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Aggravation of rat nephrotoxic serum nephritis by anti-myeloperoxidase antibodies

KENJI KOBAYASHI, TAKANORI SHIBATA, and TETSUZO SUGISAKI

Department of Nephrology, Showa University School of Medicine, Tokyo, Japan

Aggravation of rat nephrotoxic serum nephritis by anti-myeloperoxidase antibodies. To investigate a possible role of anti-neutrophil cytoplasmic antibodies directed against myeloperoxidase (MPO-ANCA) in glomerulonephritis, we prepared anti-rat MPO antiserum by immunization of rat MPO into a rabbit. Then we administered anti-rat MPO antiserum (group 1) or normal rabbit serum (NRS) (group 2) into rats before injection of nephrotoxic serum (NTS), which induced nephrotoxic serum nephritis (NTN). Other groups of rats received either anti-rat MPO anti-serum (group 3) or NRS (group 4) before injection of NRS but not NTS. Rats in group 1 and group 2 were sacrificed at either 3 hours, 15 hours, or 14 days after NTS injection. Rats in group 3 and group 4 were sacrificed at 15 hours after the last NRS injection. By light microscopy, in rats with NTN sacrificed at 3 hours, counts of polymorphonuclear leukocytes (PMN) per glomerulus were 21.6 ± 3.5 in group 1 and 8.4 ± 1.7 in group 2 ($P < 0.01$). At 15 hours, massive glomerular fibrin deposits were observed in group 1 rats (fibrin score, 131 ± 8), but not in group 2 rats (fibrin score, 27 ± 21; $P < 0.01$). By direct immunofluorescence microscopy, rat MPO was found along glomerular capillary walls more intensely in group 1 rats than in group 2 rats. No pathological alterations were found in group 3 and group 4 rats. Further, renal elution studies revealed that eluted rabbit IgG contained anti-rat MPO antibodies in group 1 rats but not in group 3 rats. These results suggest that the anti-MPO antibodies are directly involved in the more severe glomerular lesions in group 1 rats via interactions with MPO itself or activation of PMN, which release various kinds of mediators including MPO.

It has been shown that anti-neutrophil cytoplasmic antibodies (ANCA) are frequently detected in sera from patients with Wegener's granulomatosis, microscopic polyarteritis nodosa (PN) and idiopathic pauci-immune crescentic glomerulonephritis (CGN) [1–5]. ANCA are divided into two major subsets according to immunofluorescence findings: cytoplasmic (C)-ANCA and perinuclear (P)-ANCA. C-ANCA are usually specific for 29 kD serine proteinase; proteinase-3, and P-ANCA are usually specific for myeloperoxidase (MPO).

Among ANCA, MPO-ANCA directed against myeloperoxidase is closely associated with the occurrence of idiopathic CGN and microscopic PN [2, 3]. Although the pathogenic role of MPO-ANCA is still unclear, a recent report on the biological activities *in vitro* support the idea that it may be involved in the development of necrotizing vascular or glomerular lesions via activation of polymorphonuclear leukocytes (PMN) primed by proinflammatory cytokines such as tumor necrosis factor-α [6]. Hypothetically, ANCA related glomerulonephritis may develop after local release of cationic lysosomal enzymes, such as MPO, which released from PMN activated by undefined etiology [4, 5]. In the present study, we administered rabbit anti-rat MPO antiserum previously and induced nephrotoxic serum nephritis (NTN) by the injection of rabbit anti-rat glomerular basement membrane (GBM) antiserum as nephrotoxic serum (NTS) into normal Wistar rats, so that we studied the effects of anti-MPO antibodies on acute PMN-mediated glomerular injury. The rats that received both anti-MPO antiserum and NTS developed acute and more severe exudative glomerulonephritis characterized by extensive infiltration of PMN, followed by glomerular intracapillary fibrin thrombosis as compared to rats that received both normal rabbit serum (NRS) and NTS.

Here we report that rabbit anti-rat MPO antibodies clearly aggravated rat NTN. We will also discuss the possible role of MPO and anti-MPO antibodies interactions.

Methods

Animals

Female Wistar rats weighing 150 g were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). They were fed standard rat chow and received water *ad libitum.* Japanese white rabbits were purchased from a local breeder.

MPO

Rat MPO was purified according to the method by Kariya et al [7]. Briefly, hemoglobin-free white blood cells from bone marrow were homogenized in 0.25 M sucrose by electric homogenizer (Polytron, Kinematica, Luzern, Switzerland), then centrifuged at 105,000 g for 60 minutes. The precipitate was stirred with 0.5% cethyltrimethylammonium bromide (CETAB) in 10 mM potassium phosphate buffer (pH 7.0) at 4°C overnight. The supernatant of CETAB extract was collected by centrifugation and dialyzed against 25 mM acetate buffer containing 0.2 M NaCl (pH 4.7). Then the dialyzed solution was applied to CM-Sephadex C-50 column (Pharmacia Fine Chemicals, Uppsala, Sweden) and collected with 0.2 to 2.0 M NaCl gradient in acetate buffer. Protein concentration of each fraction was monitored by optical density (OD) at 280 nm and enzyme activity was tested by guiacol as substrate [7]. The MPO active fractions were pooled and dialyzed against 100 mM acetate buffer (pH 5.6) containing 5 mM $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.05% CETAB. After dialysis, crude MPO was further purified by Con A-Sepharose column (Pharmacia Fine

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Chemicals). MPO active fractions eluted by 0.2 M Methyl-α-D-glucoside were pooled and concentrated. Finally, protein concentration of MPO in 10 mM phosphate buffer containing 0.15 M NaCl, 0.02% CETAB and 2% glycerol was determined by BCA protein assay reagent (Pierce, Rockford, IL, USA) and the purity was checked by SDS-PAGE.

Human neutrophil MPO was purchased from Calbiochem Corp. (La Jolla, CA, USA).

Preparation of rabbit anti-rat MPO antiserum

Fifty micrograms of rat MPO in complete Freund's adjuvant were injected intradermally into a rabbit every other week for a total of three injections. One week later, 10 μg MPO in saline was i.v. injected. The rabbit was bled four days after the injection. An i.v. challenge and bleeding was repeated for a total of three times. The antisera were pooled and used for this study.

ELISA

Anti-MPO antibody was assessed by standard ELISA procedure. Microtiter plate (Nunc, Roskilde, Denmark) was coated with rat MPO at protein concentration of 5 μg/ml in borate buffered saline (pH 8.8). After saturation with 0.5% BSA in PBS, the plates were incubated with rabbit serum diluted appropriately with 2% BSA in PBS containing 0.05% Tween-20 (PBS-Tw). After washing bound antibody was detected by alkaline-phosphatase labeled goat anti-rabbit IgG (Cappel Laboratories, West Chester, PA, USA) and p-nitrophenyl-phosphate disodium as a substrate. Results are expressed as OD at 405 nm. Rabbit IgG concentration was determined by sandwich ELISA using goat anti-rabbit IgG (Cappel Laboratories). Standard curves were established for each assay by using purified rabbit IgG (ZYMED Laboratories, San Francisco, CA, USA).

Isolation of peritoneal PMN

Rat PMN was prepared from peritoneal exudate cells by the method described by Kudo, Nakamura and Koyama [8]. Saline containing 3% casein Na was injected i.p. into rats, and after 15 hours the peritoneal exudate cells were harvested by Krebs-Ringer phosphate buffer (KRPB, pH 7.2). The cell suspension was centrifuged 1500 rpm for five minutes at 4°C. To remove tissue debris, the cells were suspended in 2.47% gelatin-containing saline for 30 minutes. Then supernatant containing cells were centrifuged and 20 ml of 0.2% NaCl was added to cell pellets to lyse red blood cells for 10 seconds. After the hypotonic treatment, an equal volume of 1.6% NaCl was added to the cell suspension to return physiological condition immediately. The cells were washed with KRPB repeatedly. The cells thus prepared were used as PMN rich peritoneal cells for the preparation of cell smear by cytocentrifuge. The purity of PMN-rich peritoneal cells was checked by Giemsa-staining kit (Diff-Quik; Kokusaishiyaku, Kobe, Japan). The PMN was usually more than 80% of cells obtained by this method.

ANCA assay by immunofluorescence

The PMN was suspended in PBS to a concentration of 2×10^5 cells/ml and an aliquot of 300 μl was pelleted onto glass slides using cytocentrifuge (Cytospin 3, Shandon Scientific LTD., Chesire, UK). The cells were fixed in absolute ethanol at −20°C for five minutes [2]. Dried cell preparations were washed and incubated with appropriately diluted serum samples and washed in PBS. They were then incubated with FITC-labeled goat anti-rabbit IgG (Cappel Laboratories). After washing, the slides were mounted for immunofluorescence microscopy.

SDS-PAGE

SDS-PAGE was carried out according to the discontinuous buffer procedure of Laemmli [9]. After electrophoresis the gel was stained with Coomassie blue and the molecular weight was estimated by molecular weight standard (Bio-Rad Laboratories, Richmond, CA, USA). For the staining of glycoprotein, the gel was stained with periodic acid Schiff (PAS) reagent [10].

Western blotting

After SDS-PAGE, MPO was transferred onto nitrocellulose membrane by the method of Towbin, Staehelin and Gordon [11]. After saturation with 3% BSA in PBS, membrane was cut into several strips and incubated with rabbit serum 1/1000 diluted with 2% BSA in PBS-Tw at 4°C overnight. After washing with PBS-Tw, the strips were incubated with alkaline-phosphatase labeled goat anti-rabbit IgG for an additional five hours. Then they were washed with PBS-Tw and soaked in alkaline phosphatase buffer: 0.1 M Tris-HCl, pH 9.5 containing 0.1 M NaCl and 5 mM $MgCl_2$. Finally they were developed with alkaline phosphatase buffer containing Nitro Blue Tetrazolium, NBT and 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt, BCIP [12].

Labeling procedures

Goat anti-rabbit or rat IgG specific antibodies (Cappel Laboratories) were labeled with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, USA) according to the method of Engvall and Perlmann [13]. Rabbit IgG fraction was purified from rabbit anti-rat MPO antiserum by Protein A sepharose column (Pharmacia Fine Chemicals), then labeled with fluorescein isothiocyanate (FITC) (Cappel Laboratories) by the method of Hudson and Hay [14].

Preparation of rabbit anti-rat GBM antiserum

Rat glomeruli were isolated from Wistar rat kidneys by differential sieving method described by Spiro [15], then the isolated glomeruli were sonicated repeatedly and washed by centrifugation to purify GBM. The purified GBM containing 60 mg in wet weight was emulsified in complete Freund's adjuvant and injected intradermally into rabbits every other week for a total of four injections. One week later, they were bled and sacrificed by heart puncture. The serum anti-GBM antibody activities were tested by indirect immunofluorescence microscopy on sections of rat kidney. Sera of high responder rabbits were pooled and used as NTS in this study. NRS was obtained from a non-immunized rabbit. Prior to injection into rats, the antiserum was decomplemented and absorbed with rat erythrocytes. To evaluate the anti-GBM antibody activities *in vivo,* 0.1, 0.2, 0.5 or 0.7 ml of NTS was diluted respectively with saline to 1 ml of final volume, and then they were i.v. injected into rats.

Experimental design

Four groups of rats were treated according to the following design. Group 1 rats received 0.8 ml of rabbit anti-rat MPO antiserum (first i.v. injection) and 0.2 ml of NTS (second i.v. injection), then they were sacrificed at either three hours (group 1-a, $N = 6$), 15 hours (1-b, $N = 6$), or 14 days (1-c, $N = 6$) after

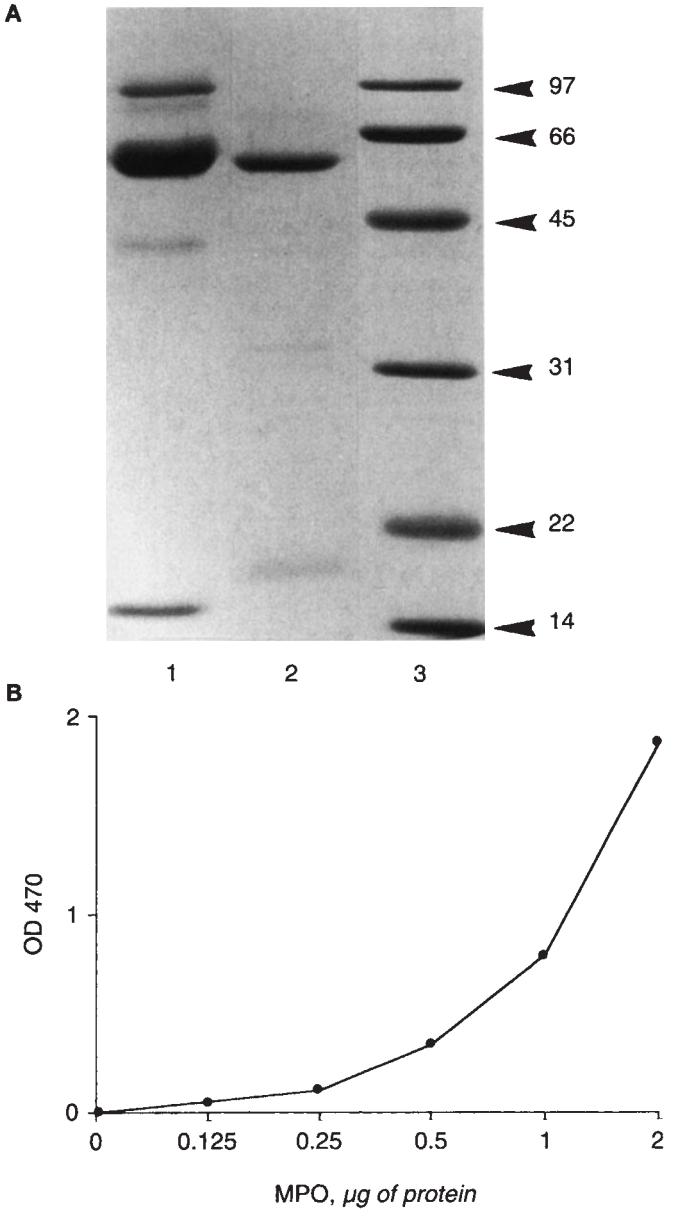

Fig. 1. *Biochemical characteristics of purified rat MPO.* **A.** SDS-PAGE (12.5%) analysis of purified rat MPO under reducing condition. lane 1, 5 μg of human MPO; lane 2, 10 μg of rat MPO; lane 3, molecular weight marker protein. Numbers shown on the right side indicate molecular weight (kD) obtained by marker protein. **B.** MPO activities in fluid phase using guaiacol as substrate. Different doses of rat MPO were incubated with 1 ml of reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 0.33 mM H_2O_2 and 33 mM guaiacol. Enzyme activities are expressed as OD at 470 nm.

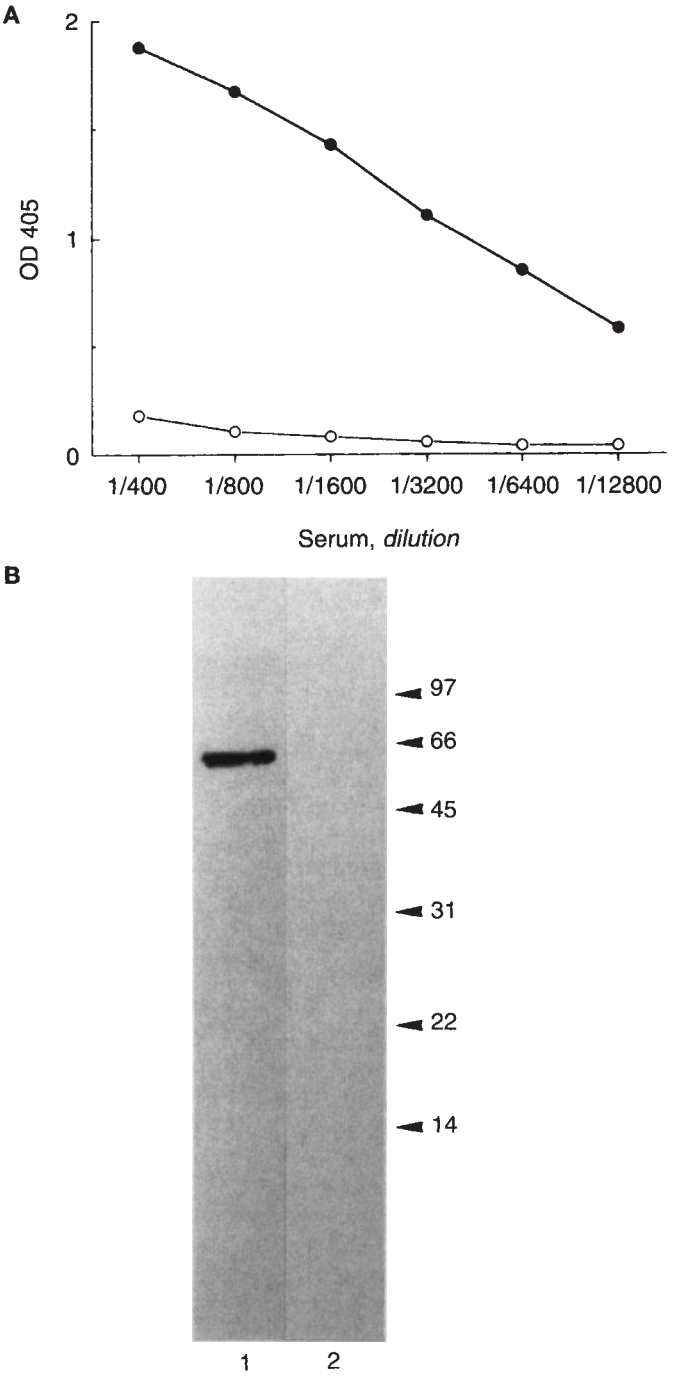

Fig. 2. *Reactivity of rabbit anti-rat MPO antibodies.* **A.** Anti-rat MPO ELISA. Dilution curves of pooled rabbit serum immunized with rat MPO (•) or NRS (○). **B.** Rabbit anti-rat MPO antibody activities detected by Western blotting. Five micrograms of rat MPO was subjected to SDS-PAGE (12.5%) under reducing condition, then reactivity of 1/1000 diluted rabbit anti-rat MPO antiserum (lane 1) or NRS (lane 2) with rat MPO were visualized by alkaline phosphatase labeled goat anti-rabbit IgG. Numbers shown on the right side indicate molecular weight (kD) obtained by marker protein.

NTS injection. Group 2 rats received 0.8 ml of NRS and 0.2 ml of NTS, and they were then sacrificed at either three hours (group 2-a, $N = 6$), 15 hours (2-b, $N = 6$), or 14 days (2-c, $N = 6$) after NTS injection. Group 3 rats received 0.8 ml of rabbit anti-rat MPO antiserum and 0.2 ml of NRS, and then they were sacrificed at 15 hours ($N = 6$) after NRS injection. Group 4 rats received 0.8 ml of NRS and an additional 0.2 ml of NRS, and then they were sacrificed at 15 hours ($N = 6$) after the last NRS injection. Injection of NTS or NRS for second injection was consistently performed seven hours after first injection. All rats were bled from abdominal aorta when they were sacrificed.

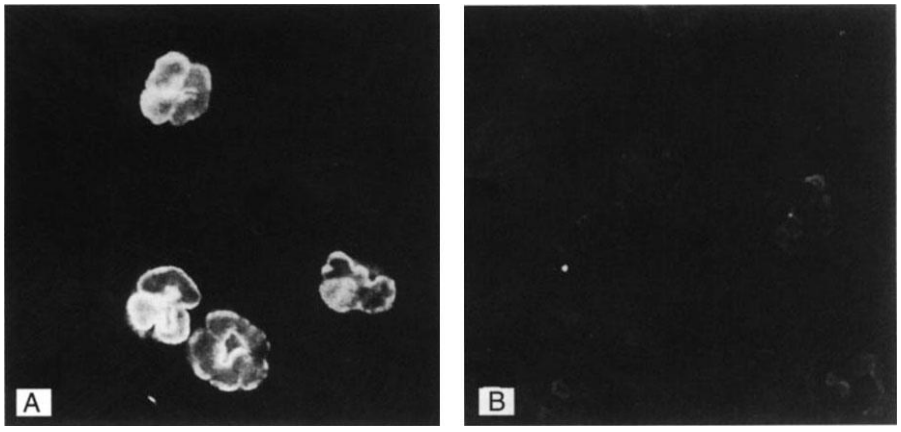

Fig. 3. *Immunostaining of neutrophils on indirect immunofluorescence microscopy.* **A.** Perinuclear staining of fixed PMN produced by a 1/40 diluted rabbit serum immunized with MPO, which is similar to so-called "P-ANCA" fluorescence (×400). **B.** Negative staining of fixed PMN produced by a 1/40 diluted NRS (×400).

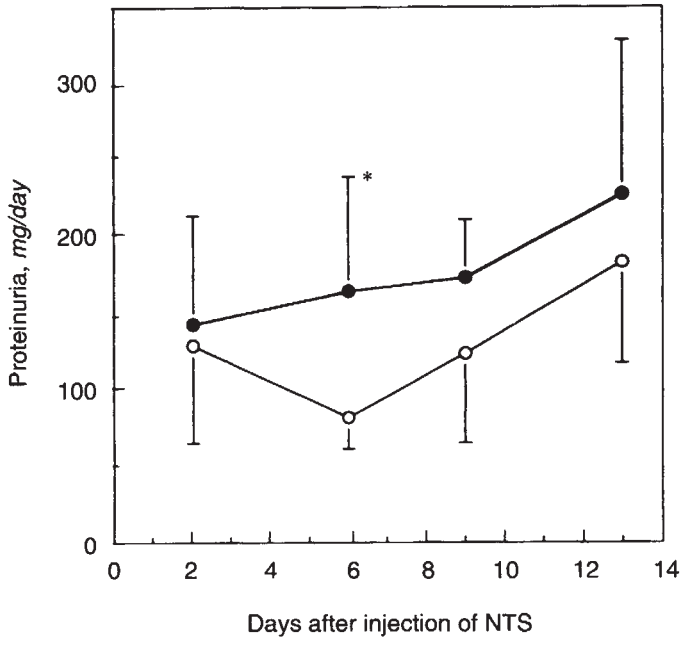

Fig. 4. *Kinetic studies on proteinuria in group 1-c (●) and group 2-c (○) rats.* The values represent mean ± 1 SD. The proteinuria of group 1-c rats was significantly higher than that of group 2-c rats on day 6 ($^*P < 0.05$).

Urinalysis

For estimation of proteinuria, urine was collected in metabolic cages for 24 hours on day 2, day 6, day 9 and day 13 after injection of NTS. Urinary protein was determined by the Biuret method.

Histopathology

Renal tissue was fixed in 2% paraformaldehyde/PBS and embedded in parafin for light microscopy. Four micrometer sections were stained with hematoxylin-eosin (HE), PAS and periodic acid-silver methenamine (PASM). Counts of PMN were made in at least 15 glomeruli according to the method of Cochrane, Unanue and Dixon [16]. The average counts per glomerulus are reported.

For immunofluorescence, the tissues were snap-frozen in liquid nitrogen and cut to 4 μm sections. Rabbit IgG, rat IgG, rat C_3 and rat fibrin/fibrinogen in the kidney sections were detected by immunofluorescence with FITC labeled goat anti-rabbit IgG, anti-rat IgG, anti-rat C3 and anti-rat fibrinogen (Cappel Laboratories).

Glomerular fibrin deposition was evaluated as follows. First the extent of fibrin deposits of each glomerulus was graded on 50 glomeruli by four different scales: 0, absent; 1, less than 1/3; 2, between 1/3 and 2/3 and 3, more than 2/3 of a whole glomerulus. Finally a fibrin score per rat is reported by a sum of the number of glomeruli that represents the respective grade times the corresponding grade.

The presence of MPO in glomeruli was studied by direct immunofluorescence microscopy using FITC labeled rabbit anti-rat MPO antibodies prepared in this study. The intensity of fluorescence was graded by the following scales: −; ±; +; and, 2+. Reactivity of the FITC labeled antibodies with rat MPO was substantiated by direct ANCA immunofluorescence test.

Elution study

Renal elution studies were performed by the method of Lerner, Glassock and Dixon [17]. Anti-GBM antibody activities and anti-MPO antibody activities in renal eluates were tested by indirect immunofluorescence and ELISA, respectively. Rabbit IgG concentration in eluates was determined by ELISA.

Statistical analysis

Statistical analysis was performed with the Mann-Whitney U test. Probability values greater than 5% were considered insignificant.

Results

Rat MPO

Purity of rat MPO was assesed by SDS-PAGE. In the case of reducing conditions, the major band was demonstrated at the position of 57 kD corresponding to large (α) subunits. Another band, probably a small (β) subunit was demonstrated at the position of approximately 17 kD. In addition, several minor bands were discernible (Fig. 1A). These results were almost the same as the previous report [7] except the position of β subunit. In the case of human MPO subjected to SDS-PAGE in parallel, the α and β subunits were demonstrated at the position of 57 kD and 15 kD, respectively, which were identical to the published results [18]. When SDS-PAGE (7.5%) was performed under non-reducing conditions without heating, rat MPO was stained at the position of around 116 kD. Further, the glycoprotein nature was confirmed by

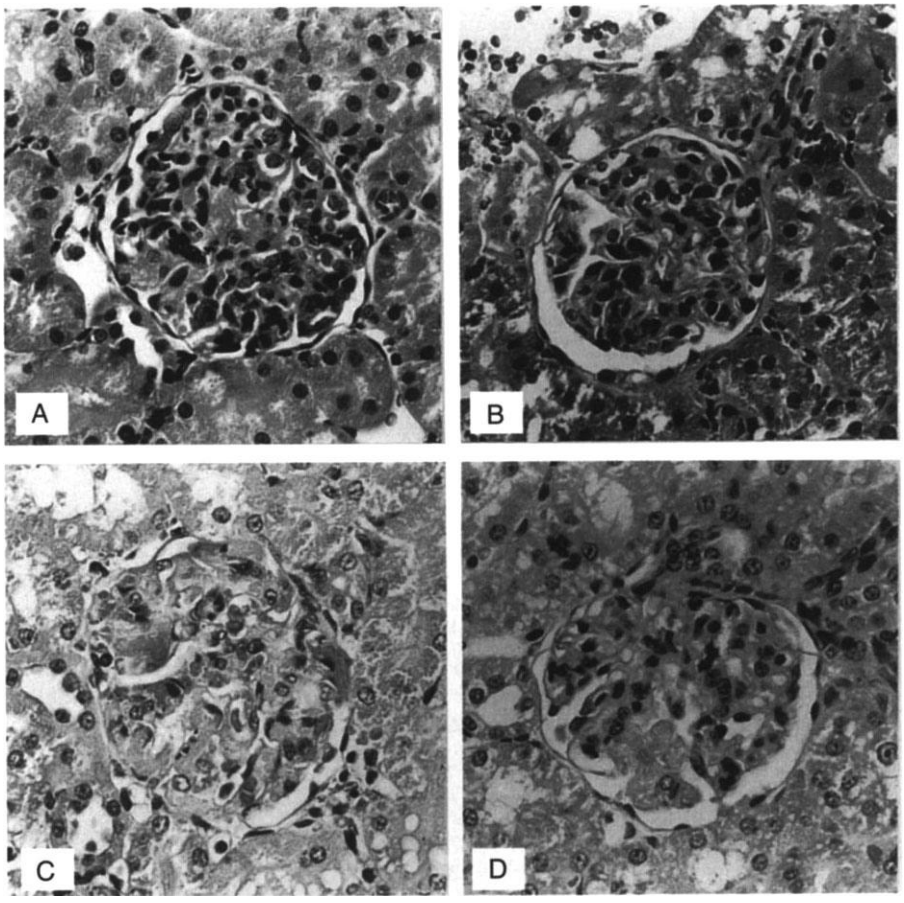

Fig. 5. *Histological findings of glomerular lesions in rats with NTN.* **A.** Enhanced infiltration of PMN in a glomerulus from a group 1-a rat (HE, ×100). **B.** Limited infiltration of PMN in a glomerulus from a group 2-a rat (HE, ×100). **C.** Representative histological appearance of a glomerulus in a group 1-b rat. Note the marked intracapillary hyaline-like materials and dilatation of mesangial areas (HE, ×100). **D.** Representative histological appearance of a glomerulus in a group 2-b rat. Minimal hyaline-like materials are seen in segmental glomerular tufts (HE, ×100).

PAS staining after SDS-PAGE (data not shown). The enzyme activity of purified MPO have been substantiated by fluid phase enzyme assay (Fig. 1B).

Rabbit anti-rat MPO antiserum

Anti-rat MPO antibody activities of immunized rabbit serum was tested by anti-MPO ELISA. The rabbit serum exhibited high titer of anti-rat MPO activities by ELISA (Fig. 2A). To define the reactive molecule, Western blotting analysis was performed after SDS-PAGE (Fig. 2B). The results indicate that rabbit anti-rat MPO antibodies react with 57 kD protein corresponding to the α-subunit of rat MPO. Although the binding activity with the β-subunit was not demonstrated, it was shown that the serum reacted with whole rat MPO molecules by Western blotting analysis after SDS-PAGE under non-reducing conditions (data not shown). The findings of the serum ANCA assay by immunofluorescence have shown the fluorescence pattern, which is similar to the so-called "P-ANCA" (Fig. 3). Notably, NRS has shown significant binding to neither rat MPO nor rat PMN. In addition, the result of indirect immunofluorescence microscopy using normal Wistar rat kidney frozen section revealed that rabbit anti-rat MPO antiserum did not have any cross reactivities against renal tissue antigens including cellular components.

Rat NTN

Rats receiving different doses of NTS were sacrificed at 3 or 15 hours after injection of NTS, then the severity of glomerulonephritis was evaluated histopathologically. Rats that received 0.2 ml of NTS showed mild glomerulonephritis, whereas rats that received 0.5 or 0.7 ml of NTS showed severe glomerulonephritis characterized by exudation of PMN at 3 hours and marked glomerular fibrin deposition at 15 hours after injection of NTS. Thus, 0.2 ml of NTS was used for the induction of NTN to study *in vivo* effect of rabbit anti-rat MPO antibodies on rat NTN.

Effect of rabbit anti-rat MPO antibodies on rat NTN

Exacerbation of proteinuria. Rats developed proteinuria after injection of NTS in group 1-c and group 2-c rats. The proteinuria increased gradually from 142 ± 71 mg on day 2 to 164 ± 75 mg on day 6 in group 1-c rats, whereas the proteinuria decreased from 127 ± 63 mg on day 2 to 80 ± 21 mg on day 6 in group 2-c rats (Fig. 4). Although the proteinuria increased finally in both groups of rats, the proteinuria in group 1-c rats was consistently higher than that of group 2-c rats.

Aggravation of rat NTN by rabbit anti-rat MPO antibodies. Rats were sacrificed at either 3 hours, 15 hours, or 14 days after injection of NTS. By light microscopy, in group 1-a rats sacrificed at 3 hours, predominant glomerular lesions were characterized by exudation of PMN. However, in group 2-a rats, PMN exudation was less prominent (Fig. 5 A,B). To determine the degree of PMN infiltration in rats, numbers of PMN per glomerulus were counted. In group 1-a rats, the PMN counts were significantly higher than that of group 2-a rats ($P < 0.01$) (Fig. 6). In rats sacrificed at 15 hours, exudation and glomerular hypercellularity ceased, but

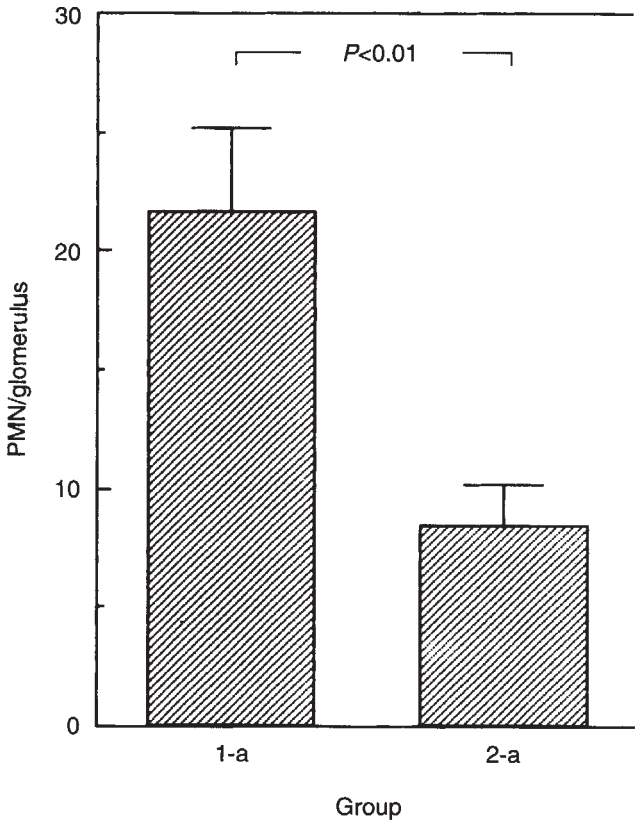

Fig. 6. *Counts of PMN in glomeruli from group 1-a and group 2-a rats.* Results are expressed as counts of PMN per glomerulus in a rat (mean ± 1 SD).

instead, a number of glomeruli showed intracapillary hyaline thrombosis and marked dilatation or distortion of mesangial areas in group 1-b rats, whereas hyaline thrombi were only found in a few segmental glomerular tufts in group 2-b rats (Fig. 5 C, D). No glomerular abnormalities were found in group 3 and group 4 rats. In rats sacrificed at 14 days, although both groups of rats that received NTS showed mild proliferative glomerulonephritis, there were no significant histopathological differences in the two groups of rats. Neither glomerular crescent formation nor tuft necrosis was found in all groups of rats.

By immunofluorescence microscopy, in all groups of rats that received NTS, strong linear binding of rabbit IgG was found along capillary walls at 3 or 15 hours after NTS injection. There were no significant differences between group 1 and group 2 rats, at least at levels of immunofluorescence microscopy. A faint linear pattern along capillary walls was found in rat C3, and rat IgG was completely negative at 3 and 15 hours after injection of NTS. Mild fibrin deposits were found in glomerular capillary lumens at 3 hours. In rats sacrificed at 15 hours, massive glomerular fibrin deposits were observed in group 1-b rats in contrast to the mild and segmental fibrin deposits in group 2-b rats (Fig. 7). Semiquantitative analysis of fibrin deposits revealed a marked difference of the fibrin score between the two groups of rats (group 1-b rats, 131 ± 8; and group 2-b rats, 27 ± 21; $P < 0.01$; Fig. 8).

Thus, the rabbit anti-rat MPO antibodies clearly aggravated rat NTN, yet the histopathology seen in group 1-a and 1-b rats was not specific pathological changes but rather severe rat NTN.

Localization of MPO. In rats sacrificed at 3 hours, the MPO was detected in interrupted pattern along the glomerular capillary walls and in exudative leukocytes in group 1-a rats by immunofluorescence microscopy. In group 2-a rats, the MPO was found in only exudative leukocytes but in neither glomerular capillary walls nor mesangial areas. At 15 hours, the presence of MPO in group 1-b rats became more intense as compared to that in group 1-a rats; however, only very weak segmental deposition of MPO was noted in group 2-b rats (Fig. 9 and Table 1). In rats sacrificed at 14 days after injection of NTS, rat MPO was almost negative in glomeruli. In group 3 and group 4 rats, MPO was completely negative in glomeruli.

Anti-rat MPO antibody activities in renal eluates. As MPO is significantly detected in group 1-a rats at 3 hours and apparently the deposition increased in group 1-b rats at 15 hours, it seemed likely that administered rabbit anti-rat MPO antibodies actively were involved in the enhanced deposition of MPO in group 1 rats. On the mechanisms of MPO localization, renal elution studies were performed to see if rabbit anti-rat MPO antibodies existed in glomeruli, because it was extremely difficult to discriminate the rabbit anti-rat MPO antibodies from the rabbit anti-rat GBM antibodies on kidney sections. Rabbit anti-rat MPO antibody activities were demonstrated in renal eluates obtained from the kidneys of both group 1-a and 1-b rats by ELISA. Representative results are shown in Figure 10. Stronger anti-MPO antibody activities were noted in the eluate obtained from the kidneys of group 1-b rats than in group 1-a rats by comparative titration assay at same rabbit IgG levels (data not shown). The eluate obtained from group 1-c rats did not show any anti-MPO antibody activities. In the eluates obtained from group 3 rats, no anti-MPO antibody activities were detected. Notably, rabbit anti-rat GBM antibody activities in eluates from group 1 and group 2 rats were almost identical levels by indirect immunofluorescence microscopy at any time sacrificed. In addition, there were no significant differences in the amounts of eluted rabbit IgG per gram of kidneys from group 1 and group 2 rats (5.0 ± 1.5 μg; mean ± 1 SD).

Discussion

In the present study, we have shown that heterologous anti-rat MPO antibodies clearly aggravated acute rat NTN by immunopathological findings and exacerbation of proteinuria. The most striking observation in the present study is the strong localization of rat MPO in glomeruli from rats treated with both anti-MPO antiserum and NTS (group 1 rats). In addition, anti-rat MPO antibody activities were demonstrated in renal eluates from the same groups of rats. These results indicate that anti-MPO antibodies are clearly involved in the more severe glomerular lesions via interactions with MPO itself or activation of PMN, which release various kinds of mediators including MPO into the extracellular microenvironment. Since administration of anti-MPO antibodies alone was not able to induce any tissue lesions in group 3 rats, the activation and degranulation of PMN are prerequisites for the more severe pathological changes induced by anti-rat MPO antibodies.

MPO is one of the lysosomal enzymes, which released from PMN activated by various inflammatory stimuli [19]. Although the primary action of MPO is a microbicidal effect in the intracellularly, it could be involved in the tissue injury as a result of generation of reactive oxygen radicals in extracellular milieu [19, 20].

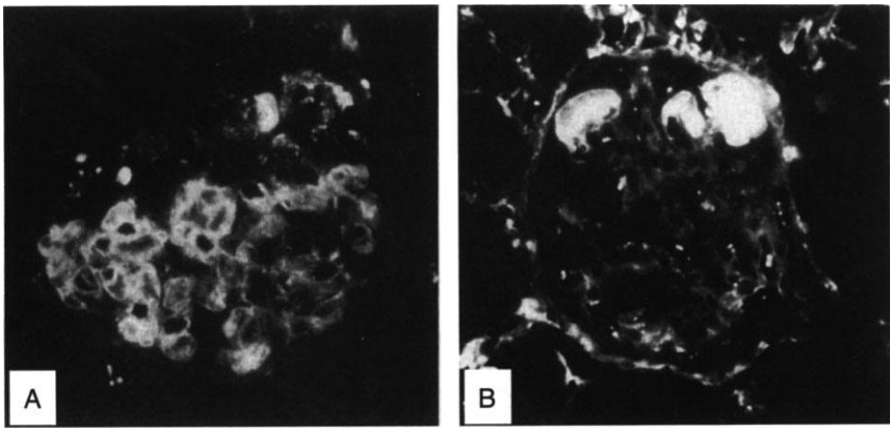

Fig. 7. *Glomerular fibrin deposition in rats with NTN.* **A.** Marked fibrin deposition in a glomerulus from a group 1-b rat (×400). **B.** Minimal fibrin deposition in a glomerulus from a group 2-b rat (×400).

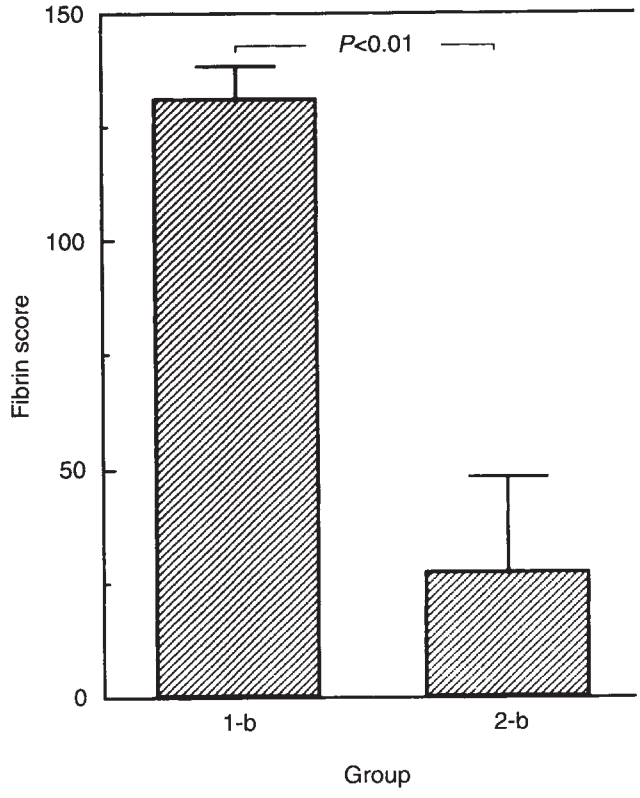

Fig. 8. *Semi-quantitative studies on glomerular fibrin deposits in group 1-b and group 2-b rats.* Results of fibrin deposits are expressed as a total of fibrin score per rat (mean ± 1 SD).

Biochemically, it has been shown that MPO is able to bind to anionic surfaces such as GBM or endothelial cells via its highly cationic charge. Based on these characteristics, Johnson et al demonstrated the development of glomerular injury induced by MPO-hydrogen peroxide-halide system *in vivo* [21]. Since it has been well established that PMN play a major role in the development of NTN especially at heterologous, that is, acute, phase [16, 22, 23], it is postulated that MPO is one of the significant enzymes leading to glomerular pathological changes. In fact, it has been reported that MPO could be detected enzymologically in renal homogenates from rats with NTN [24].

In our experimental model, the effects of anti-rat MPO antibodies on rat NTN were studied. Mild NTN was induced in rats to see *in vivo* effect of anti-rat MPO antibodies by histopathological studies and proteinuria. At the acute phase, rabbit anti-rat MPO antibodies aggravated mild glomerulonephritis to severe acute exudative glomerulonephritis. Since histopathological studies revealed that pretreatment of anti-MPO antiserum promoted the infiltration of PMN and enhanced fibrin deposition in glomeruli, which are remarkable immunopathological features of acute rat NTN, the involvement of anti-MPO antibodies is clearly indicated. These histopathological changes could explain the exacerbation of proteinuria at the heterologous phase in rats with anti-MPO pretreated NTN; that is, the course of proteinuria in group 1 rats was apparently different from group 2 rats. The fact that significantly higher amounts of proteinuria in group 1-C rats was observed on day 6 could be ascribed to the continual glomerular pathophysiological changes induced by anti-rat MPO antibodies.

To analyze more directly the mechanisms of aggravation of glomerulonephritis, the glomerular localization of MPO and the presence of rabbit anti-rat MPO antibodies in glomeruli were studied. Pre-treatment of anti-rat MPO antibodies could induce or enhance the glomerular localization of MPO in rats with NTN. As MPO is one of the lysosomal enzymes in PMN, it is most likely that glomerular MPO would be released from PMN by the cellular inflammatory reactions in NTN. Eventually the glomerular MPO localization, which may be a significant immunochemical marker of acute and severe glomerular lesions, could be augumented by anti-MPO antibodies. On the other hand, rabbit anti-rat MPO antibodies could be eluted specifically from kidneys in group 1 rats but not in group 3 rats. Therefore, the binding of anti-rat MPO antibodies to MPO localized previously in glomeruli is highly suggestive. The fact that anti-MPO antibody activities were detected in renal eluates are quite significant, because it indicates administered anti-MPO antibodies directly are involved in the glomerular lesions in group 1 rats, whatever the mechanisms may be. Further, the correlation of the intensity of glomerular MPO deposition with the levels of eluted rabbit anti-rat MPO antibodies also suggests the role of the anti-rat MPO antibodies. However, it should be noted that there was no pathological changes in group 3 and group 4 rats. Taken together, these results indicate that interactions of MPO and anti-MPO antibodies are

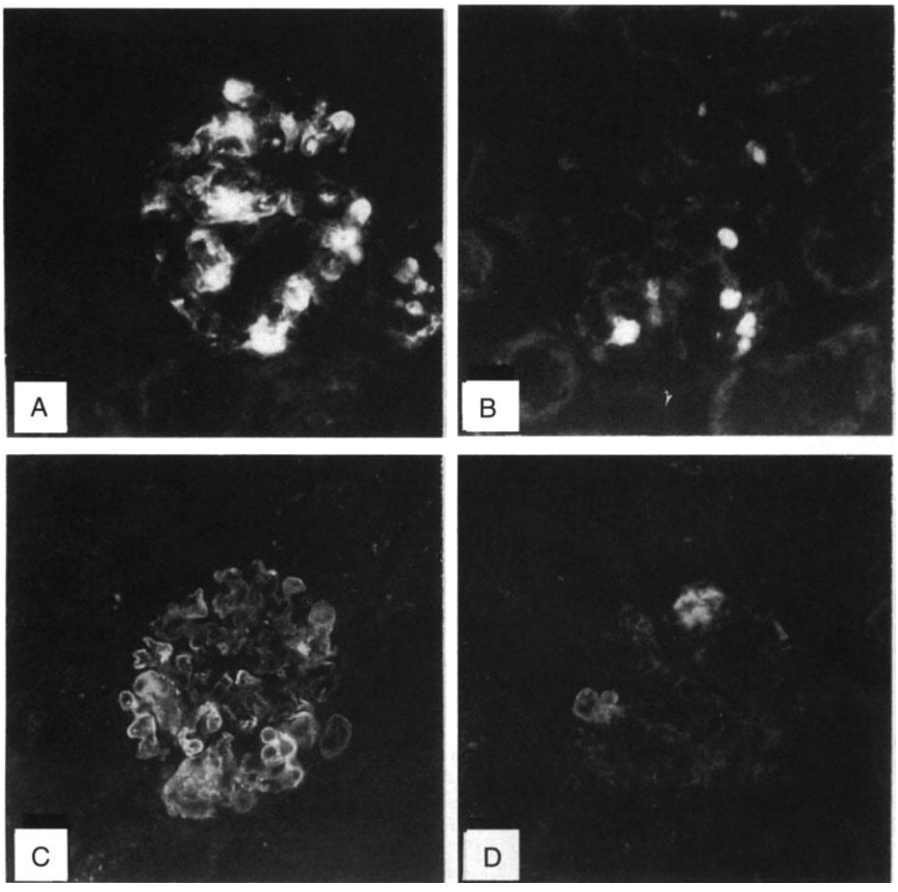

Fig. 9. *Localization of rat MPO in rats with NTN.* Rat MPO was demonstrated by direct immunofluorescence microscopy. **A.** Positive staining for rat MPO along the glomerular capillary walls and in leukocytes (group 1-a) (×400). **B.** Positive staining for rat MPO is seen only in leukocytes. Note the absence of rat MPO staining along the glomerular capillary walls (group 2-a) (×400). **C.** Strong positive staining for rat MPO both along the capillary walls and in mesangial areas (group 1-b) (×400). **D.** Very weak staining for rat MPO is seen in segmental glomerular tufts (group 2-b) (×400).

Table 1. Presence of rat MPO in kidneys from rats with NTN

Rats	N[c]	MPO in glomeruli[a]			
		−[b]	±	+	++
Group 1-b	6	0/6	0/6	4/6	2/6
Group 2-b	6	3/6	2/6	1/6	0/6
Group 3	6	6/6	0/6	0/6	0/6
Group 4	6	6/6	0/6	0/6	0/6

[a] Incidence of positive MPO staining
[b] Intensity of MPO staining was semi-quantitated by direct immunofluorescence microscopy
[c] Number of rats studied

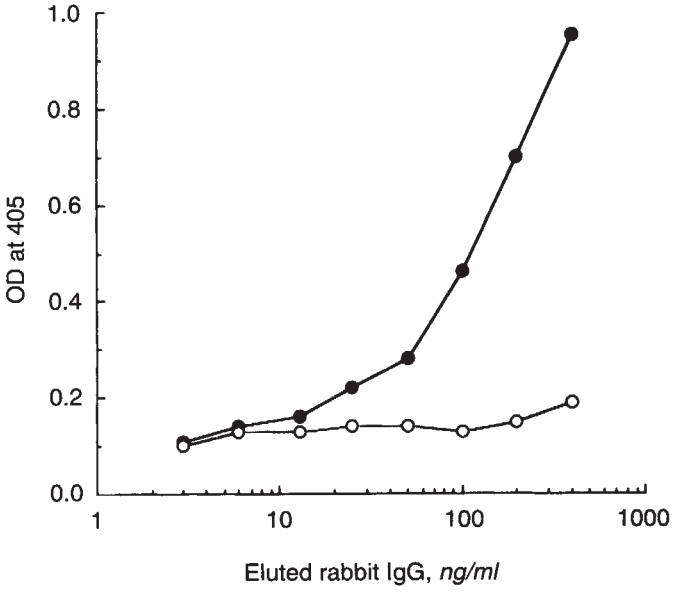

Fig. 10. *Rabbit anti-rat MPO antibody activities in renal eluates obtained from group 1-b (●) and 2-b (○) rats.* Results are expressed as OD at 405 nm.

critical events leading to more severe glomerular lesions in group 1 rats than group 2 rats.

Several mechanisms responsible for the aggravation of rat NTN may be considered. First, a possible mechanism is the formation of MPO-anti-MPO antibodies complexes in glomeruli. These complexes would promote the enhanced PMN infiltration and fibrin deposition in group 1 rats. In fact, Brouwer et al reported an experimental model developing acute necrotizing CGN as a result of the interaction between a human MPO-directed immune response and the lysosomal enzyme extract plus H_2O_2, which were infused from renal artery in human MPO immunized Brown-Norway rats [25]. Second, the prolongation of MPO enzyme activities by the binding of antibodies may be considered, as in the case of a recent observation on the inhibition of PR3 complexation with alpha 1-antitrypsin, the major inhibitor of PR3 by C-ANCA [26]. Consequently, it will activate the MPO-hydrogen peroxide-halide system which generates hypochlorous acid

(HOCl). Thus, the hypochlorous acid or a similar oxidant generated by the stimulated PMN would play a critical role in GBM degradation [20]. Alternatively, the direct activation of PMN by anti-MPO antibodies should be considered. Recently, Falk et al reported that anti-MPO antibodies stimulate PMN *in vitro* and induce the generation of reactive oxygen radicals as well as degranulation [6]. In our model, PMN activated by the inflammatory process of rat NTN may be activated further by anti-rat MPO antibodies in circulation or *in situ.* Consequently, this would enhance glomerular MPO deposition associated with anti-rat MPO antibodies, one of the inflammatory mediators, which aggravate the rat NTN. Obviously, activated PMN releases various kinds of mediators, for example, lysosomal enzymes such as neutral proteinases, which degrade human GBM *in vitro* [27, 28]. Therefore, MPO may be only one of the enzymes participating in the glomerular injury.

In our experimental condition, neither glomerular crescent formation nor tuft necrosis has been observed in group 1 rats, despite the aggravation of the histopathology. This might be partly ascribable to certain genetic factors that determine cellular immunological response in rats. Indeed, it has been known that the Wistar rat is not highly susceptible to the crescent formation, in contrast to the susceptibility to crescentic NTN in the strain of WKY rat [29].

It should be emphasized that our aim in this study was to investigate the effects of anti-MPO antibodies on PMN mediated glomerular injury. For that purpose, we injected a small dose of NTS to induce mild NTN. Accordingly, our experimental model may not be analogous to human ANCA related glomerulonephritis characterized by the paucity of visible immune deposits, although the co-existence of both ANCA and anti-GBM antibodies, which significance is still uncertain, has been recently reported in anti-GBM mediated glomerulonephritis [30–33]. Nevertheless, the data presented here strongly suggest that MPO-ANCA may be actively involved in the glomerular pathology, not solely but in concert with the pre-existence of activated PMN. This is of clinical importance, because it has been obserbed intercurrent infections, resulting in priming of PMN, frequently precede disease activity [34]. Falk et al has also reported that in the majority of patients, flu-like prodrome occurred prior to the onset of overt ANCA associated diseases [4, 35].

At present, the pathogenic role of anti-MPO antibodies for ANCA associated glomerulonephritis and vasculitis, remains unproven. Several lines of evidence support the hypothesis that ANCA and its target antigens may be implicated in the pathogenesis of these diseases [4, 5, 36, 37]. In the present study, we have demonstrated that anti-rat MPO antibodies potentially are involved in the glomerular injury when they will interact MPO molecules. Although, the *in vitro* effects of the rabbit anti-rat MPO antibodies on enzyme activities or PMN function remain to be determined, our results using an animal model suggest pathogenic significance of anti-MPO antibodies in human ANCA-associated diseases.

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Reprint requests to Takanori Shibata, M.D., Department of Nephrology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan.

Appendix. Abbreviations

ANCA	anti-neutrophil cytoplasmic antibody(ies)
CGN	crescentic glomerulonephritis
ELISA	enzyme-linked immunosorbent assay
GBM	glomerular basement membrane
MPO	myeloperoxidase
NRS	normal rabbit serum
NTN	nephrotoxic serum nephritis
NTS	nephrotoxic serum
PMN	polymorphonuclear leukocyte(s)

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