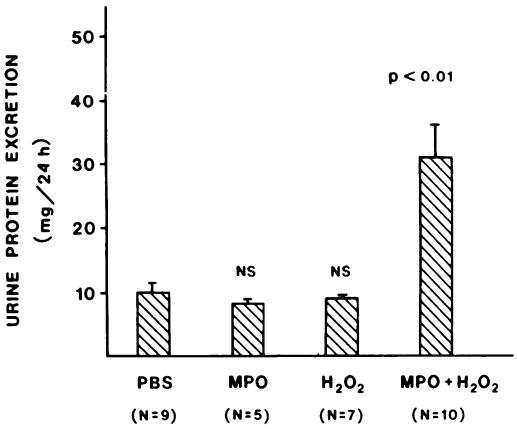

Figure 1. Proteinuria in animals receiving MPO + H_2O_2. The left kidney of rats was perfused with either PBS, MPO, H_2O_2, or MPO, followed by H_2O_2 and protein excretion was measured for the next 24 h. Data show the mean protein excretion and SEM. The significance of the difference from the PBS control is shown above the bars (NS, not significant; $P > 0.05$).

fusion of 0.5 ml of MPO (9.0 U) in PBS was followed by 2.0 ml of 10^{-3} M H_2O_2 in PBS, protein excretion during the first 24 h was increased to 31.2 mg, a value significantly different from either MPO or H_2O_2 alone. The MPO extracted from the glomeruli with CETAB following perfusion was 0.0093±0.0023 U/38,000 glomeruli (n = 4) or 0.1% of the injected dose. The CETAB extraction procedure solubilized 30–40% of the MPO bound to sonically prepared rat GBM following its addition in vitro (data not shown), suggesting that ~ 0.3% of the injected MPO was bound to the glomeruli. When the amount of MPO infused was decreased to 5.4, 4.5, 2.5, or 1.25 U before the H_2O_2 infusion, the initial 24-h protein excretion was 39, 24, 8.9, and 12.1 mg respectively (single animals), suggesting that the threshold dose of MPO required to increase protein excretion in the presence of H_2O_2 was ~ 4.5 U.

To insure that the increased protein excretion observed originated from the perfused kidney, in separate experiments rats received an infusion of MPO (9.0 U) followed by 10^{-3} M H_2O_2 in the left renal artery and immediately following the perfusion had either the normal right kidney or perfused left kidney removed. When the normal right kidney was removed, urine protein excretion was 39.1 and 30.4 mg on day 1 and 66.6 and 119.9 mg on day 3 (n = 2). In contrast, when the perfused left kidney was removed the protein excretion on day 1 was 19.9±3.6 mg and on day 3 was 7±2.2 mg (n = 4). This is comparable to the protein excretion observed if the left kidney is perfused with PBS alone before removal (18.5±1.8 mg on day 1, 4.3±0.5 mg on day 3, n = 3). Although nephrectomy alone produces low-grade proteinuria during the first 24 h (19.9 and 18.5 vs. a normal protein excretion 10.0±1.6 mg), this data suggests that the proteinuria induced by the MPO system originates in the perfused kidney.

Glomerular localization of MPO. Glomeruli in control animals receiving H_2O_2 (n = 2) were negative for peroxidase by light microscopy (Fig. 2 *A*), whereas following MPO infusion, the glomerular capillary wall was strongly peroxidase-positive (n = 2) (Fig. 2 *B*). The tubules were generally negative for peroxidase although occasional tubules in both MPO or PBS-perfused animals stained positively. By electron microscopy, MPO could be demonstrated along the glomerular basement membrane with the highest concentration in the subepithelial space, particularly along the base of the epithelial cell foot processes (Fig. 3 *B*). The glomeruli of rats perfused with PBS alone were peroxidase-negative by EM cytochemistry (Fig. 3 *A*).

Glomerular morphology. Biopsies in the control rats 4 and 24 h after perfusion with MPO alone (n = 2) or H_2O_2 alone (n = 2) revealed normal glomerular architecture by light (Fig. 4, *A* and *C*) and electron microscopy. In contrast, biopsies from rats perfused with MPO and H_2O_2 exhibited significant glomerular injury (Fig. 4, *B* and *D*). At 4 h there was a reduction in nuclear staining of resident glomerular cells, cell swelling, and the GBM often appeared wrinkled (Fig. 4 *A*). Capillary loops were fre-

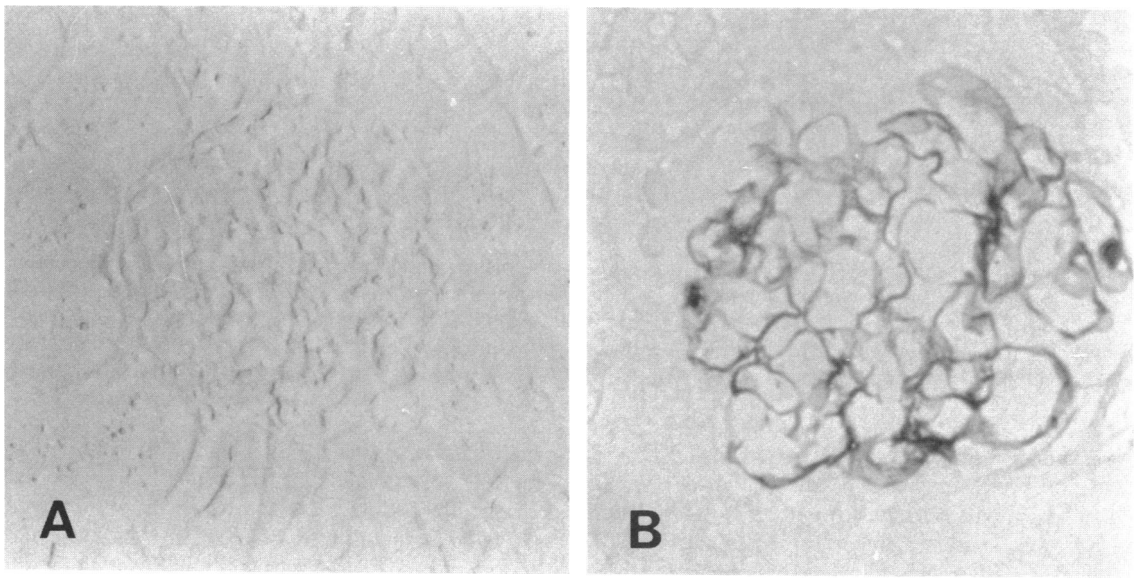

Figure 2. MPO localization by light microscopy. (*A*) Light microscopic demonstration of negative glomerular staining at 1 min in a control rat receiving H_2O_2 alone (200 ×). (*B*) Positive DAB staining for MPO along the glomerular capillary wall 1 min following MPO perfusion. The biopsy was taken during the subsequent H_2O_2 perfusion (400 ×).

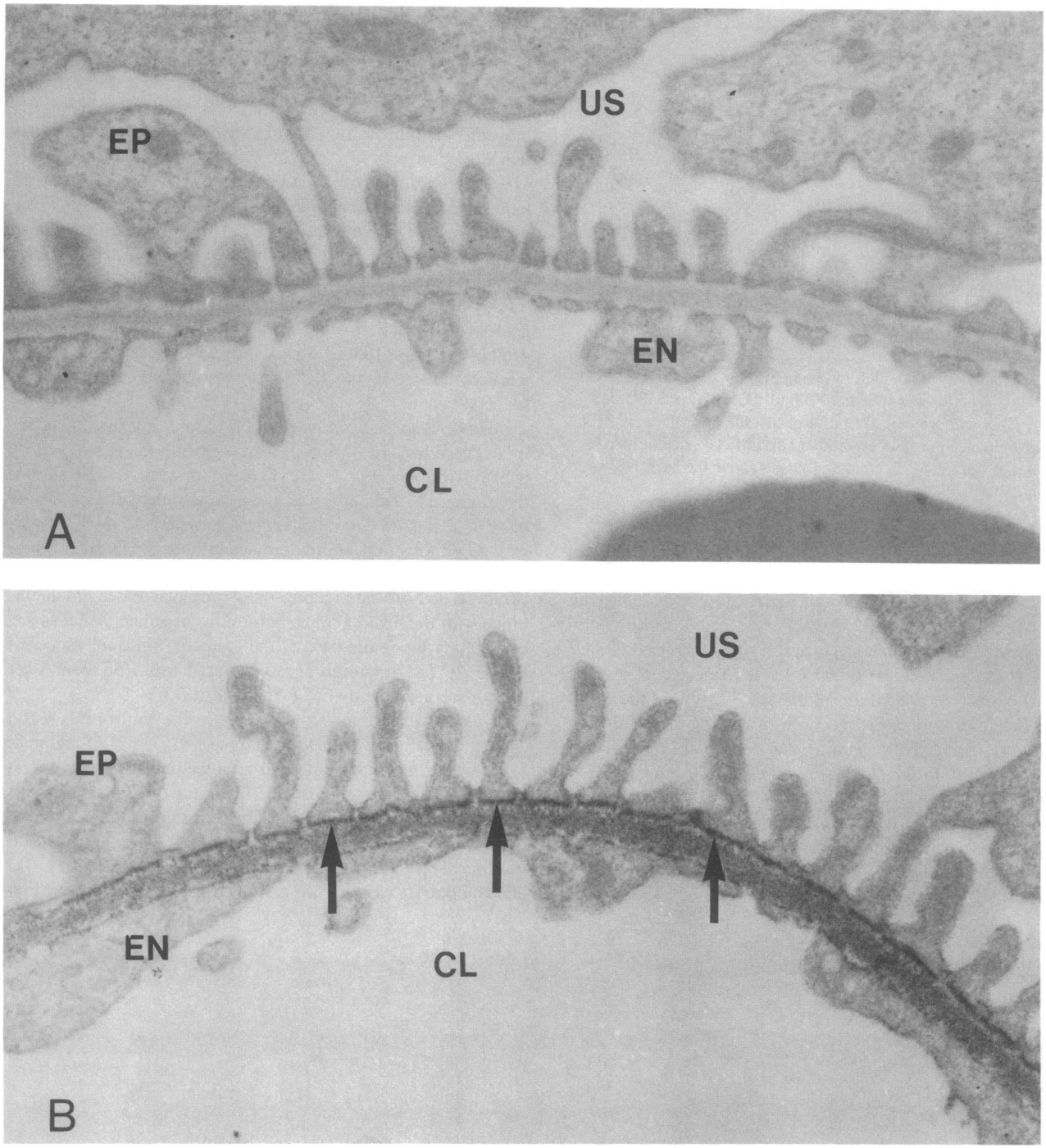

Figure 3. MPO localization by electron microscopy. (*A*) Electron micrograph demonstrating the absence of DAB-positive material in the glomerular basement membrane of animals infused with PBS alone (40,000 ×). (*B*) Electron micrograph of the glomerular capillary wall of an animal receiving MPO and PBS, demonstrating the presence of DAB-positive material in the basement membrane with concentration in the subepithelial space along the base of the epithelial cell foot processes (40,000 ×). *CL,* capillary lumen; *EN,* endothelial cell; *EP,* epithelial cell; *US,* urinary space.

quently occluded by a granular weakly eosinophilic material. At 24 h these changes were more prominent and appeared to involve primarily endothelial and mesangial cells. Electron microscopy at 4 h demonstrated endothelial cell swelling with occasional denudation (Fig. 5). Platelets were frequently present although infiltrating leukocytes were not common. Focal epithelial cell foot process effacement was evident, but there were no discontinuities in the GBM.

In vivo iodination by the MPO-H_2O_2-halide system. The ability of the MPO-H_2O_2-halide system to cause in vivo iodination was assessed by adding ^{125}I to the last perfusion. Between 24,000 and 35,000 glomeruli were isolated per rat kidney, and the numbers of cpm of ^{125}I was corrected to 38,000 glomeruli (25). The number of cpm of ^{125}I in glomeruli isolated from rats receiving both MPO and H_2O_2 was markedly increased compared with controls receiving either PBS, H_2O_2, or MPO alone

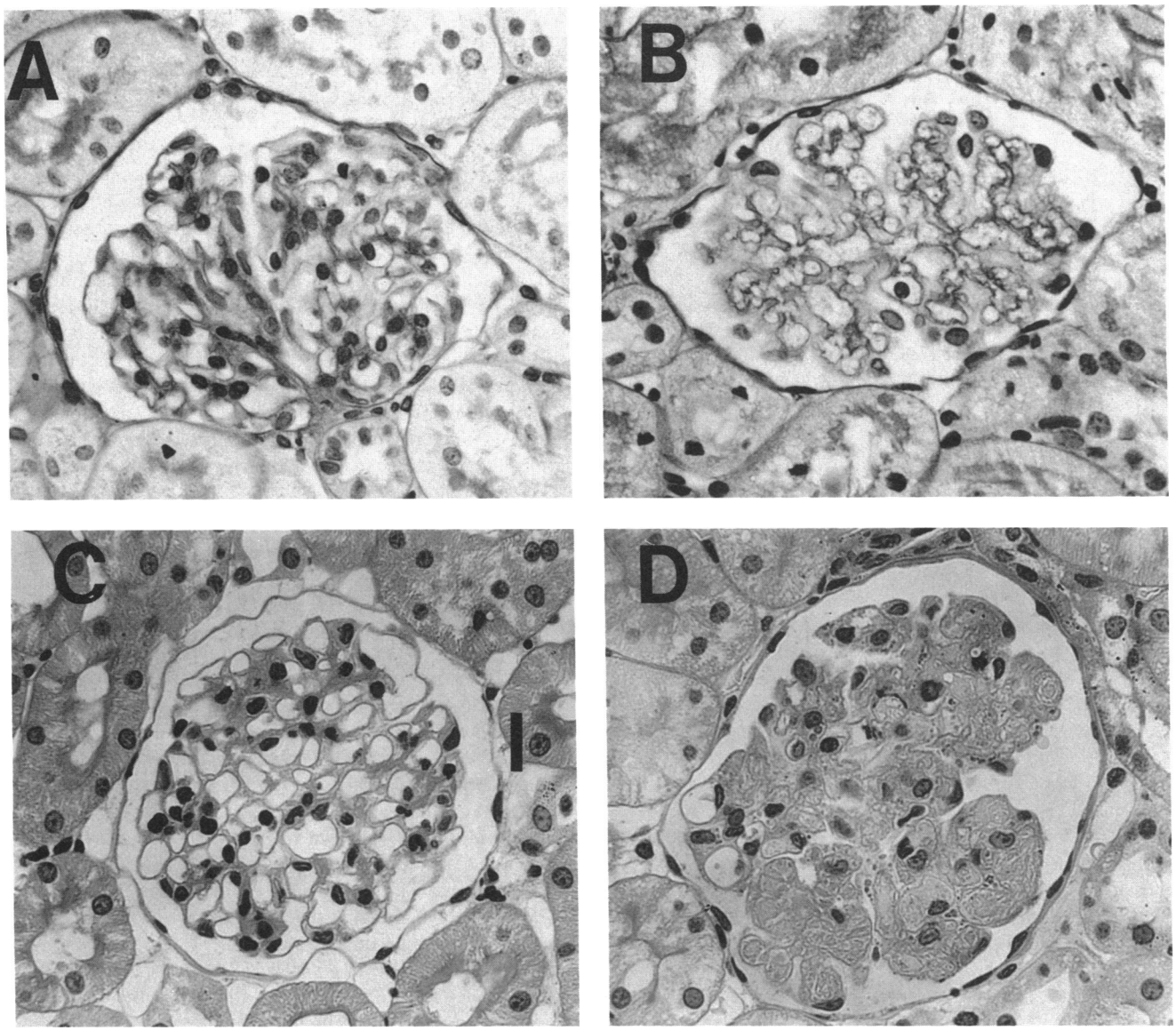

Figure 4. Glomerular morphology by light microscopy. A glomerulus from a rat 4 h (*A*) and 24 h (*C*) after being perfused with H_2O_2 alone demonstrated normal glomerular morphology (400 ×, periodic acid Schiff stain). In contrast, a glomerulus from a rat 4 h after receiving both MPO and H_2O_2 (*B*) showed endothelial cell swelling and a loss of nuclear staining and at 24 h (*D*) demonstrated, in addition, the occlusion of many capillary loops with a weakly eosinophilic granular material (400 ×).

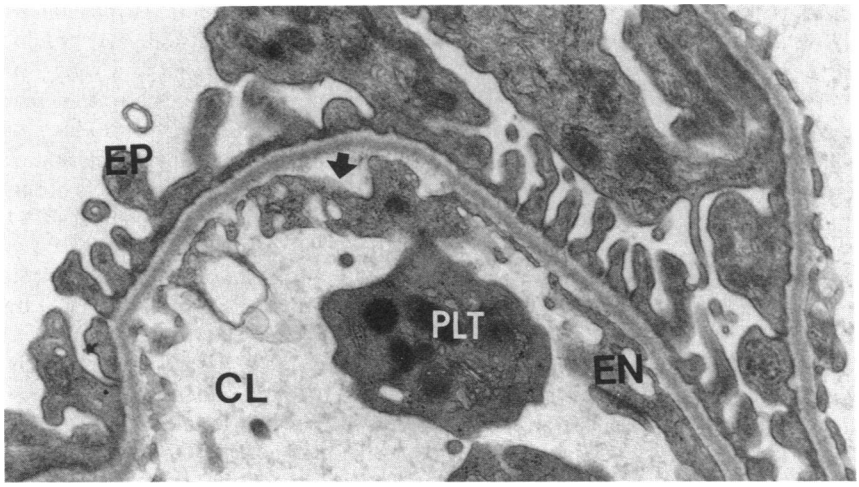

Figure 5. Glomerular morphology by electron microscopy. An EM of a glomerular capillary wall from a rat 4 h after being perfused with MPO and H_2O_2 demonstrated endothelial cell injury with sloughing into the capillary lumen (*arrow*) and focal epithelial cell foot process fusion. A platelet is apparent in the capillary lumen. *CL*, capillary lumen; *EN*, endothelial cell; *EP*, epithelial cell; *PLT*, platelet (28,000 ×).

Table I. Iodination of Glomeruli

Perfusion	Iodination	% TCA precipitable
	cpm per total glomeruli	
PBS	24±3	ND
MPO	14±4	ND
H_2O_2	130±20	ND
MPO and H_2O_2*	24,132±1502	38.7±1.6

The renal artery of rats were perfused with either PBS alone, MPO alone, H_2O_2 alone, or MPO followed by H_2O_2. The last perfusion contained 16 nmol (0.25 μCi, 500,000 cpm) of $Na^{125}I$. Glomeruli were isolated and the cpm/total glomeruli determined, assuming 38,000 glomeruli per kidney. The results are the mean of four animals in each group. ND, not done.
* $P < 0.001$ relative to the other groups.

despite perfusion with equivalent amounts of ^{125}I (Table I). Perfusion with H_2O_2 alone resulted in a small but statistically significant amount of iodination relative to the PBS perfused control ($P < 0.005$). ~ 0.7 nmol of iodine was incorporated into glomeruli in rats receiving both MPO and H_2O_2. After the glomeruli were solubilized, 38.7±1.6% of the radioactivity was detected in the TCA-precipitable fraction.

EM autoradiography. The sites of iodination were clearly demonstrated by EM autoradiography. Essentially no silver grains (demonstrating tissue iodination) were present in the glomeruli of three control rats that received H_2O_2 and Na ^{125}I but no MPO. In contrast, the glomeruli from the six rats receiving MPO, Na ^{125}I and H_2O_2 demonstrated a high silver grain density that localized to the capillary wall (Fig. 6), and mesangium. Silver grains were not present in the tubules and interstitium. When the position of > 500 silver grains in the peripheral capillary wall was determined, it was evident that they centered closely around the basement membrane with an HD of 150 nm (Fig. 7). The distribution of silver grain density was very similar to the theoretical distribution of silver grains about a radioactive solid band (30) with a width of 150 nm (i.e., the width of the GBM). This suggests that the primary target of the halogenation reaction catalyzed by MPO in the capillary wall is the basement membrane.

Discussion

Our results demonstrate that exposure to small amounts of MPO followed by nontoxic concentrations of H_2O_2 and halide results in halogenation of the glomerular capillary wall accompanied by a marked increase in urine protein excretion and morphologic evidence of glomerular injury. Electron microscopy of DAB-treated sections of perfusion-fixed tissue revealed localization of MPO throughout the capillary wall but concentrated in the subepithelial space at the base of epithelial cell foot processes. This pattern is similar to that described previously by Graham and Karnovsky following intravenous administration of MPO to mice (28). MPO is a highly cationic protein (pI > 10) (18) of ~ 160 kD that presumably localizes in glomeruli on the basis of charge interactions with glomerular anionic sites including sialoglycoproteins, heparan sulfate proteoglycans and perhaps other charged structural components (31). Other neutrophil cationic proteins have previously been demonstrated to localize in glomeruli in both human and experimental forms of immune complex nephritis (19). Platelet cationic proteins exhibit similar properties and have been postulated to increase glomerular deposition of immune complexes by altering the size and charge selective properties of the capillary wall (20, 32). Although alterations in glomerular permeability to proteins can result from charge neutralization produced by polycations (33), MPO in the microgram quantities utilized here did not alter glomerular barrier function as judged by lack of proteinuria and absence of epithelial cell foot process effacement in animals perfused with MPO alone. Infusion of H_2O_2 is not directly toxic to glomeruli in the concentration used here. Similar or higher concentrations of H_2O_2 have been shown by others to depress the glomerular filtration rate and tubular reabsorption of sodium in the isolated perfused rat kidney (34). We did not measure these parameters. Only when H_2O_2 was perfused into kidneys containing glomerular-bound MPO did we detect glomerular injury as evidenced by marked endothelial cell swelling, epithelial cell foot process effacement, and a three to four-fold increase in protein excretion over control values.

The kidney is exposed for a short period to ischemic conditions during the perfusion procedure. Although ischemia alone does not appear to be toxic under our conditions as indicated by the normal morphology and absence of proteinuria in control animals, it is possible that ischemia may act synergistically with the MPO system to induce glomerular injury.

The mechanism of peroxidase-mediated injury to glomerular structures is unknown. H_2O_2 reacts with MPO to form a complex with strong oxidizing activity. Among the substances oxidized by the MPO-H_2O_2 complex are halides, and the products formed (hypohalous acids, halogens) are toxic to tissues by a combination of oxidation and halogenation reactions. Thus the MPO-H_2O_2-halide system has been shown to have powerful microbicidal activity and to contribute to the killing of microorganisms ingested by neutrophils (35). When released into the extracellular milieu, reaction products of this system may directly injure a variety of mammalian cells and can inactivate certain soluble mediators of inflammation as well as other compounds (35, 36). That MPO, H_2O_2, and a halide can interact in the glomeruli was shown here by the iodination of glomerular structures, a reaction known to be a consequence of the oxidation of iodide by peroxidase and H_2O_2. Autoradiography demonstrated that the basement membrane was a major site of iodination, although silver grains were also prominent over endothelial, epithelial, and mesangial cells.

Chloride is the more important halide in most biologic systems. The primary product of chloride oxidation is hypochlorous acid and this powerful reagent can oxidize sulfhydryl groups, sulfur ether groups, iron sulfur centers, as well as a variety of other structures and can form nitrogen-chloride derivatives that retain oxidizing activity (35). The reaction of the products of chloride (or other halide) oxidation with glomerular structures thus may contribute to the injury. Other indirect effects of this system may also be operative. For example, hypohalous acid generated by the MPO-H_2O_2-halide system has been shown to potentiate protease action by activating a latent neutrophil collagenase (37) and a latent metalloenzyme protease (38) and by inactivating alpha 1 proteinase inhibitor (36).

Glomerular injury secondary to ROS may not be entirely dependent on circulating inflammatory cells. Resident glomerular mesangial cells have also been shown to generate O_2^- and H_2O_2 in response to phagocytic and nonphagocytic stimuli such as platelet-activating factor and the C5b-9 membrane attack

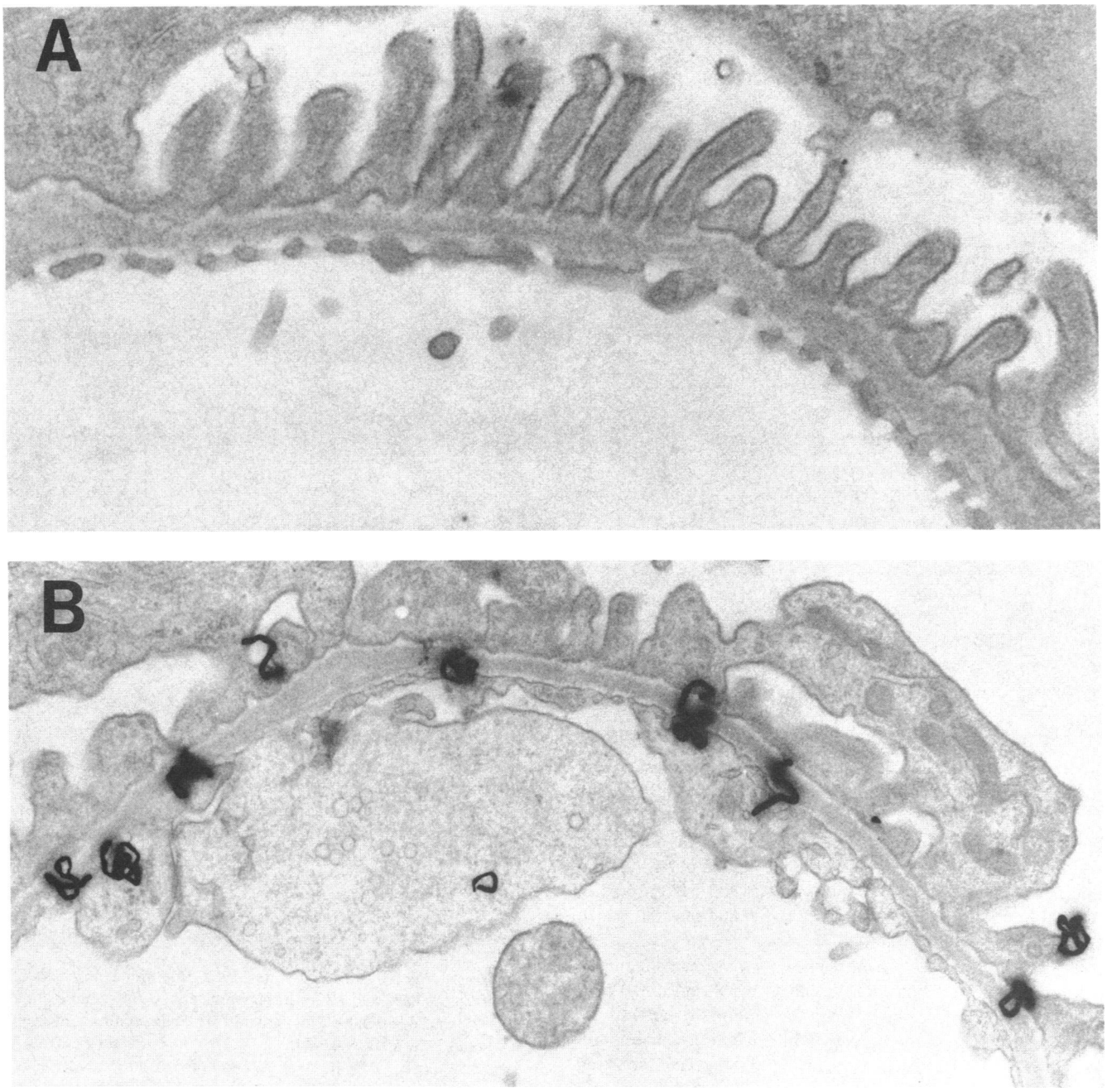

Figure 6. EM autoradiography. (*A*) is an EM autoradiograph of a glomerular capillary wall from a control rat that was perfused with H_2O_2 and Na ^{125}I. Essentially no silver grains could be demonstrated (36,000 ×). (*B*) is an EM autoradiograph of a rat that was perfused with MPO followed by H_2O_2 and Na ^{125}I (21,000 ×). Numerous silver grains are present along the capillary wall. Endothelial cell swelling and focal epithelial cell foot process effacement are also prominent.

complex of complement (39–41). The H_2O_2 so formed may affect glomerular structures either directly or following interaction with peroxidase released by granulocytes.

It is not known whether the amount of MPO and H_2O_2 used in our studies can be released by neutrophils in vivo. The amount of MPO that was bound to the glomeruli was ~ 0.3% of the injected dose. When rat neutrophils are similarly treated with CETAB to extract MPO, the amount of MPO bound by 38,000 glomeruli (the total number per kidney) was that present in 10^4 neutrophils, or one neutrophil could provide the MPO found in four glomeruli (data not shown). Although this calculation assumes the same efficiency for extraction of MPO from neutrophils as from GBM, it suggests that the amount of MPO bound in our studies could be achieved in vivo. It is nonetheless difficult to extrapolate in this way because degranulation is generally incomplete with the amount of MPO released extracellularly being a variable proportion of the total intracellular pool. Further, the MPO released from neutrophils in vivo would not be expected to be distributed evenly over the basement membrane as with perfusion of MPO but would be at very high concentration at the point of degranulation. Because neutrophils have a short lifetime in tissues, a lesion that develops over a period of days or weeks may acquire MPO from neutrophils that arrive at different times, discharge their granule contents, and are dis-

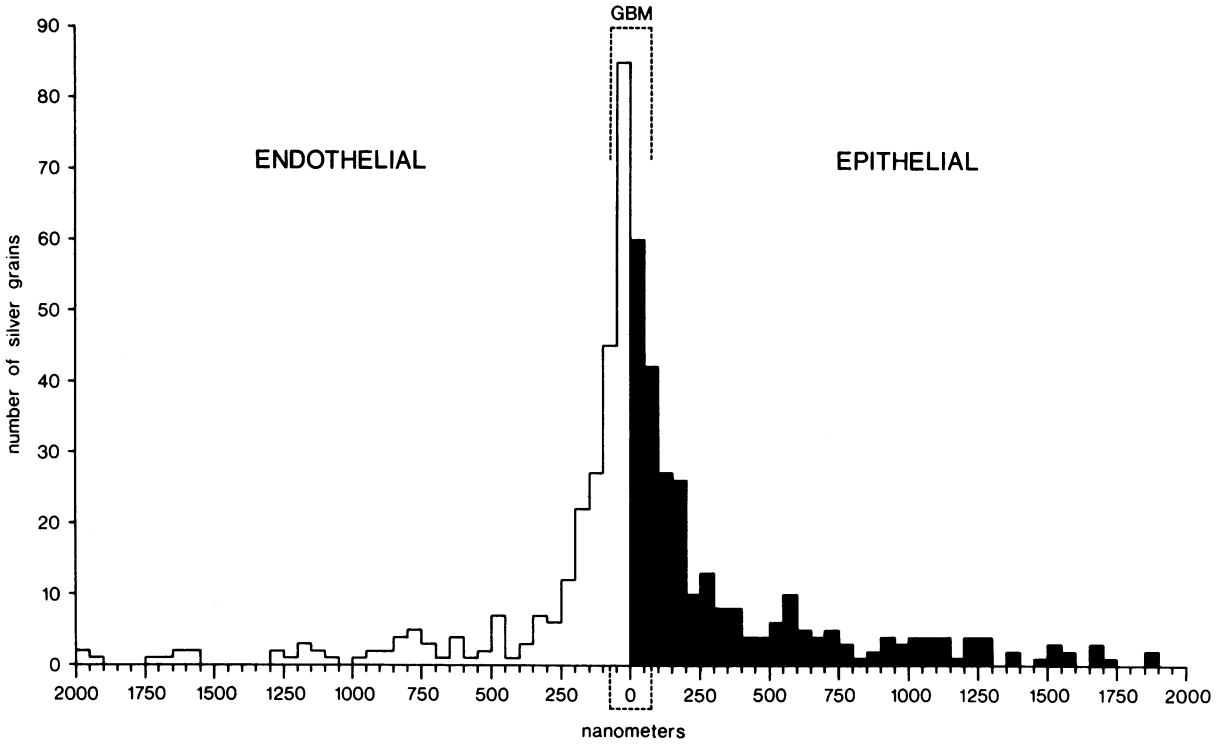

Figure 7. Silver grain position in a rat receiving MPO followed by H_2O_2 and Na ^{125}I. The position of 538 silver grains was determined relative to the center of the basement membrane and the number of grains in each 50-nm interval was plotted. The majority of the grains were centered around the basement membrane (*dashed line*), with a HD of 150 nm.

sipated. Similarly, it is difficult to relate the concentration of infused H_2O_2 (10^{-3} M) to that which may be produced locally by granulocytes at the site of degranulation. In one study, the concentration of H_2O_2 formed by granulocytes was said to vary from 10^{-3} to 10^{-1} M depending on the degree of stimulation and the pH (42). Thus the amount of MPO and H_2O_2 used in our study do not appear to be very different from that which might be produced by granulocytes.

Whereas our studies provide support for a mechanism of neutrophil-mediated glomerular injury that involves release of both MPO and H_2O_2 by the neutrophil, the binding of MPO to the glomerular basement membrane and the reaction of the bound MPO with H_2O_2 and a halide to form a toxic species damaging to the capillary wall, they do not document that this system is operative in glomerulonephritis mediated by neutrophils in the intact animal. However our recent observation that iodination of glomeruli occurs in a neutrophil-mediated model of experimental glomerulonephritis suggests a role for this system in vivo (43). More studies are clearly required to establish the importance of this system in immune tissue injury and to further define the mechanisms by which oxidation/halogenation of glomeruli leads to morphologic and functional glomerular disease.

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