Antimyeloperoxidase-associated Proliferative Glomerulonephritis: An Animal Model

By Elisabeth Brouwer,* Minke G. Huitema,* Pieter A. Klok,‡
Henk de Weerd,§ Jan Willem Cohen Tervaert,* Jan J. Weening,||
and Cees G. M. Kallenberg*

From the Departments of *Clinical Immunology, ‡Pathology, and §Electronmicroscopy, University
Hospital, 9713 EZ Groningen; and the ||Department of Pathology, Academic Medical Center,
1105 AZ Amsterdam, The Netherlands

Summary

To develop an animal model for antimyeloperoxidase (MPO)-associated necrotizing crescentic glomerulonephritis (NCGN), we immunized Brown Norway rats with MPO and localized a neutrophil lysosomal enzyme extract, primarily consisting of MPO and elastinolytic enzymes, plus H_2O_2, the substrate of MPO, to the glomerular basement membrane (GBM). Upon immunization rats developed antibodies and positive skin tests to MPO. After unilateral perfusion of the left kidney with the lysosomal enzyme extract and H_2O_2, MPO and immunoglobulin (Ig)G localized transiently along the GMB. At the time of maximal inflammation, at 4 and 10 d after perfusion, MPO, IgG, and C3 could not be detected anymore. MPO-immunized rats perfused with the lysosomal enzyme extract and H_2O_2, in contrast to control-immunized and/or control-perfused rats, developed a proliferative GN characterized by intra- and extracapillary cell proliferation, ruptured Bowman's capsule, periglomerular granulomatous inflammation, and formation of giant cells. Monocytes, polymorphonuclear leukocytes (PMN), and to a far lesser extent T cells were found in the glomeruli. Interstitial infiltrates consisted of monocytes, PMN, and T cells. Granulomatous vasculitis of small vessels was found at 10 d after perfusion. The proliferative NCGN in this rat model closely resembles human anti-MPO-associated pauci-immune NCGN, and enables the study of the pathophysiology of anti-MPO-associated NCGN.

Necrotizing crescentic glomerulonephritis (NCGN),[1] a clinically rapidly progressive form of glomerular disease, can be associated with multisystem disorders (infectious or noninfectious), or may occur in patients without a definite diagnosis of systemic illness (1). NCGN can be classified into three immunopathogenic categories: (a) anti–glomerular basement membrane (GBM) antibody mediated; (b) immune complex mediated; and (c) antineutrophil cytoplasmic antibody (ANCA) associated (2). ANCA in patients with ANCA-associated NCGN are either directed to proteinase 3 (Pr3) (3–5) or myeloperoxidase (MPO) (2, 6, 7), both myeloid lysosomal enzymes. NCGN associated with anti-MPO antibodies is characterized by segmental fibrinoid necrosis of the GBM, marked infiltration of neutrophils and mononuclear cells, and paucity of IgG deposits (8–12). NCGN can be accompanied by granuloma formation (13, 14). The pathogen-

esis of ANCA-associated NCGN has not been elucidated so far. The close association between pauci-immune NCGN and anti-MPO antibodies (6, 7) suggests a pathogenetic role for an anti-MPO-directed immune response. In vitro studies showed that anti-MPO antibodies can activate PMN to cause a respiratory burst and to mediate degranulation (15). We hypothesized that NCGN may develop after focal release of lysosomal enzymes and production of H_2O_2 by activated neutrophils in the presence of an anti-MPO-directed immune response. To test this hypothesis we developed an animal model in which we perfused a lysosomal enzyme extract and H_2O_2 into the kidney of Brown-Norway (BN) rats immunized with MPO. BN rats were chosen because they develop both humoral and cellular responses to MPO. Rats immunized with MPO and perfused with the lysosomal enzyme extract and H_2O_2 were found to develop glomerular intracapillary thrombosis followed by a proliferative glomerulonephritis characterized by glomerular capillary wall necrosis, extracapillary cell proliferation, infiltration of neutrophils and monocytes, ruptured Bowman's capsule, periglomerular granuloma formation, vasculitis, and extensive interstitial mononuclear infiltrates. Ig deposits were absent or scanty, similar to human

---

[1] *Abbreviations used in this paper:* ANCA, antineutrophil cytoplasmic antibody; GBM, glomerular basement membrane; MPO, myeloperoxidase; NCGN, necrotizing crescentic glomerulonephritis; Pr3, proteinase 3.

NCGN. The development of this first experimental model of pauci-immune NCGN was found to be dependent on the presence of the lysosomal enzyme extract and H_2O_2 along the GBM and a MPO-directed immune response.

Materials and Methods

Rats. All experiments were performed in conventionally housed, 3-mo-old BN rats, fed ad libitum with standard chow (Hope Farms, Woerden, The Netherlands).

Lysosomal Enzyme Extract. Human PMN were isolated from buffy coats on a Ficoll density gradient followed by dextran sedimentation. After sedimentation the remaining erythrocytes were removed by hypotonic lysis. MPO was extracted from PMN by dissolution of the cells in cetyltrimethyl ammonium bromide (CETAB) and sonification. Nuclei and membrane fragments were discarded by ultracentrifugation and the extract was absorbed to a Con A Sepharose gel and eluted with α-methyl-D-mannoside. Eluted fractions with a ratio (OD 428/280) >0.5, measured as the ratio between the OD obtained at 428 nm (showing a specific spectral band for MPO) and the OD obtained at 280 nm, were pooled and extensively dialyzed to sodium acetate buffer, pH 6.0 (16). The resulting lysosomal extract had an OD 428/280 ratio >0.5 and contained mainly MPO, Pr3, and trace amounts of elastase, but no lactoferrin as determined by sandwich ELISA (3). In accordance with the results of ELISA, specific bands for MPO (at 15, 39, 58 kD) and Pr3 (at 30 kD) were found by gel electrophoresis (results not shown). Peroxidase activity of the extract was assayed spectrophotometrically by measuring the increase in absorbance at 470 nm upon oxidation of guaiacol (Sigma Chemical Co., St. Louis, MO). In brief, 10 μl of the MPO preparation was added to a 1-cm light path cuvette containing 3 ml of the assay solution. The solution consisted of 29.6 ml H_2O, 3 ml 0.1 M sodium phosphate buffer, pH 7.0, 0.1 ml 0.1 M H_2O_2, and 48 μl guaiacol. 1 U of peroxidase activity is defined as the amount that consumes 1 μmol/min of H_2O_2 (17). 100 μg of the lysosomal extract contained ~58 μg MPO with an activity of 30 U.

Myeloperoxidase. Human MPO was purchased from Calbiochem-Behring Co. (La Jolla, CA). This MPO was dissolved in sodium acetate buffer and had an OD 428/280 ratio >0.7.

Immunization Procedure. BN rats were immunized with human MPO in CFA supplemented with H37Ra (5 mg/ml; Difco Laboratories, Detroit, MI). Rats received 10 μg MPO or control solution without MPO (sodium acetate buffer) subcutaneously in 0.2 ml at two sites near the tailbase. At different intervals levels of anti-MPO antibodies were measured by ELISA. The cellular response was assessed by skin testing.

Detection of Anti-MPO Antibodies by ELISA. In short, Labstar (Greiner, Kremsmünster, Austria) microtiter plates were coated overnight with human MPO (isolated as described before) at a protein concentration of 20 μg/ml in 0.1 M carbonate buffer, pH 9.6. The plates were incubated with rat plasma, diluted in 0.05 M Tris, 0.05% Tween, 2% BSA, and 0.3 M NaCl, pH 8.0, starting at a dilution of 1:100 in PBS. Antibody binding was detected with sheep anti-rat Ig linked to alkaline phosphatase (Serotec, Oxford, England), followed by p-nitrophenyl-phosphate-disodium as a substrate. The OD was read at 405 nm, and a standard curve was prepared from a reference serum. Antibody concentrations were computed from the linearized titration curve obtained after log-logit transformation of the absorbances of the respective dilutions of the reference serum. The concentration of the reference serum was set at 100 U. The specificity of the antibodies for MPO was confirmed by immunoprecipitation from a total neutrophil extract.

Detection of Anti-MPO by Immunoprecipitation. Human PMN were isolated by Ficoll, and a crude granule extract was obtained by nitrogen cavitation and ureum extraction in the presence of protease inhibitors (3). Nuclei and membrane fragments were discarded by centrifugation. The antigens were radiolabeled with ^{125}I by the iodogen method (3), and diluted in PBS containing 4% BSA, 1% Triton X-100, 0.5% SDS, and 0.5 M NaCl (immunoprecipitation [IPP] buffer). Antibody-bound antigen complexes were precipitated with protein A CL-4B (Pharmacia, Uppsala, Sweden), 5 mg/plasma sample, washed with IPP buffer, PBS, and 10 mM Tris-HCl, pH 6.8, and centrifuged through a sucrose gradient. The samples were boiled for 1 min in β-mercaptoethanol/SDS and subjected to electrophoresis in a 12.5% acrylamide gel. After electrophoresis, gels were vacuum dried and exposed to a Kodak XAR5 film.

Skin Tests. To test the cellular response to MPO, heat-inactivated human MPO was applied to the inner ear pinna subcutaneously in MPO-immunized and control rats at 1, 5, and 10 wk after immunization. All measurements were done in triplicate. The increase in ear thickness was measured with an engineer's micrometer (Tesa, Switzerland) at 0, 4, 24, and 48 h after application.

Renal Perfusion. Unilateral perfusion of the left kidney was performed according to a modification of the method of Hoyer et al. (18). Rats were anesthetized with halothane, O_2, and NO_2. Through a midline incision, the aorta and vena cava were exposed by blunt dissection. After ligation of the tributaries, temporary clamps were placed on the aorta above the left renal artery, leaving the circulation of the right renal artery undisturbed. Through a puncture hole in the aorta, a needle was inserted and advanced up to the level of the left renal artery. The left renal vein was punctured to let blood and perfused fluids escape, which were collected in cotton wool. PBS (37°C, pH 7.3) was infused until the kidney became pale and no visible blood escaped from the punctured renal vein. Subsequently 100 μg of the lysosomal enzyme extract in 0.5 ml PBS containing ~58 μg MPO with an activity of 30 U, or 58 μg MPO alone in PBS, or PBS (see experimental design) alone were perfused for 1 min. After an additional exposure time of 2 min the kidney was again perfused with PBS or with 2 ml PBS containing 1 mM H_2O_2 (nontoxic concentration) followed by PBS according to Johnson et al. (19). After perfusion the needle was removed, the aorta was closed with atraumatic sutures, the clamps were removed, and the kidney was allowed to reperfuse. Total ischemia time was always <12 min. Only rats were studied in which the kidney regained normal perfusion and color. After surgery the rats were placed under a heat lamp for 2 h and received an injection with buprenorfine (Reckitt and Coleman, Hull, England) subcutaneously as a pain reliever.

For estimation of proteinuria, urine was collected in metabolic cages 24 h after perfusion from all rats and before the day of death at 4 and 10 d after perfusion. The protein content was estimated with buprenorfine (Reckitt and Coleman, Hull, England) subcutaneously as a pain reliever.

Histological Examination. At different time intervals, rats were perfused with PBS at 4°C to remove the blood from both kidneys and killed. Specimens from both kidneys were obtained and prepared for light microscopy, immunofluorescence, and immunohistochemistry. For light microscopy, renal tissue was fixed in 2% paraformaldehyde/PBS and embedded in plastic. 4-μm sections were stained with hematoxylin-eosin, periodic acid Schiff, or periodic acid silver methamine. For immunofluorescence (IF) and immunoperoxidase staining (IP), material was snap frozen in freon or isopenthane (isobutane), and 2- and 4-μm sections were cut. Sections were standard fixed with acetone. For detection of MPO, sections were fixed with acetone-buffered formalin, 9% (20). After

fixation, sections were incubated with 10% normal rabbit, goat, or swine serum, depending on the conjugate used. Detection of MPO, Ig, and complement was tested by IF using mouse anti–human MPO (Table 1), rabbit anti–rat IgG/A/M, F(ab′)₂ goat anti–rat IgG, and F(ab′)₂ goat anti–rat C3, respectively (all from Cappel Laboratories, West Chester, PA), followed by FITC-labeled conjugates. The following conjugates were used: rabbit anti–mouse FITC (Dakopatts, Glostrup, Denmark), F(ab′)₂ goat anti–rabbit FITC, and F(ab′)₂ rabbit anti–goat FITC (both from Cappel Laboratories). Granulocytes, monocytes, and T cells were detected by IP using the mAbs ED1, His48, and OX19, respectively (Table 1), followed by peroxidase (PO)-labeled conjugates. We used rabbit anti–mouse PO (Dakopatts) with aminoethylcarbimazole as a substrate. Endogenous peroxidase activity was blocked with 0.05% H_2O_2 in PBS. Sections were counterstained with hematoxylin. All necessary dilutions were made in PBS, pH 7.2. The amount of MPO, IgG, and complement deposition was estimated on a semiquantitative (3+) scale: –, absent; ±, weak; +, mild; + +, intermediate; + + +, strong. To estimate the number of ED1-, His48-, and OX19-positive cells, we calculated the average number of cells per glomerulus by counting all positive cells present within 50 glomeruli and dividing the total number by 50. Interstitial leukocytes were quantified by using a semiquantative method: –, absent; ±, minimal; +, mild; + +, moderate, and + + +, severe. For immunofluorescence, sections were scored by two blinded persons.

Electron Microscopy. Renal tissue was fixed in 2% paraformaldehyde (PF), 0.05% glutaraldehyde in PBS, pH 7.3, at 4°C for 1 h. After fixation tissue was stored in PBS, containing 1% PF and 1% sucrose. Before processing sections of 1 mm³ were cut and immersed in 50 mM NH_4Cl in PBS for 1 h at 4°C. The sections were dehydrated, after several wash steps, by overnight incubation in absolute ethanol at progressively lowered temperatures (PLT method) ranging from 0 to –35°C. After dehydration the sections were infiltrated with Lowicryl (Bio-Rad Laboratories, Richmond, CA) at –35°C. Lowicryl was refreshed three times before the resin was allowed to polymerize under ultraviolet light (360 nM) for 2 d at –40°C followed by 2 d polymerization at room temperature (RT).

Lowicryl ultrathin sections on formvar-coated nickel grids (200 mesh) were floated on drops of 20 µl 5% normal goat serum/Tris-buffered saline (TBS), pH 7.4. Grids were washed four times in 1% TBS/BSA by transferring and floating on four droplets for 5 min each. The sections were subsequently incubated in 20-µl droplets of the primary antiserum (anti-MPO or anti-IgG) for 2 h at RT in a humidity chamber. The antiserum was washed off with 1% TBS/BSA. The sections were then immersed in 20-µl droplets of goat anti-rabbit–labeled with 15 nm immuno-gold (Auroprobe, Amersham, UK) in 1% TBS/BSA, supplemented with 1% normal rat serum for 1 h at RT. After gold labeling, grids were washed with TBS/BSA (once for 5 min), TBS (twice for 5 min), and bidest (three times for 5 min). The sections were counterstained with 1.8% uranylacetate in 0.2% methylcellulose (15 centipose) in bidest for 5 min. Specificity of the staining was controlled by use of a mAb of the IgG1 subclass directed against pollen.

Statistical Analysis. All values are expressed as the mean ± SEM and were analyzed for statistical differences by a nonparametric one-way analysis of variance (anova) for unpaired data. Correlations within groups for different values were assessed with Spearman's rank correlation test.

Experimental Design. Seven groups of experiments were performed according to the following design: (I) Renal perfusion with the lysosomal enzyme extract and H_2O_2 in MPO-immunized rats. The lysosomal enzyme extract and H_2O_2 were perfused into the

left kidney in MPO-immunized rats, 5 wk after immunization. Rats were killed at 4 h (n = 2), 24 h (n = 3), 4 d (n = 6), and 10 d (n = 8) after perfusion. (II) Renal perfusion with the lysosomal enzyme extract and H_2O_2 in control-immunized rats. The same procedure was followed for control-immunized rats as described in Exp. I. (III) Renal perfusion with the lysosomal enzyme extract in MPO-immunized rats. The same procedure was followed as described in Exp. I. Rats were killed at 4 h (n = 2), 24 h (n = 2), 4 d (n = 2), and 10 d (n = 3) after perfusion. (IV) Renal perfusion with purified MPO and H_2O_2 in MPO-immunized rats. The same procedure was followed as described in Exp. I. Rats were killed at 4 h (n = 2), 24 h (n = 2), 4 d (n = 4), and 10 d (n = 4) after perfusion. (V) Renal perfusion with H_2O_2 in MPO-immunized rats. The same procedure was followed as described in Exp I. Rats were killed at 10 d (n = 4) after perfusion. (VI) Renal perfusion with MPO in MPO-immunized rats. The same procedure was followed as described in Exp. I. Rats were killed at 4 h (n = 2), 24 h (n = 2), 4 d (n = 2), and 10 d (n = 2) after perfusion. (VII) Renal perfusion with MPO in control-immunized rats. The same procedure was followed as described in Exp. VI.

Results

Humoral and Cellular Immune Response to MPO

BN rats immunized with human MPO developed antibodies reacting with MPO (Fig. 1). The antibodies specifically precipitated MPO from a crude granule extract. The precipitate shows the 58-kD MPO heavy chain and a 39-kD heat degradation product. The polyclonal anti-MPO antibody (Table 1) precipitated the same bands, whereas control rat plasma did not precipitate any granule proteins (Fig. 2). The skin tests used to document the cellular response to MPO showed an increase in ear thickness developing from 4 to 24 h (Fig. 3) with a gradual decrease thereafter. Immunohistological examination of the ears at 24 h showed an infiltrate consisting of monocytes and neutrophils. Control rats showed only a slight increase in ear thickness at 4 h, probably due to a transient reaction to the injected MPO solution. For per-
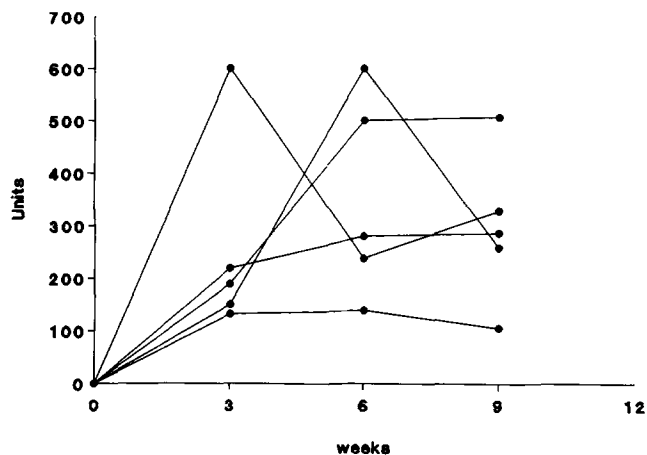

Figure 1. Development of anti-MPO antibody titers in five representative rats immunized with human MPO. Antibodies to human MPO were measured by ELISA at 0, 3, 6, and 9 wk after immunization. Results are expressed as arbitrary units. Negative controls were <5 U (mean ± 2 SD).

Table 1. *Antibodies Used for Immunofluorescence and Immunohistochemistry on Frozen Tissues*

Code	Antigen recognized	Ig(sub-)class	Specificity	Source
MPO	Human MPO	Poly	PMN/monocytes	Dakopatts, Glostrup, Denmark
4.15	Human MPO	IgG1	PMN/monocytes	CLB*
ED1	Cytoplasmic	IgG1	Macrophages	Reference 30
His 48	Membrane	IgG1	Neutrophils	Reference 31
OX 19	CD5/69 kD	IgG1	T cells/subpopulation of B cells	Oxford‡

* Central Laboratory for the Blood Transfusion Service, Amsterdam, The Netherlands.
‡ MCR Cellular Immunology Unit, William Dunn School of Pathology, University of Oxford, Oxford, UK.

fusion studies, rats 5 wk postimmunization were used, i.e., at a time when the humoral response was maximal.

Renal Perfusion in MPO-immunized and Control-immunized Rats

Urine Analysis. Rats perfused with the lysosomal extract with or without H_2O_2 or MPO in combination with H_2O_2 (groups I-IV) had proteinuria (Fig. 4), and severe hematuria (80% of all those rats) or erythrocyturia (20% of all those rats) during the first 24 h after perfusion. There were no significant differences in levels of proteinuria between groups

I-IV by anova. At 4 and 10 d after perfusion rats from groups I-IV were no longer proteinuric (Fig. 4), but severe hematuria and erythrocyturia remained in 20–40%, respectively, of all those rats. Rats perfused with H_2O_2 alone (group V; Fig. 4) and rats perfused with MPO alone (groups VI and VII; results not shown) had no proteinuria.

Deposition of MPO, IgG, and Complement. At 4 h after perfusion MPO was present in a granular pattern along the GBM (Fig. 5 *A*) in all MPO-immunized rats (Table 2). By immunoelectron microscopy (IEM) we observed that at 4 h after perfusion MPO was localized at the subendothelial and subepithelial side of the GBM (Fig. 5 *B*). In control-immunized rats (groups II and VII) MPO could not be detected along the GBM. In MPO-immunized rats IgG and complement were present along the GBM in a granular pattern along the GBM at 4 h after perfusion. At 24 h MPO was no longer present in group I rats, whereas IgG and complement C3 could still be detected in a diffuse granular pattern along the GBM (Fig. 5, *C* and *D*). In the remaining MPO-immunized rats (groups III, IV, and VI) MPO as well as IgG and C3
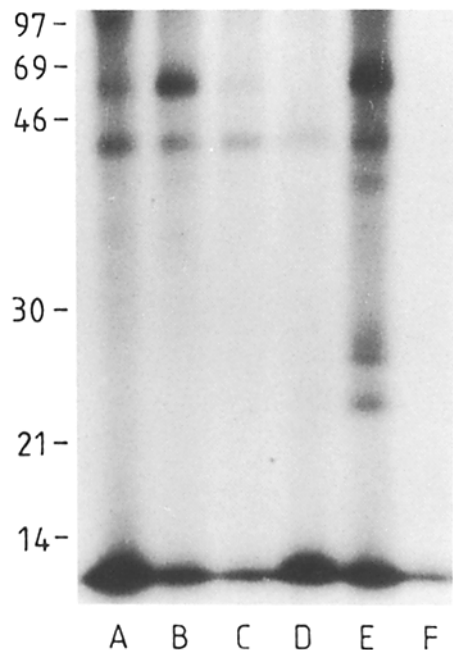
Figure 2. Immunoprecipitation studies with a crude neutrophilic protein extract. Lanes *A–D* contain the characteristic MPO bands (55 and 39 kD) precipitated by rat IgG, obtained at 10 wk after immunization with MPO. Sera from control immunized rats did not precipitate any proteins (lane *F*). Lane *E* shows also the 55- and 39-kD bands precipitated by a polyclonal anti-MPO antibody. This polyclonal antibody also recognizes smaller MPO fragments.
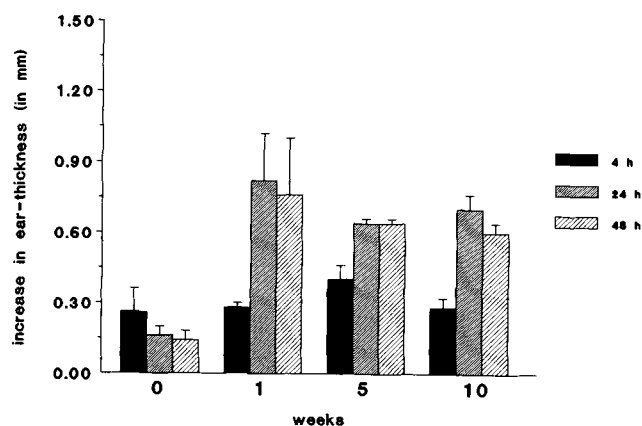
Figure 3. Skin tests in control (*n* = 3) and MPO-immunized rats (*n* = 9) at 1, 5, and 10 wk after immunization. Ear thickness was measured in triplicate at 4, 24, and 48 h after injection of 10 μl heat-inactivated MPO solution subcutaneously in the inner ear pinna. Increase in ear thickness was measured with an engineer's micrometer. Results represent mean ± SEM.
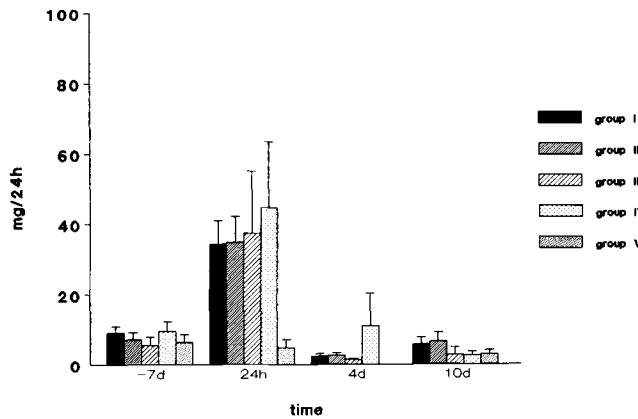
Figure 4. Urine analysis performed 1 wk before perfusion, and 24 h, 4 d, and 10 d after perfusion. Proteinuria was estimated with the biuret method and results are presented as mean ± SEM. Group I, MPO immunized and perfused with the lysosomal enzyme extract and H_2O_2 (n = 17); group II, control immunized and perfused with the lysosomal enzyme extract and H_2O_2 (n = 9); group III, MPO immunized and perfused with the lysosomal enzyme extract (n = 5); group IV, MPO immunized and perfused with MPO and H_2O_2 (n = 8); and group V, MPO immunized and perfused with H_2O_2 (n = 4).

Histopathology. Severe lesions were only found in rats that were immunized with MPO and perfused with the lysosomal enzyme extract or MPO and H_2O_2 (groups I and IV). At 24 h the main abnormalities detected in the perfused left kidney consisted of wrinkling of the GBM, accumulation of eosinophilic proteinaceous material into the capillary lumen and Bowman's space, glomerular capillary wall necrosis, PAS-positive reabsorption droplets in the cytoplasm of proximal convoluted tubules, and numerous urinary casts in tubular lumina (Fig. 6 *A*). At day 4, severe changes were seen in the renal cortex. 30–80% of the glomeruli showed fibrinoid necrosis of glomerular capillary walls, intra- and extracapillary cell proliferation, ruptures in Bowman's capsule, and influx of mononuclear cells and PMN. Periglomerular 'granuloma-like' infiltrates were seen consisting of mononuclear cells, PMN, and giant cells. At day 10 active, inflammatory lesions were still present. Intra- and extracapillary cell proliferation was found in 30–70% of glomeruli and was accompanied by ruptures in Bowman's capsule, periglomerular cell infiltration, and giant cell formation (Fig. 6, *B–D*). The interstitium showed chronic infiltrates consisting of mononuclear cells and PMN that surrounded tubuli and vessels at day 4 and even more at day 10 (Fig. 6 *E*). In addition vasculitis of small venules and arteries was observed (Fig. 6 *F*). In one biopsy we found a larger vessel surrounded by PMN and karyorrhectic fragments (Fig. 6 *G*).

In control-immunized rats perfused with the lysosomal enzyme extract and H_2O_2 (group II), we also found accumulation of eosinophilic proteinaceous material into the capillary lumen, at 24 h after perfusion, but no development of NCGN. In MPO-immunized rats perfused with the lysosomal enzyme extract or MPO alone (groups III and VI), no significant lesions were observed, except for periodic acid Shiff-positive droplets in the cytoplasm of proximal tubuli and a slight interstitial infiltrate. MPO-immunized rats perfused with

were still detectable (Table 2). At 4 and 10 d after perfusion deposits of IgG and complement were scanty or had disappeared completely in rats immunized with MPO and perfused with the lysosomal extract and H_2O_2 (group I; Table 2 and Fig. 5, *E* and *F*). In contrast, deposits of IgG and C3 were still present in MPO-immunized rats perfused with the lysosomal extract without H_2O_2 (group III), MPO and H_2O_2 (group IV), and MPO alone (group VI) at 4 and 10 d after perfusion (Table 2). The right nonperfused kidneys were always negative for MPO, IgG, and C3, except for a weak mesangial staining for IgG in some of the biopsies.

Table 2. *Intraglomerular Deposition of MPO, IgG, and C3 in Rats Killed 24 h, 4 d, and 10 d after Perfusion*

Groups	MPO				IgG				C3			
	4 h	24 h	4 d	10 d	4 h	24 h	4 d	10 d	4 h	24 h	4 d	10 d
I	+	−	−	−	+ +	+	−	−	+	+	−	−
II	−	−	−	−	−	−	−	−	−	−	−	−
III	+	+	−	−	+ +	+ +	+	±	+	+ +	+	±
IV	+	±	−	−	+ +	+ +	+ +	+	+ +	+	+ +	+
V	ND	ND	ND	−	ND	ND	ND	−	ND	ND	ND	−
VI	+ +	+	±	−	+ +	+ + +	+ +	+ +	+ +	+ +	+ +	+ +
VII	−	−	−	−	−	−	−	−	−	−	−	−

Group I, MPO immunized and perfused with the lysosomal enzyme extract and H_2O_2 (n = 19); group II, control immunized and perfused with the lysosomal enzyme extract and H_2O_2 (n = 11); group III, MPO immunized and perfused with the lysosomal enzyme extract (n = 9); group IV, MPO immunized and perfused with MPO and H_2O_2 (n = 12); group V, MPO immunized and perfused with H_2O_2 (n = 4); group VI, MPO immunized and perfused with MPO (n = 8); group VII, control immunized and perfused with MPO (n = 5). The amount of MPO, IgG, and complement deposition was estimated on a semiquantitative (3+) scale: −, absent; ±, weak; +, mild; + +, intermediate; + + +, strong.
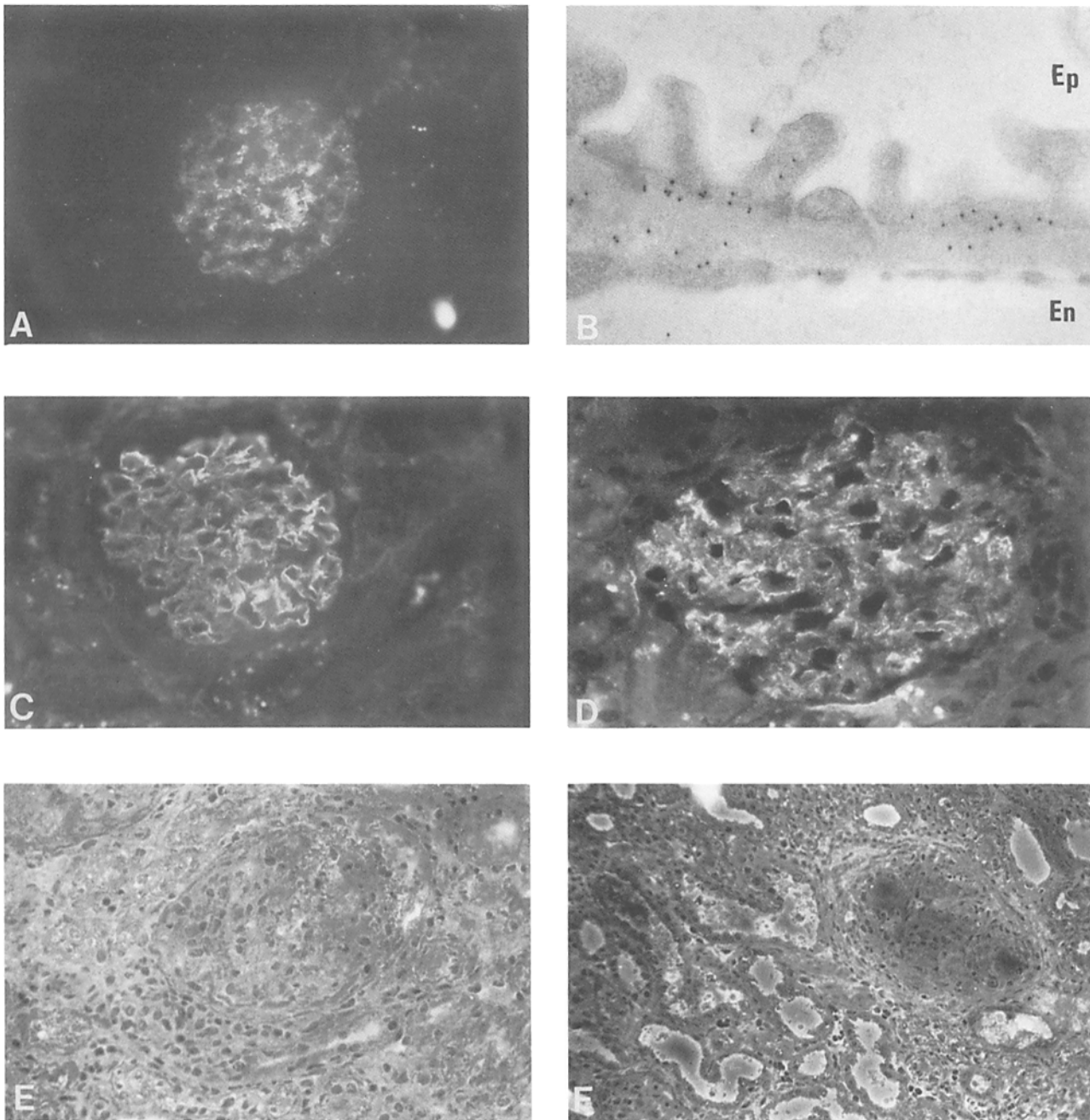
Figure 5. Localization of MPO by immunofluorescence and electron microscopy. (*A*) Staining for MPO in a granular pattern along the GBM, 4 h after perfusion with MPO alone in MPO-immunized rats (×320). (*B*) Electron microscopic localization of MPO, 4 h after perfusion with MPO alone in MPO-immunized rats, on the subendothelial and subepithelial sides of the GBM (*Ep*, epithelial side; *En*, endothelial side) (×30,000). (*C*) Staining for IgG in a granular pattern along the GBM, 24 h after perfusion with the lysosomal extract and H_2O_2 in MPO-immunized rats (×320). (*D*) Staining for complement in a granular pattern along the GBM, 24 h after perfusion with the lysosomal extract and H_2O_2 in MPO-immunized rats (×320). (*E*) Absence of IgG deposits in the presence of active proliferative lesions, 10 d after perfusion with the lysosomal extract and H_2O_2 in MPO-immunized rats (×320). (*F*) Overview shows a glomerulus negative for IgG and tubuli with IgG-positive casts, 10 d after perfusion with the lysosomal extract and H_2O_2 in MPO-immunized rats (×200).

H_2O_2 (group V) or control-immunized rats perfused with MPO (group VII) developed no lesions at all.

Immunophenotyping. In accordance with the findings by light microscopic examination, immunophenotyping of renal tissue from group I and IV rats showed larger numbers of intraglomerular, endo-, and extracapillary macrophages and PMN at days 4 and 10 (Table 3). The amount of macrophages found in the interstitium gradually increased (Table 3). T cells were observed in small numbers within the glomeruli and more abundantly within the interstitium at day 10 (Table 3).

Table 3. *Intraglomerular and Interstitial Presence of Macrophages, Neutrophils, and T Cells, as Detected by the mAbs ED1, His 48, and OX19, respectively*

Infiltrating cells	Groups	Intraglomerular cells*			Interstitial cells[‡]		
		24 h	4 d	10 d	24 h	4 d	10 d
PMN	I	1.9 ± 0.2	7.4 ± 4.2	4.1 ± 2.3*	+ +	+ + +	+ ±
	II	0.8 ± 0.2	0.9 ± 0.5	0.8 ± 0.2	±	±	±
	III	1.7 ± 0.1	0.6 ± 0.1	0.4 ± 0.2	+ +	–	–
	IV	1.3 ± 0.1	6.3 ± 3.2	1.3 ± 1.1	+ +	+ + +	+
	V	ND	ND	0.6 ± 0.2	ND	ND	–
Mono's	I	3.0 ± 0.3	16.0 ± 8.1[§]	12.4 ± 6.3*	±	+ + +	+ + +
	II	0.5 ± 0.2	1.2 ± 0.2	2.1 ± 0.7	–	±	+
	III	1.5 ± 0.3	1.4 ± 0.1	0.2 ± 0.2	+	+ +	±
	IV	0.8 ± 0.1	15.1 ± 9.6	15.6 ± 6.4	+	+ + +	+ + +
	V	ND	ND	0.3 ± 0.3	ND	ND	–
T cells	I	0	0.1 ± 0.1	0.2 ± 0.1[§]	–	–	+ +
	II	0	0	0	–	–	–
	III	0	0	0	–	–	±
	IV	0	0	0.6 ± 1.0	–	–	+ +
	V	ND	ND	0	ND	ND	–

Rats were killed at 24 h, 4 d, and 10 d after perfusion. Group I, MPO immunized and perfused with the lysosomal enzyme extract and H_2O_2 (*n* = 17); group II, control immunized and perfused with the lysosomal enzyme extract and H_2O_2 (*n* = 9); group III, MPO immunized and perfused with the lysosomal enzyme extract (*n* = 7); group IV, MPO immunized and perfused with MPO and H_2O_2 (*n* = 10); and group V, MPO immunized and perfused with H_2O_2 (*n* = 4).

* For estimation of the number of PMN, monocytes, and T cells, we calculated the mean number of cells per glomerulus by counting all positive cells present within 50 glomeruli and dividing the total number by 50. Numbers represent mean ± SEM.

‡ Labeled interstitial leukocytes were quantified by using a semiquantative method: –, absent; ±, minimal; +, mild; + +, moderate; and + + +, severe.

§ p <0.05 compared with groups II, III, and IV.

Mild infiltration of inflammatory cells was found in group VI at 24 h and 4 d after perfusion (data not shown). In accordance with the absence of IgG or complement deposits at the time of active fulminant NCGN, we found no correlation between the presence of IgG or complement and the amount of infiltrated macrophages and PMN.

Discussion

This study describes the first animal model that allows us to study the pathogenesis of anti-MPO-associated pauci-immune NCGN. The model is based on immunization of rats with MPO and subsequent localization of a lysosomal extract, primarily consisting of MPO and elastinolytic enzymes, in combination with H_2O_2, the substrate of MPO, along the GBM. The lesions found in this model closely resemble human anti-MPO-associated NCGN, characterized by a lack of Ig deposition at the time of fulminant glomerulonephritis (9, 10, 12).

BN rats immunized with human MPO developed anti-MPO antibodies. The presence of an anti-MPO-directed im-

mune response alone did, however, not result in the development of NCGN, as shown by the absence of lesions in the nonperfused kidneys. In human pathology, also, anti-MPO antibodies can be present without disease activity (21). Intercurrent infections, resulting in priming of PMN followed by further activation and release of enzymes and oxygen radicals in the presence of anti-MPO antibodies (15), frequently precede disease activity (22).

In our hypothesis the (focal) presence of active enzymes at the site of the GBM, possibly as a result of focal neutrophil activation and degranulation, is a prerequisite for the development of NCGN. For this reason, we chose to perfuse the rat kidney with a lysosomal extract, containing MPO and elastinolytic enzymes, and H_2O_2. Proteinuria was found in rats during the first 24 h after perfusion with the lysosomal extract and H_2O_2 (groups I and II), and MPO and H_2O_2 (group IV), and was independent of the presence of antibodies to MPO. In accordance, intracapillary eosinophilic deposits were found both in MPO-immunized (group I) and control-immunized rats (group II) as reported also by Johnson et al. (19, 23). Perfusion with the lysosomal extract (group III), containing elastinolytic enzymes capable of GBM degrada-
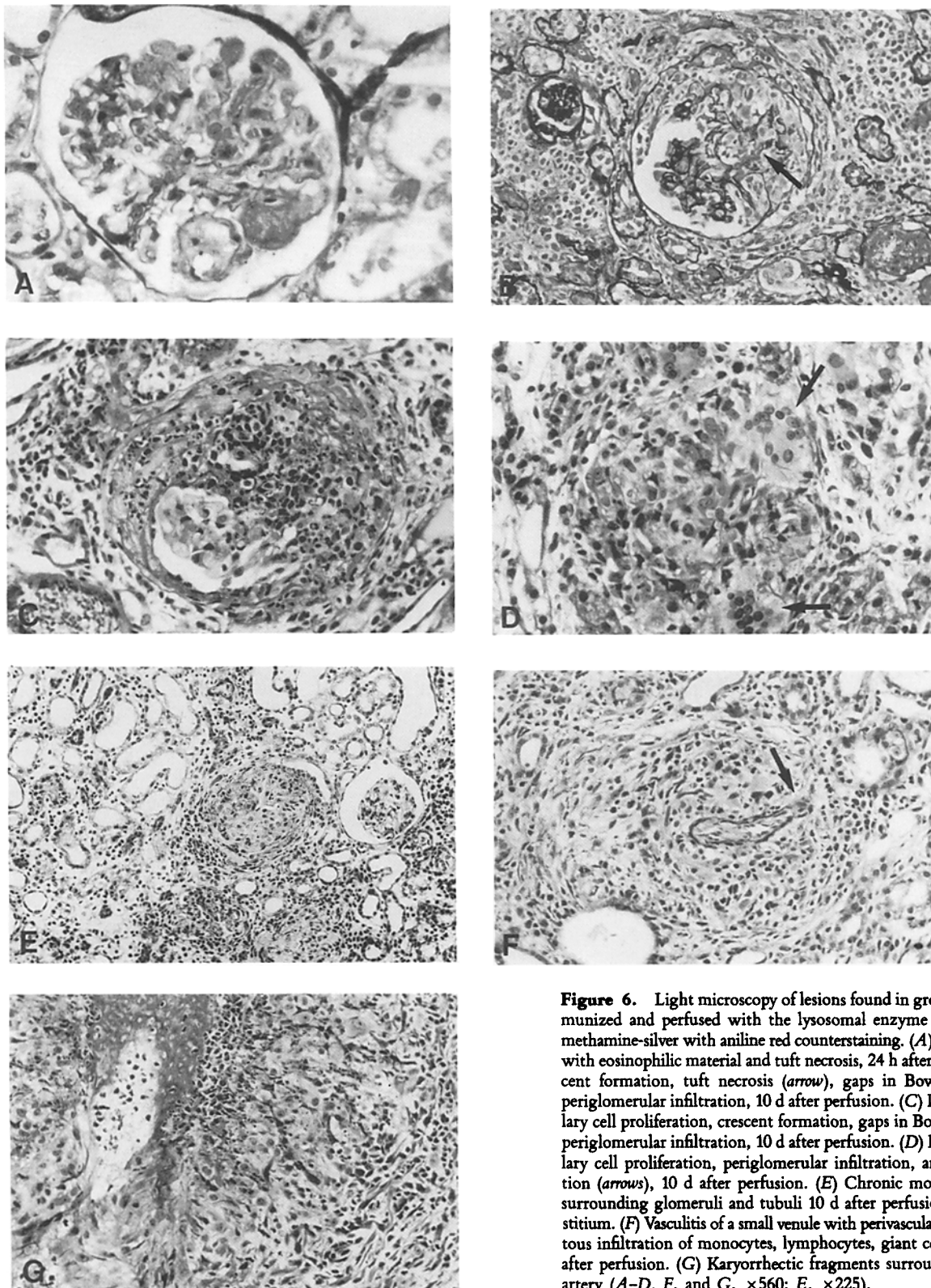
Figure 6. Light microscopy of lesions found in group I rats (MPO immunized and perfused with the lysosomal enzyme extract and H_2O_2); methamine-silver with aniline red counterstaining. (*A*) Capillaries occluded with eosinophilic material and tuft necrosis, 24 h after perfusion. (*B*) Crescent formation, tuft necrosis (*arrow*), gaps in Bowman's capsule, and periglomerular infiltration, 10 d after perfusion. (*C*) Intra- and extracapillary cell proliferation, crescent formation, gaps in Bowman's capsule, and periglomerular infiltration, 10 d after perfusion. (*D*) Intra- and extracapillary cell proliferation, periglomerular infiltration, and giant cell formation (*arrows*), 10 d after perfusion. (*E*) Chronic mononuclear infiltrates surrounding glomeruli and tubuli 10 d after perfusion within the interstitium. (*F*) Vasculitis of a small venule with perivascular slightly granulomatous infiltration of monocytes, lymphocytes, giant cells, and PMN, 10 d after perfusion. (*G*) Karyorrhectic fragments surrounding a larger renal artery (*A–D*, *F*, and *G*, ×560; *E*, ×225).

tion (24), alone also resulted in proteinuria during the first 24 h after perfusion. A direct toxic effect of MPO/H_2O_2 or elastinolytic degradation of the GBM by perfused elastinolytic enzymes seems to be responsible for the proteinuria found in those groups of rats. Only rats immunized with MPO and perfused with the lysosomal enzyme extract or MPO plus H_2O_2 developed proliferative lesions. The earliest lesions consisted of intracapillary deposition of eosinophilic material. Subsequently glomerular capillary wall necrosis, fibrinoid necrosis, intra- and extracapillary cell proliferation, fragmentation of Bowman's capsule, periglomerular "pseudogranuloma" formation, and chronic interstitial inflammation and vasculitis were found. IgG and C3 deposits were no longer present at the time when these active inflammatory lesions had developed in MPO-immunized rats perfused with the lysosomal extract and H_2O_2. Control-immunized rats, perfused with the lysosomal enzyme extract and H_2O_2, developed only intracapillary eosinophilic deposits and mild glomerular infiltration, but no proliferative lesions in accordance with Johnson et al. (19, 23). Active MPO, which produces, in concert with H_2O_2, toxic hydroxylradicals and hypochlorite (25, 26), was shown to be present for a longer time period

in MPO-immunized rats than in control-immunized rats (27). This probably contributes to the more extensive morphologic damage found in MPO-immunized rats. Whereas significant lesions were observed also in MPO-immunized rats perfused with MPO and H_2O_2, IgG and C3 were still present in these rats 10 d after perfusion. Probably, the presence of lytic enzymes in the perfusate is related to the disappearance of IgG and C3 from the GBM. Since toxic oxygen metabolites locally inactivate α-1-antitrypsin, the major inhibitor of elastinolytic enzymes (26, 28), those enzymes have the opportunity to degrade the GBM focally at the site of their release and are probably responsible for the fast removal of IgG and C3 in rats perfused with the lysosomal extract and H_2O_2 resulting in the pauci-immune aspect of NCGN. The contribution of MPO-specific T cells to the progression of lesions still has to be investigated (29).

In conclusion, we have developed the first model for anti-MPO-associated pauci-immune glomerulonephritis, in which the focal presence of products from activated neutrophils (i.e., MPO, its substrate H_2O_2, and lytic enzymes) in the presence of an anti-MPO-directed immune response is a prerequisite for the development of pauci-immune NCGN.

References

1. Couser, W.G. 1982. Idiopathic rapidly progressive glomerulonephritis. *Am. J. Nephrol.* 2:57.
2. Falk, R.J. 1990. ANCA-associated renal disease (clinical conference). *Kidney Int.* 38:998.
3. Goldschmeding, R., C.E. van der Schoot, D. ten Bokkelhuinink, C.E. Hack, C.E. van den Ende, C.G.M. Kallenberg, and A.E.G. Kr. von dem Borne. 1989. Wegener's granulomatosis autoantibodies identify a novel diisopropylfluorophosphate-binding protein in the lysosomes of normal human neutrophils. *J. Clin. Invest.* 84:1577.
4. Niles, J.L., R.T. McCluskey, M.F. Ahmad, and M.A. Arnaout. 1989. Wegener's Granulomatosis autoantigen is a novel neutrophil serine protease. *Blood.* 74:1888.
5. Lüdemann, J., B. Utecht, and W.L. Gross. 1990. Antineutrophil cytoplasm antibodies in Wegener's Granulomatosis recognize an elastinolytic enzyme. *J. Exp. Med.* 171:357.
6. Falk, R.J., and J.C. Jennette. 1988. Anti-neutrophil cytoplasmic antibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N. Engl. J. Med.* 318:1651.
7. Cohen Tervaert, J.W., R. Goldschmeding, J.D. Elema, M. van der Giessen, M.G. Huitema, G.K. van der Hem, T.H. The,

A.E.G. Kr. von dem Borne, and C.G.M. Kallenberg. 1990. Autoantibodies against myeloid lysosomal enzymes in crescentic glomerulonephritis. *Kidney Int.* 37:799.
8. Horn, R.G., A.S. Fauci, A.S. Rosenthal, and S.M. Wolff. 1974. Renal biopsy pathology in Wegener's Granulomatosis. *Am. J. Pathol.* 74:423.
9. Germuth, F.G., Jr., A.J. Valdes, J.J. Taylor, O'L. Wise, and E. Rodriguez. 1975. Fatal immune complex glomerulonephritis without deposits. *John Hopkins Med. J.* 136:189.
10. Stilmant, M.M., W.K. Bolton, B.G. Sturgill, G.W. Schmitt, and W.G. Couser. 1979. Crescentic glomerulonephritis without immune deposits; Clinicopathologic features. *Kidney Int.* 15:184.
11. Ronco, P., P. Verroust, F. Mignon, O. Koruilsky, Ph. Vanhille, A. Meyrier, J. Ph. Mery, and L. Morel-Maroger. 1983. Immunopathological studies of polyarteritis nodosa and Wegener's granulomatosis: a report of 43 patients with 51 renal biopsies. *Q. J. Med.* 206:212.
12. Weiss, M.A., and J.D. Crissman. 1989. Renal pathologic features of Wegener's Granulomatosis: a review. *Semin. Resp. Med.* 10(2):141.
13. Godman, G.C., and J. Churg. 1954. Wegener's Granulomatosis; Pathology and a review of the literature. *Arch. Pathol.*

58:533.

14. Bhatena, D.B., S.D. Migdal, B.A. Julian, J.G. McMorrow, and R.W. Baehler. 1987. Morphologic and immunohistochemical observations in granulomatous glomerulonephritis. *Am. J. Pathol.* 126:581.

15. Falk, R.J., R.S. Terrell, L.A. Charles, and J.C. Jennette. 1990. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc. Natl. Acad. Sci. USA.* 87:4115.

16. Merrill, D.P. 1980. Purification of human myeloperoxidase by concavalin A-sepharose affinity chromatography. *Prep. Biochem.* 10(2):133.

17. Klebanoff, S.J., A.M. Waltersdorph, and H. Rosen. 1984. Antimicrobial activity of myeloperoxidase. *Methods Enzymol.* 105:399.

18. Hoyer, J.R., S.M. Mauer, and A.F. Michael. 1975. Unilateral renal disease in the rat. I. clinical, morphologic and glomerular mesiangial functional features of the experimental model produced by renal perfusion with aminonucleoside. *J. Lab. Clin. Med.* 85:756.

19. Johnson, R.J., S.J. Guggenheim, S.J. Klebanoff, R.F. Ochi, A. Wass, P. Baker, M. Schulze, and W.G. Couser. 1988. Morphologic correlates of glomerular oxidant injury induced by the myeloperoxidase-hydrogen peroxide-halide system of the neutrophil. *Lab. Invest.* 5(3):294.

20. Pryzwansky, K.B., L.E. Martin, and J.K. Spitznagel. 1978. Immunocytochemical localization of myeloperoxidase, lactoferrin, lysozyme and neutral proteases in human monocytes and neutrophilic granulocytes. *J. Reticuloendothel. Soc.* 24:295.

21. Cohen Tervaert, J.W., R. Goldschmeding, J.D. Elema, P.C. Limburg, M. van der Giessen, M.G. Huitema, M.I. Koolen, R.J. Hené, T.H. The, G.K. van der Hem, A.E.G. Kr. von dem Borne, and C.G.M. Kallenberg. 1990. Association of antibodies to myeloperoxidase with different forms of vasculitis. *Arthritis Rheum.* 33:1264.

22. Pinching, A.J., A.J. Rees, B.A. Pussel, C.M. Lockwood, R.S.

Mitchison, and D.K. Peters. 1980. Relapses in Wegener's Granulomatosis: the role of infection. *Br. Med. J.* 281:836.

23. Johnson, R.J., W.G. Couser, E.Y. Chi, S. Adler, and S.J. Klebanoff. 1987. New mechanism for glomerular injury myeloperoxidase-hydrogen peroxide-halide system. *J. Clin. Invest.* 79:1379.

24. Johnson, R.J., W.G. Couser, C.E. Alpers, M. Vissers, M. Schulze, and S.J. Klebanoff. 1988. The human neutrophil serine proteases, elastase and cathespin G, can mediate glomerular injury in vivo. *J. Exp. Med.* 168:1196.

25. Klebanoff, S.J. 1980. Oxygen metabolism and the toxic properties of phagocytes. *Ann. Int. Med.* 93:480.

26. Henson, P.M., and R.B. Johnston, Jr. 1987. Tissue injury in inflammation; oxidants, proteinases, and cationic proteins. *J. Clin. Invest.* 79:669.

27. Fleuren, G.J., J. Grond, and Ph. J. Hoedemaeker. 1980. in situ formation of subepithelial glomerular immune complexes in passive serum sickness. *Kidney Int.* 17:631.

28. Weiss, S.J. 1989. Tissue destruction by neutrophils. *N. Engl. J. Med.* 320:365.

29. Kallenberg, C.G.M., J.W. Cohen Tervaert, F.J. van der Woude, R. Goldschmeding, A.E.G. Kr. von dem Borne, and J.J. Weening. 1991. Autoimmunity to lysosomal enzymes: new clues to vasculitis and glomerulonephritis? *Immunol. Today.* 12:61.

30. Dijkstra, C.D., E.A. Döpp, P. Joling, and G. Kraal. 1985. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3. *Immunology.* 54:589.

31. Kampinga, J., and R. Aspinoll. 1990. Thymocyte differentiation and thymic microenvironment development in the fetal rat thymus: an immunological approach. *In* Thymus Update. Vol 3. M. Kendall and M. Ritters, editors. Harwood Academic Publishers GmbH, Berks, UK. 149–186.