Immune complex glomerulonephritis is induced in rats immunized with heterologous myeloperoxidase

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SUMMARY

Anti-neutrophil cytoplasmic antibodies (ANCA), including anti-myeloperoxidase (MPO) antibodies, are associated with pauci-immune necrotizing small vessel vasculitis or glomerulonephritis. In order to substantiate a pathogenic role for ANCA, an animal model of pauci-immune ANCA-induced glomerulonephritis or vasculitis is required. Brouwer et al. reported pauci-immune glomerulonephritis in rats immunized with human MPO followed by perfusion of kidneys with lysosomal enzyme extract combined with H2O2, and suggested that this could serve as a model of ANCA-induced disease. We repeated these studies in spontaneously hypertensive rats (SHR) and Brown Norway rats (BNR). We immunized rats with human MPO. When circulating anti-MPO antibodies were detectable by indirect immunofluorescence microscopy and ELISA, blood pressure was measured, then perfusion of the left kidney of each rat was done via the renal artery in a closed, blood-free circuit with either MPO + H2O2, MPO, H2O2 alone or MPO + H2O2+neutral protease. Rats were killed on day 4 or day 10 after perfusion, and specimens were examined by light and immunofluorescence microscopy. Pathological lesions and deposits of IgG, C3, and MPO were found in immunized rats perfused with MPO + H2O2 with or without neutral protease, or MPO alone, in both rat strains and on both day 4 and day 10. The degree of histologic injury was proportional in intensity to the amount of IgG immune deposits. Spontaneously hypertensive rats sustained more damage and higher blood pressure than Brown Norway rats. No lesion was observed in immunized rats perfused with H2O2 or in the non-perfused right kidneys. Some of the non-immunized rats perfused with MPO + H2O2 developed pathological lesions. In conclusion, these rat models are examples of immune complex-mediated glomerulonephritis, and therefore are not similar to human ANCA-associated disease.

Keywords myeloperoxidase anti-neutrophil cytoplasmic antibodies glomerulonephritis immune complex animal model

INTRODUCTION

The serologic association of anti-neutrophil cytoplasmic antibodies (ANCA) with systemic necrotizing vasculitis or glomerulonephritis is well established [1]. However, the pathogenic role of ANCA in these diseases has not been established. Several in vitro studies have demonstrated that ANCA can activate neutrophils, resulting in a respiratory burst or degradation of azurophilic granule constituents. This process causes neutrophil adhesion to, and damage of, endothelial monolayers [2,3]. In order to substantiate the pathogenic role for ANCA, an animal model that closely resembles human pauci-immune ANCA-induced vasculitis is required. Recently, Brouwer et al. reported that pauci-immune glomerulonephritis and vasculitis were produced using a model in which kidney of rats immunized with human myeloperoxidase (MPO) were perfused with lysosomal enzyme extract containing MPO, protease 3 (PR3) and elastase in combination with H2O2, and suggested that this could serve as a model of ANCA-induced disease [4]. We sought to repeat these studies in spontaneously hypertensive rats (SHR) and Brown Norway rats (BNR). The evidence presented here demonstrates that rats immunized with human MPO and perfused with MPO + H2O2 with or without neutral protease, or MPO alone, develop an immune complex-mediated proliferative glomerulonephritis rather than a pauci-immune necrotizing glomerulonephritis.

MATERIALS AND METHODS

Isolation of granule protein
Human polymorphonuclear leucocytes (PMN) were obtained
from leukaemic human donors. Blood was drawn into a heparinized container and the erythrocytes sedimented with 'Plasmagel' (Cellular Products, Buffalo, NY). The PMN were separated with a Ficoll–Hypaque gradient (Sigma, St Louis MO) and the remaining erythrocytes removed with hypotonic lysis. PMN were suspended in 0.34 M sucrose and homogenized at 4°C with a homogenizer. The homogenate was centrifuged at 200 g for 10 min at 4°C and the supernatant was removed and centrifuged at 8700 g for 20 min at 4°C. The pellet containing the mixed granule fraction was extracted with 0.2 M sodium acetate buffer pH 4.0 containing 10 mM calcium chloride, sonicated and then centrifuged at 100,000 g for 1 h at 4°C. The supernatant fluid contained granule protein [5,6].

Isolation of neutral protease and myeloperoxidase

The granule protein was chromatographed on FPLC Superose 12 gel filtration column, HR 16/50 (Pharmacia, Uppsala, Sweden), equilibrated with degassed, filter-sterilized 0.2 M sodium acetate buffer pH 4.0. The neutral proteases used in these studies were obtained from the peak of molecular weight approximately 29 Kd. When this peak was applied to a Mono S cation exchange column, HR 5/5 (Pharmacia, Uppsala, Sweden), with 0.08 M citrate phosphate buffer pH 4.0, PR3, elastase and cathepsin G were separable by a linear salt gradient from 0 to 1-2 M NaCl. These neutral proteases were identified by ELISA using polyclonal and monoclonal antibodies to elastase, PR3 and cathepsin G.

The peak of molecular weight approximately 140 kD from Superose 12 Column containing MPO activity was applied to FPLC Mono S column, HR 5/5 with 0.02 M sodium acetate buffer pH 5.0, and MPO was isolated by a linear salt gradient from 0 to 1 M NaCl. The peaks that contained MPO activity were pooled, dialysed extensively against PBS pH 7.4, and stored frozen at −70°C [6,7]. MPO activity was measured spectrophotometrically by using 4-aminophenazinium as the hydrogen donor. Activity was recorded as an increase in A510 per minute. One unit was defined as a change in absorbance of 1/0 optical density unit per minute at 510 nm [7,8]. The purity of MPO isolated by our laboratory was documented by an optical density A430/A280 ratio of 0.73, activity of 192 U/mg protein, and showed one band of molecular weight approximately 140 kD on SDS-gel electrophoresis. The recovery of MPO was 78% for Superose 12 column and 91% for Mono S column.

Immunization of rats

Spontaneously hypertensive male rats (n = 34) weighing 150–200 g obtained from Harlan Inc. (Madison, WI) and BN male rats (n = 39) weighing 75–150 g obtained from Charles River, Inc. (Raleigh, NC) were fed ad libitum with standard chow (Granville Milling, Creedmoor, NC). Twenty-eight SHR and 33 BNR were immunized with human MPO emulsified with TiterMax (CytRx, Inc. Norcross, GA). Each rat was injected with 25 μg MPO emulsion intramuscularly. Six SHR and six BNR remained non-immunized. Anti-MPO antibodies were detectable by indirect immunofluorescence microscopy and ELISA 1 and 2 months after perfusion.

Detection of anti-myeloperoxidase by ELISA

Costar high-binding type II microtitre strips (Costar, Inc. Cambridge, MA) were coated overnight with human MPO (Calbiochem, La Jolla, CA) at a protein concentration of 12.5 μg/ml in 50 mm sodium acetate, 100 mm NaCl buffer at pH 6.0. The strips were blocked with PBS containing 0.2% normal goat serum and 0.05% Tween 20 at pH 7.4. The strips were then incubated for 1 h at room temperature with rat serum diluted 1:100 in the blocking buffer. Antibodies were detected with an alkaline phosphatase-conjugated goat anti-rat immunoglobulin (heavy and light chain specific) adsorbed against human serum proteins (Tago Inc., Camarillo, CA) followed with an alkaline phosphatase substrate kit (Biorad, Inc., Hercules, CA). The optical density was read at 405 nm, and a positive optical density value of 0.5 was arbitrarily chosen. Serum from a normal rat was used as a negative control, and a highly reactive serum was used as a positive control to monitor reproducibility of the assay.

Detection of anti-myeloperoxidase antibodies by indirect immunofluorescence microscopy

Commercially prepared slides coated with human PMN (INOVA Diagnostics, Inc., San Diego, CA) were incubated with a 1:10 screening dilution of rat serum in sterile-filtered PBS for 30 min in a humid chamber. The slides were then soaked in two washes of PBS for 10 min each. A secondary antibody, fluorescein-conjugated affinity-purified goat anti-rat IgG (heavy and light chain specific) (Jackson Immunoresearch, Inc., West Grove, PA) was diluted to 1:320 and applied to the slides for 20 min. The slides were soaked in fresh PBS to remove unbound antibody, coverslipped, and examined with a fluorescence microscope. Sera from non-immunized rats were used as negative controls.

Measurement of blood pressure

Rats were anaesthetized with sodium phenobarbital 60 mg/kg intraperitoneally. The right femoral artery was cannulated (PE-50 tubing) and mean arterial blood pressure was measured with a Statham p23 Db pressure transducer (Statham Instruments, Oxnard, CA). The tubing was removed, the right femoral artery was ligated, and muscle and skin were closed.

Perfusion of rat kidney

Animals were used for experiments when circulating anti-MPO antibodies were detectable by both indirect immunofluorescence microscopy and ELISA. A moderate to intense perineural staining pattern on indirect immunofluorescence microscopy was consistently observed from rat serum diluted 1:10. Similarly, rats were used when significant anti-MPO antibodies were detectable on ELISA using MPO as the target antigen, and the rat serum diluted 1:100 (optical density 0.96±0.35 (mean ± s.d.)).

The abdominal cavities of rats were exposed through a midline incision. In all studies, the left kidney was isolated and perfused according to a modification of the method of Hoyer et al. [9]. For perfusion, the left spermatic, adrenal vessels and lumbar arteries were ligated with 5–0 silk. Clamps were placed across the aorta above and below the left renal artery and a 30 G needle was entered into the aorta below the renal artery. PBS pH 7.5 was perfused until the kidney and renal vein became pale. Then the left renal vein was closed by a clamp and a hole was punctured in the anterior wall of the left renal vein. The left kidney was perfused in the closed, blood-free circuit with either MPO (100 μg) + neutral protease (100 μg) + 2 ml 10−3 M H2O2,
MPO+H₂O₂, MPO, or H₂O₂ alone. Finally, 2 ml PBS were perfused. The rate of perfusion was 0.5 ml/min. After perfusion, the needle and the clamps were removed, and the bleeding was stopped with gelfoam (Upjohn, Inc., Kalamazoo, MI). Total ischaemia time was less than 10 min. The abdomen was closed in two layers with 5–0 sutures. In each animal, the right kidney served as the non-perfused control.

**Histological examination by light microscopy**
Rats were killed on day 4 or day 10 after perfusion. Specimens from both kidneys were obtained and prepared for light microscopy and immunofluorescence microscopy. For light microscopy, renal tissue was fixed in B5/10% formaldehyde and embedded in paraffin. Sections (2–3 μm) were cut and stained with haematoxylin–eosin and examined by light microscopy. Pathological lesions were scored as follows: 0 = no lesion; 1 = mesangial hypercellularity only; 2 = focal inflammatory glomerular injury without necrosis or crescents; 3 = diffuse inflammatory glomerular injury without necrosis or crescents; 4 = severe inflammatory glomerular injury with necrosis and/or crescents.

**Immunopathological examination by immunofluorescence microscopy**
For direct immunofluorescence microscopy, renal tissue was embedded in cryomolds containing O.C.T. Compound (Miles, Elkhart, IN) and frozen at −20°C. Then, 2–4 μm sections were cut and adhered to microscope slides coated with 3-aminopropyltriethoxysilane/acetone. To detect immunoglobulin and complement (C), direct immunofluorescence was performed using FITC-conjugated sheep antibodies to rat IgG, IgM, IgA and C3 (The Binding Site, San Diego, CA). To detect MPO, the sections were incubated with 1:200 monoclonal mouse anti-human MPO (Dako, Carpinteria, CA) for 30 min, then soaked in PBS and counterstained with fluorescein-conjugated goat anti-mouse IgG + IgM (heavy and light chain specific) (Jackson Immunoresearch). The amount of deposition was estimated on a semi-quantitative scale:

![Fig. 1. Light microscopy of the non-perfused right kidney (a) and perfused left kidney (b and c) of a rat with circulating myeloperoxidase (MPO) antibodies examined 4 days after perfusion of the left kidney with MPO and H₂O₂. The photomicrograph of the right kidney (a) shows no histologic abnormalities, whereas that of the left kidney (b) reveals necrosis and crescent formation in the glomerulus and thickening of capillary walls and mesangial matrix expansion in the other, and (c) reveals proliferative glomerulitis, which is the most common lesion in this experiment model. (Haematoxylin and eosin stain.)](image1)

![Fig. 2. Photomicrograph of arteritis with fibrinoid necrosis in the left kidney of a rat with circulating myeloperoxidase (MPO) antibodies examined 4 days after perfusion of the kidneys with MPO and H₂O₂. (Haematoxylin and eosin stain.)](image2)
IC glomerulonephritis in rats induced by MPO

RESULTS

Histopathology
In those kidneys that developed histologic changes, there was a proliferative glomerulonephritis that ranged in severity from mild mesangial hypercellularity (1+ injury) to severe inflammation with focal necrosis and occasional crescent formation (4+ injury) (Fig. 1). Several kidneys also had segmental necrotizing vasculitis affecting arterioles and small arteries (Fig. 2). There was focal tubulointerstitial injury commensurate with this glomerular injury.

The semiquantification of pathologic lesions after left

Statistical analysis
Analysis of variance for unbalanced data was used to test the overall effect of rat strains, perfusion materials, and days to sacrifice. Terms were tested for interaction. The least-squares means test was used to test the differences between rats and perfusion materials, while controlling for the other variables in the model. A Bonferroni correction was used for the P value to control for multiple testing. A prespecified cut-off of significance was set at $P < 0.006$.

0 = absent; 1 = weak; 2 = moderate; 3 = moderately intense; 4 = intense.
kidney perfusion in SHR and BNR is shown in Tables 1 and 2, and Fig. 4. By light microscopy, pathologic lesions were found in MPO-immunized rats perfused with MPO + H₂O₂ with or without neutral protease or MPO alone in both rat strains. Lesions were observed on both day 4 and day 10. In immunized SHR, 83% of kidneys revealed pathologic injury and the average severity was 2.7 ± 1.7 on day 4, and 100% of kidneys revealed pathologic injury and the average severity was 2.8 ± 1.2 on day 10 in the rats perfused with MPO combined with H₂O₂. Glomerular injury was 71% and 1.7 ± 1.4 on day 4, and 86% and 2.1 ± 1.5 on day 10 in the rats perfused with MPO alone. In immunized BNR, 100% of kidneys revealed pathologic injury and average severity was 2.7 ± 0.5 on day 10 in the rats perfused with MPO + neutral protease + H₂O₂. Glomerular injury was 67% and 1.6 ± 1.4 on day 4, and 100% and 1.8 ± 1.0 on day 10 in the rats perfused with MPO combined with H₂O₂, and was 17% and 0.2 ± 0.4 on day 4 and 67% and 0.8 ± 0.8 on day 10 in the rats perfused with MPO alone.

SHR had more damage than BNR. The difference between the two strains of rat perfused by MPO with H₂O₂ on day 4 was statistically significant (Table 3). The lesions in rats perfused with MPO + H₂O₂ with or without neutral protease appeared to be more severe than those in rats perfused with MPO alone, but the difference was not statistically significant.

No pathological lesion was observed in immunized rats perfused with H₂O₂. Lesions were observed in about half of the non-immunized rats perfused with MPO + H₂O₂. The scores were 0.8 ± 1.2 in SHR and 1.0 ± 1.0 in BNR. No pathologic lesion was observed in any of the non-perfused right kidneys.

**Immunofluorescence**

The semiquantification of immune deposition after left kidney perfusion in SHR and BNR is shown in Tables 1 and 2, and Fig. 5. By immunofluorescence microscopy, immunized rats

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**Fig. 3.** Direct immunofluorescence microscopy of glomeruli from a rat immunized with myeloperoxidase (MPO). The left kidney was perfused with MPO + H₂O₂ + neutral protease, and the right kidney was not perfused. After 10 days, the kidneys were removed for examination. The perfused kidney demonstrates global capillary wall staining with fluoresceinated antibodies specific for rat IgG (a), rat IgM (b), rat C3 (c) and human MPO (d). There is trace to no staining of glomeruli in the non-perfused control kidney with antisera specific for rat IgG (e), rat IgM (f), rat C3 (g) and human MPO (h).

**Fig. 4.** Score of pathologic lesions in left kidney after perfusion. ▲, Spontaneously hypertensive rats; ○, Brown Norway rats. The order from left to right is immunized rats perfused with myeloperoxidase (MPO) + H₂O₂ + neutral protease (NP), MPO + H₂O₂, MPO, H₂O₂ and non-immunized rats perfused with MPO + H₂O₂, respectively.

**Fig. 5.** Score of IgG deposition in left kidney after perfusion. ▲, Spontaneously hypertensive rats; ○, Brown Norway rats. The order from left to right is immunized rats perfused with myeloperoxidase (MPO) + H₂O₂ + neutral protease (NP), MPO + H₂O₂, MPO, H₂O₂ and non-immunized rats perfused with MPO + H₂O₂, respectively.
deposits of perfused MPO + H₂O₂ with or without neutral protease and MPO alone (Fig. 3c). The average of scores ranged from 0.4 to 2.1 in SHR, and from 0.6 to 2.0 in BNR. No C3 deposit was observed in immunized rats perfused with H₂O₂, in non-immunized rats perfused with MPO + H₂O₂, nor in non-perfused right kidneys (Fig. 3g).

Weak, linear to granular basement membrane deposits of MPO were observed in immunized rats perfused with MPO + H₂O₂ with or without neutral protease and MPO alone (Fig. 3d). No MPO deposit was found in immunized rats perfused with H₂O₂, in non-immunized rats perfused with MPO + H₂O₂, nor in non-perfused right kidney (Fig. 3h).

No or low intensity deposits of IgM and IgA were observed in these rats (Fig. 3b, f).

There was no statistical difference in immune deposition between different strains of rat, perfusion materials or days to sacrifice.

**The relation between pathologic lesions and immune deposition**

In immunized rats, 93% of the glomeruli that developed proliferative glomerulonephritis had IgG depositions, and 88% of them had C3 depositions. The IgG immune deposits were proportional in intensity to the degree of histologic injury in both rat strains (Fig. 6).

**Blood pressure**

The blood pressure was 76.5 ± 11.1 mmHg (60–92 mmHg) in BNR before perfusion; however, it was 122.0 ± 26.3 mmHg (86–160 mmHg) in SHR. The values were significantly different (P < 0.005) (Table 3).

**DISCUSSION**

Necrotizing crescentic glomerulonephritis can be divided into three categories based on immunofluorescence microscopy: (i) immune complex-mediated glomerulonephritis; (ii) anti-glomerular basement membrane-mediated glomerulonephritis; and (iii) pauci-immune necrotizing glomerulonephritis [10]. There are several experimental models of both immune complex and anti-glomerular basement membrane glomerulonephritis [11]. To date, however, there is no animal model of pauci-immune necrotizing glomerulonephritis similar to that found in humans with systemic vasculitis. The development of such a model will allow a better understanding of this most common

**Table 1. Pathologic lesions and immune deposits in spontaneously hypertensive rats**

<table>
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<tr>
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<th>Perfused</th>
<th>MPO + H₂O₂</th>
<th>MPO</th>
<th>H₂O₂</th>
<th>None</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sacrificed</td>
<td>Day 4</td>
<td>Day 10</td>
<td>Day 4</td>
<td>Day 10</td>
</tr>
<tr>
<td>LM</td>
<td></td>
<td>2.7 ± 1.7</td>
<td>2.8 ± 1.2</td>
<td>1.7 ± 1.4</td>
<td>2.1 ± 1.5</td>
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<tr>
<td>IgG</td>
<td></td>
<td>1.5 ± 0.8</td>
<td>1.3 ± 0.7</td>
<td>1.2 ± 1.1</td>
<td>2.5 ± 1.0</td>
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<tr>
<td>C3</td>
<td></td>
<td>0.8 ± 0.6</td>
<td>0.9 ± 0.6</td>
<td>0.4 ± 0.4</td>
<td>2.1 ± 1.0</td>
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<tr>
<td>MPO</td>
<td></td>
<td>0.3 ± 0.4</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.3</td>
<td>0.3 ± 0.4</td>
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<td>(n = 6)</td>
<td></td>
<td>(n = 6)</td>
<td>(n = 7)</td>
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Values are mean ± s.d. MPO, Myeloperoxidase; LM, light microscopy.
form of human aggressive glomerulonephritis, and will allow elucidation of the underlying pathogenic mechanism. Recently, Brouwer et al. reported an animal model of proliferative glomerulonephritis that was distinguished by the presence of anti-MPO antibodies and by the paucity of immune complexes after perfusion of the left kidney with lysosomal enzyme extract containing MPO, PR3 and elastase in combination with hydrogen peroxide in rats previously immunized with MPO. These authors suggested that this was a model of ANCA-induced disease. Our study was performed in an attempt to duplicate the model described by Brouwer et al. Our study reproduced the antigen, route of administration, method of perfusion, perfusion materials, and days to sacrifice of the Brouwer study. Brouwer et al. found immune deposits along the basement membrane at 4 h and 24 h, but at the time of maximal inflammation, 4 and 10 days after perfusion, IgG, C3 and MPO could no longer be detected. Unfortunately, we could not corroborate the results of Brouwer et al. When we duplicated the experiments of Brouwer et al., the immunohistologic observations indicated induction of a model of in situ immune complex-mediated disease. In our hands, the linear and granular staining along the glomerular basement membrane did not dissipate by 10 days. This model does not resemble the human form of ANCA-associated glomerulonephritis, which is a pauci-immune necrotizing and crescentic glomerulonephritis and is characterized by a lack of immunoglobulin deposition at the time of fulminant glomerulonephritis [12,13].

Our study indicates that this model is an in situ immune complex glomerulonephritis, which does not come as a surprise. Myeloperoxidase is an extremely cationic protein with an isoelectric point of more than 10 [14]. Several studies have demonstrated that there is glomerular localization of neutrophil and platelet-derived cationic proteins in glomerulonephritis [15,16]. Studies by Johnson et al. reported that very small quantities of MPO were capable of localizing along the glomerular capillary wall. In their model, no demonstrable injury was associated with MPO localization alone until there was exposure to non-toxic concentrations of hydrogen peroxide; at that time, severe glomerular damage was observed [17]. It was suggested that the MPO–hydrogen peroxide–halide system caused glomerular injury as a mechanism for glomerulonephritis. Our observations indicate that MPO binds to glomerular basement membranes, where it acts as a nidus for in situ immune complex formation with circulating anti-MPO antibodies in rats that have previously been immunized with MPO. In non-immunized rats, the perfusion with MPO and hydrogen peroxide into left kidneys resulted in mild glomerular injury in one-half of the rats. This would support the contention by Johnson et al. that the MPO–hydrogen peroxide–halide system is capable of inducing a neutrophil-mediated glomerulonephritis in rats. Johnson et al. have shown that when infusing microgram quantities of elastase or cathepsin G into renal arteries of rats, both localized to the glomerular capillary wall and mediated glomerular injury [18].

It is not clear why our results differ from those Brouwer et al. In our model, the glomerular lesion is made worse in animals that are hypertensive (spontaneously hypertensive rats). Despite the changes in the aggressiveness of the proliferative glomerulonephritis, immune complex deposition was the same in both Brown Norway and spontaneously hypertensive rats.

Table 3. The relation between pathologic lesions and blood pressure in rats immunized with myeloperoxidase (MPO)

<table>
<thead>
<tr>
<th>Perfused</th>
<th>MPO + H2O2</th>
<th>MPO</th>
</tr>
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<td>Sacrificed</td>
<td>Day 4</td>
<td>Day 10</td>
</tr>
<tr>
<td>SHR</td>
<td>2.7 ± 1.7*</td>
<td>2.8 ± 1.2</td>
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<tr>
<td>BNR</td>
<td>1.6 ± 1.4</td>
<td>1.8 ± 1.0</td>
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</table>

*P < 0.005.

Values are mean ± s.d. SHR, spontaneously hypertensive rats; BNR, Brown Norway rats.
In conclusion, the model described in this study is not an animal model of anti-neutrophil cytoplasmic antibody-associated pauci-immune necrotizing glomerulonephritis. Further efforts must be directed at developing such a model, either by manipulating this model or by developing a model in a different animal system.

REFERENCES


