Nephrotoxic Serum-Induced Nephritis in Wistar-Kyoto Rats: A Model to Evaluate Antinephritic Agents

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ABSTRACT—We investigated nephrotoxic serum (NTS)-induced glomerulonephritis in Wistar-Kyoto (WKY) rats as a model to evaluate antinephritic agents. WKY rats required only a small amount of NTS to induce crescentic glomerulonephritis and the rats progressively lost their renal function in a few weeks. In a comparative study with WKY and Sprague-Dawley (SD) rats, WKY rats showed a normal distribution pattern in the severity of proteinuria with a small variance. While SD rats needed a much higher amount of NTS to exhibit a comparable proteinuria which was not normal and had a large variance. The effects of clinically available antinephritic drugs, methylprednisolone, cyclophosphamide and cyclosporin A, were studied in both strains. In WKY rats, these drugs significantly inhibited the proteinuria, glomerular histological changes and decrease in creatinine clearance. On the other hand, such significant inhibitory effects on proteinuria were not observed with any of these drugs in SD rats. In conclusion, NTS nephritis in WKY rats may prove to be a useful model for studying antinephritic agents.

Keywords: Glomerulonephritis, Nephrotoxic serum, Wistar-Kyoto rat, Antinephritic agent, Proteinuria

It is well understood that the development of a certain type of human glomerulonephritis is associated with various immunological reactions such as deposition of the immune complex in the glomerulus, activation of the platelet and complement, and leukocyte migration into the glomerulus (1). Therefore, powerful therapeutic drugs such as corticosteroids and immunosuppressants are needed for the treatment of this disease. These drugs, however, have limitations because of the serious adverse effects (2–5), and development of innovative drugs is anticipated in this area. The pharmacological evaluation of antinephritic agents using animal models has been difficult. First of all, nephritis is a disease involving numerous components, such as those described above, that contribute to the disease state. Second, nephritis is a chronic disease, and it takes a long period of time to assess drug efficacy in animal models. Third, although there are many procedures to induce nephritis in animals, considerable variation in severity exists among individual animals in each procedure. The lack of good animal models with high reproducibility and low variation may account for the difficulty in evaluating antinephritic agents.

Nephrotoxic serum (NTS)-induced nephritis in rats is a basic experimental model involving immune processes (6). It has been used to study the cause and developing mechanisms of glomerulonephritis (7) and to evaluate antinephritic effects of various agents (8–11). Some investigators, however, have reported that clinically available drugs, such as corticosteroids and immunosuppressants, do not show significant antinephritic effects on this model (9, 10). This led us to suspect that NTS nephritis might not always allow precise evaluation for antinephritic agents. Kawasaki et al. (12) have reported that the Wistar-Kyoto (WKY) rats given a dose of NTS subnephritogenic to other strains, such as Sprague-Dawley (SD), Lewis and SHR, showed severe proliferative and necrotizing glomerulonephritis with crescent formation. In our preliminary study, we tried to produce NTS-induced nephritis in seven rat strains, Brown Norway (BN), Lewis, SD, SHR, SHRSP, Wistar and WKY. As in the previous report (12), only WKY rats with 25 μl/kg of NTS developed glomerulonephritis without the heterologous phase. The other strains all needed more than 600 μl/kg of NTS to exhibit a significant proteinuria and showed both heterologous and autologous phases. These results suggested that the pathologic patterns and the underlying mechanisms of NTS nephritis in WKY rats might

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have some properties different from those in other rat strains.

In the present study, we used WKY and SD rats to produce NTS nephritis and examined differences in proteinuria between both the strains. In addition, we also investigated the effects of clinically available drugs on NTS nephritis in WKY and SD rats. We chose SD rats as the reference strain because this strain has been widely recognized as the global standard and used various examinations including experimental nephritis. We used methylprednisolone (MP), cyclophosphamide (CP) and cyclosporin A (CsA) as the standard corticosteroid, cytotoxic agent and immnosuppressant, respectively, to study their antinephritic effects.

MATERIALS AND METHODS

Animals

Seven-week-old male WKY and SD rats (Charles River Japan, Inc., Kanagawa) were used. They were fed with standard rat chow and given free access to water.

Drugs

MP, CsA and CP were purchased from Sigma (St. Louis, MO, USA), Sandoz (Basel, Switzerland) and Nacalai Tesque (Kyoto), respectively.

Nephrotoxic serum

Glomerular basement membrane (GBM) from WKY rats was prepared by the method of Spiro (13). Ten Japanese white rabbits weighing 2.0–2.3 kg were immunized subcutaneously with 10 mg each of the GBM emulsified in an equal volume of complete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA). Boosters of the same immunogen were given in the 2nd, 4th and 6th weeks after the initial immunization. Sera were prepared from the blood drawn on the 10th day after the last booster. Antisera were heat-decomplemented and stored at −80°C until use. Antisera against SD rat GBM were also prepared by the same method.

Comparative study of urinary protein excretion in WKY and SD rats

A dose-response study of NTS nephritis was carried out in both strains (data not shown). The average of proteinuria obtained at 50-fold diluted NTS in WKY rats and at twofold diluted NTS in SD rats were comparable, but their distributions seemed not to be. We, therefore, studied the frequency distribution of proteinuria in both WKY and SD rats. Sixty WKY rats and 22 SD rats were injected with 2.5 ml/kg of NTS against their respective GBM diluted with saline 50-fold and twofold, respectively. On day 8, 24-hr urine samples were collected in individual metabolic cages and urinary protein excretion was determined.

Drug effects on NTS nephritis in WKY and SD rats

In this experiment, we used NTS prepared from SD rat GBM. WKY rats were divided into the following eight groups: Normal group, Control group, MP groups at doses of 1 or 3 mg/kg, CsA groups at doses of 10 or 30 mg/kg and CP groups at doses of 1 or 5 mg/kg. All groups except for the normal group consisted of six rats. All rats except for the normal rats were injected with 50-fold diluted NTS at a volume of 2.5 ml/kg on day 0. Four normal rats were injected with the same volume of saline.

SD rats were divided into the following five groups: Normal group, Control group, MP group at a dose of 3 mg/kg, CsA group at a dose of 30 mg/kg and CP group at a dose of 5 mg/kg. All groups consisted of six rats. All rats except the normal rats were injected with non-diluted NTS at a volume dose of 2.5 ml/kg on day 0. The other protocols were the same as those used for WKY rats.

Each drug was suspended in purified water with Tween 80 and orally administered daily at a volume of 10 ml/kg. Normal and control rats were daily given the same volume of vehicle. Twenty-four-hour urinary samples were collected in metabolic cages on days 4, 8 and 14 in WKY rats and on days 1, 4, 8 and 14 in SD rats. On the last day of the experiment, blood samples were obtained from the abdominal aorta under pentobarbital anesthesia (50 mg/kg, i.p.), and centrifuged to separate the serum. Each kidney specimen was fixed in buffered formalin and stained with the periodic acid-Schiff reagent for light-microscopic examination.

Measurements

Urinary protein excretion was determined by the sulphasalicylic acid method. Serum concentrations of urea nitrogen, albumin and cholesterol were measured by routine methods using an autoanalyzer (type 705; Hitachi, Tokyo). Serum and urinary creatinine concentrations were determined by the enzymatic method using the autoanalyzer. Creatinine clearance (Ccr) was calculated from urinary and serum creatinine concentration and urine volume. Serum anti-rabbit IgG antibody titers were determined by the sandwich-ELISA technique.

Statistical analyses

Data were shown as means ± S.E.M. Student’s t-test was used for comparison of means between normal and control. Analysis of variance (ANOVA) followed by Dunnett’s test was used to calculate statistical significance from the control values.
RESULTS

Comparative study of urinary protein excretion in WKY and SD rats

As shown in Fig. 1, the frequency distribution of proteinuria on day 8 in 60 WKY rats with 50-fold diluted NTS (2.5 ml/kg) was nearly normal. In contrast, the proteinuria of 22 SD rats injected with twofold diluted NTS (2.5 ml/kg) did not show a normal distribution, and the animals were divided into the following two groups: one showed proteinuria with protein excretion less than 55 mg/day and the other showed protein excretion higher

![Graph A](image1)

![Graph B](image2)

**Fig. 1.** Frequency plots of the level of proteinuria in NTS nephritis in WKY (A) and SD (B) rats. Sixty WKY rats were injected with 2.5 ml/kg of 50-fold diluted NTS and 22 SD rats were injected with 2.5 ml/kg of twofold diluted NTS. The amount of urinary protein excretion per 24 hr was determined on day 8 after NTS injection.

![Graph C](image3)

**Fig. 2.** Effects of methylprednisolone (MP), cyclophosphamide (CP) and cyclosporin A (CsA) on proteinuria in NTS nephritis in WKY rats. Fifty-fold diluted NTS at 2.5 ml/kg was injected into all groups of rats except for the normal group (n=4). **P < 0.01 and *P < 0.05, compared with the normal group by the t-test. **P < 0.01 and *P < 0.05, compared with the corresponding control by Dunnett’s test. []: normal, ■: control, □: MP (1 mg/kg), ◼: MP (3 mg/kg), □: CP (1 mg/kg), □: CP (5 mg/kg), ■: CsA (3 mg/kg), ◼: CsA (10 mg/kg).
<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Cholesterol (mg/dl)</th>
<th>Albumin (g/dl)</th>
<th>Urea nitrogen (mg/dl)</th>
<th>IgG titer (OD&lt;sub&gt;450&lt;/sub&gt;)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>83 ± 3</td>
<td>3.90 ± 0.04</td>
<td>20.8 ± 0.5</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Control</td>
<td>175 ± 8†</td>
<td>2.35 ± 0.05&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>39.3 ± 3.6&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>1.76 ± 0.14&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>100 ± 5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3.02 ± 0.07&lt;sup&gt;**&lt;/sup&gt;</td>
<td>18.5 ± 1.1&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.48 ± 0.14&lt;sup&gt;**&lt;/sup&gt;</td>
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<td></td>
<td>66 ± 2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3.77 ± 0.10&lt;sup&gt;**&lt;/sup&gt;</td>
<td>19.0 ± 0.5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.33 ± 0.16&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>172 ± 14</td>
<td>2.47 ± 0.18</td>
<td>27.0 ± 6.8</td>
<td>1.26 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>97 ± 11&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3.30 ± 0.11&lt;sup&gt;**&lt;/sup&gt;</td>
<td>16.9 ± 2.1&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.55 ± 0.12&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>159 ± 7</td>
<td>2.62 ± 0.05&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25.4 ± 2.7</td>
<td>1.55 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>83 ± 3&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3.63 ± 0.03&lt;sup&gt;**&lt;/sup&gt;</td>
<td>20.5 ± 0.8&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.72 ± 0.11&lt;sup&gt;**&lt;/sup&gt;</td>
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Vehicle or a drug was orally administered once a day from day 0 to 13. Each data value represents the mean ± S.E.M. of 6 animals except for the normal group (n = 4) on day 14. <sup>‡</sup>P < 0.01, compared with the normal group by the t-test. <sup>**</sup>P < 0.01 and <sup>*</sup>P < 0.05, compared with the control by Dunnell's test.

than 130 mg/day. The mean, S.D. and coefficient of variation (C.V.) of protein excretion for WKY rats were 79.9 mg/day, 18.2 mg/day and 22.7%, respectively; and those for SD rats were 108.3 mg/day, 95.3 mg/day and 88.0%, respectively.

Drug effects on NTS nephritis in WKY and SD rats

Since the first experiment showed that SD rats consist of a considerable proportion of non-responders to NTS at the dose used, we used a two times higher dose for SD rats in this experiment. In WKY rats, MP at 1 and 3 mg/kg, CP at 5 mg/kg and CsA at 10 mg/kg significantly inhibited proteinuria on days 4, 8 and 14 (Fig. 2). On day 14, these drug dosages also significantly inhibited the increase in serum cholesterol and urea nitrogen concentration, and the decrease in serum albumin concentration, which were observed in the control animals. The anti-rabbit IgG titer was significantly inhibited by CP at 5 mg/kg or CsA at 10 mg/kg (Table 1). Ccr in control rats significantly decreased to approximately 45% that of normal rats during two weeks. CP and CsA at these doses significantly prevented the deterioration of Ccr and MP tended to improve it (Fig. 3A). Marked crescent formation was diffusely observed in the control rats. Treatment with each drug, at the doses exhibiting an antiproteinuric effect, also inhibited these crescentic proliferative glomerular lesions (Fig. 4). Although SD rats with NTS nephritis also showed clear proteinuria, hypercholesterolemia and hypoalbuminemia, they did not show a significant decrease in Ccr and detectable glomerular lesions.

![Fig. 3](image-url)

Fig. 3. Effects of methylprednisolone (MP), cyclophosphamide (CP) and cyclosporin A (CsA) on creatinine clearance (Ccr) in NTS nephritis in WKY (A) and SD (B) rats. WKY and SD rats were injected with 2.5 ml/kg of 50-fold diluted and non-diluted NTS, respectively. Vehicle or a drug was orally administered once a day from day 0 to 13. Each column represents the mean ± S.E.M. of 6 animals except for the normal group of WKY (n = 4). <sup>‡</sup>P < 0.01, compared with the normal group by the t-test. <sup>**</sup>P < 0.01, compared with the control by Dunnell's test. NS, not significant. , normal, ■: control, : MP (1 mg/kg), : MP (3 mg/kg), : CP (1 mg/kg), : CP (5 mg/kg), : CsA (3 mg/kg), : CsA (10 mg/kg).
The treatment with MP, CP and CsA, at doses high enough to exhibit antinephritic action in WKY rats, showed no inhibitory effects on the nephrotic symptoms. The anti-rabbit IgG titer, however, was inhibited by these drugs (Figs. 3B and 5 and Table 2).

The rats showed favorable body weight gains during the experiment, although the body weight did not increase in the MP-treated groups in either strain (data not shown).

DISCUSSION

In this study, we proved the antinephritic effects of three typical clinically available antinephritic agents against NTS nephritis in WKY rats. All drugs inhibited the pathological changes and prevented the decrease in Ccr in this model. In contrast, with NTS nephritis in SD rats, none of these drugs showed any antinephritic effects at the dose effective in WKY rats. However, these drugs did show some immunosuppressive effects because they significantly decreased the anti-rabbit IgG titer. Development of the NTS-induced nephritis model has been generally believed to involve the following two phases (7): a heterologous phase where the immunoreactions occur due to the binding of the exogeneous anti-GBM antibody to the host's GBM and an autologous phase where the reactions take place due to the host's antibody binding to the anti-GBM antibody. We speculate that SD rats with NTS nephritis have a clear heterologous phase, and GBM gets damaged so quickly and severely in this period that these drugs might not exert a protective effect. We confirmed that WKY rats injected with NTS did not show proteinuria on day 1 which was heterologous phase. In Lewis rats with NTS nephritis, which also showed both

Fig. 4. Light micrographs of glomeruli from WKY rats with NTS nephritis on day 14 after NTS injection. Glomeruli from normal rats (a). Severe proliferative glomerular lesions with crescent formation (arrows) were globally observed in the glomeruli from control rats (b). The glomeruli from the rats treated with 3 mg/kg of methylprednisolone (c), 5 mg/kg of cyclophosphamide (d) or 10 mg/kg of cyclosporin A (e) showed little of those histological changes.
heterologous and autologous phases such as in SD rats, neither MP nor CP could inhibit the proteinuria (data not shown). These observations support the speculation.

Why WKY rats given NTS easily show the characteristic symptoms is not clear. In our therapeutic experiment, we used NTS prepared from the GBM of SD rats. However, the severity of glomerulonephritis and the drug effects were almost the same when we injected NTS, prepared from the GBM of WKY rats, to WKY or SD rats. This observation suggests that the distinctive symptoms of NTS-induced glomerulonephritis in WKY rats are attributable to the differences of the immune system between WKY and other strains. Several reports have demonstrated the strain-related differences in the severity of proteinuria or pathologic responses exist in rat glomerulonephritis (14–16). WKY rats have higher levels of natural killer (NK) cell activity and antibody-dependent cell-mediated cytotoxicity than BN rats (17). Furthermore, CD8-positive NK cells and monocytes/macrophages play important roles in the development of NTS-induced glomerulonephritis in WKY rats (12, 18, 19). On the other hand, Lelongt et al. (15) have reported that the SD rats with the administration of anti-heparan sulfate-proteoglycan antibodies showed the least proteinuric response among several rat strains. Furthermore, SD rats with nephritis showed the increase in anti-rabbit IgG titer, but the level was lower than WKY rats (Tables 2 and 3). These observations suggest that the immune responsiveness of SD rats may be rather lower than those in other strains and that the susceptibility of WKY rats to NTS and characteristic symptoms depend on the immunogenetic background, which may be due to the increased activity of cell-mediated immunity of this strain.

In the course of the experiments, we observed not only strain differences but also individual differences in each rat strain in the severity of nephritis. Marked individual differences often make it difficult to analyze the results. Therefore, we compared the individual differences in the

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**Table 2. Effects of methylprednisolone, cyclophosphamide and cyclosporin A on serum cholesterol, albumin and urea nitrogen concentration, and anti-rabbit IgG titer in SD rats with NTS nephritis**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Cholesterol (mg/dl)</th>
<th>Albumin (g/dl)</th>
<th>Urea nitrogen (mg/dl)</th>
<th>IgG titer (OD$_{405}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>69±5</td>
<td>3.77±0.05</td>
<td>22.1±2.0</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>145±12#</td>
<td>2.83±0.09#</td>
<td>20.4±1.0</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>3</td>
<td>196±28</td>
<td>2.22±0.10</td>
<td>25.7±2.2</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>5</td>
<td>148±22</td>
<td>3.01±0.15</td>
<td>23.2±2.0</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>10</td>
<td>139±17</td>
<td>3.05±0.09</td>
<td>26.7±1.3</td>
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</table>

Vehicle or a drug was orally administered once a day from day 0 to 13. Each data value represents the mean±S.E.M. of 6 animals on day 14. #P<0.01, compared with the normal group by the t-test. **P<0.01, compared with the control by Dunnett's test.
severity of proteinuria between WKY and SD rats that differed in susceptibility to NTS. The results demonstrated that levels of proteinuria in the WKY rats injected with NTS showed a normal distribution pattern with a smaller variance than in the SD rats. The large variance of proteinuria in SD rats may be due to the genetic heterogeneity of the animals, because SD rats are raised in a closed colony, yet marked individual differences were observed among rats. In contrast, WKY rats are an inbred strain that is limited in genetic individual differences. In the preliminary study, C.V. for the severity of proteinuria in inbred Lewis rats was approximately 10%. In contrast, that in Wistar rats, which came from closed colony strains, was more than 35%. In a previous report, MP depressed protein excretion more than 40% in SD rats with NTS nephritis, but no statistical significance was detected (20). The large variance of proteinuria in SD rats would make it difficult to obtain a statistically significant result. In the present study of NTS nephritis in WKY rats, more than 30% inhibition of urinary protein excretion was detected with statistical significance. It is suggested that NTS nephritis in WKY rats may provide a more reliable model for evaluating the efficacy of antinephritic agents.

In conclusion, this is the first report that the drugs used for human nephritis apparently show antinephritic effects in NTS nephritis in WKY rats. This model has proved to be a useful evaluating system for studying antinephritic agents because of the following reasons: 1) NTS nephritis in WKY rats requires only a small amount of NTS to induce severe necrotizing nephritis. This enables us to carry out a large series of experiments with the same NTS preparation. 2) The severity of nephritis assessed by proteinuria showed a normal distribution pattern with a small C.V. 3) The prominent histological manifestations, such as crescent formation, make it easy to assess the disease states by microscopic observation. 4) The current clinically available antinephritic agents, MP, CP and CsA, were all effective in this model.

Acknowledgments

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