Studies on Antinephritic Effect of TJ-8014, a New Japanese Herbal Medicine (4): Effects on Accelerated Passive Heymann Nephritis in Rats

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Accepted July 31, 1990

Abstract—We investigated the antinephritic effect of TJ-8014, in comparison to dipyridamole, on accelerated passive Heymann nephritis in rats. TJ-8014 (4.0 g/kg/day, p.o.) given from the heterologous phase (from the day of injection of the antiserum against Fx1A) markedly inhibited the urinary protein excretion and the elevation of plasma cholesterol levels as well as glomerular histopathological changes. When the treatment was started from the autologous phase (from the 22nd day) after proteinuria was fully developed, TJ-8014 also showed a beneficial effect. Dipyridamole (0.4 g/kg/day, p.o.) had no effect when the treatment was started either from the heterologous or autologous phase. TJ-8014 decreased glomerular rat IgG and rat C3 deposits, although it affected neither the plasma antibody titer against rabbit γ-globulin nor the plasma complement level. TJ-8014 markedly prevented the reduction of plasma and adrenal corticosterone level as well as the reduction of renal blood flow of rats with nephritis. These results suggest that TJ-8014 may be a useful drug against idiopathic membranous nephropathy and the beneficial effect of this drug may be caused by the elimination of glomerular immune deposits and C3 through the increase in renal blood flow related to the enhanced release of adrenal corticosterone.

TJ-8014 is a new Japanese herbal medicine prepared by extracting and lyophilizing a mixture of eight crude drugs consisting of Bupleuri radix, Pinelliae tuber, Scutellariae radix, Glycyrrhizae radix, Zizyphi fructus, Ginseng radix, Coptidis rhizoma and Hoelen. We have already reported that TJ-8014 is markedly effective in preventing urinary protein excretion as well as glomerular histopathological changes in original-type and crescentic-type anti-glomerular basement membrane (anti-GBM) nephritis in rats, which resemble mild proliferative glomerulonephritis and rapidly progressive glomerulonephritis in humans, respectively (1, 2).

Of the glomerular diseases other than proliferative glomerulonephritis, idiopathic membranous nephropathy (MN) is characterized by diffuse GBM thickening with spike formation without the proliferation of glomerular cells as well as nephrotic syndrome such as heavy proteinuria and hypercholesterolemia (3). There are no drugs that have a clear effect on human idiopathic MN. Although steroids and immunosuppressive agents have been often used for the treatment of various types of human glomerulonephritis, both types of agents have only a slight effect on human idiopathic MN.

Recently, we established an experimental model of accelerated passive Heymann nephritis (APHN), which closely simulates human idiopathic MN, by i.v. injections of rabbit antiserum against an antigen from the brush border of the proximal tubules of rat kidney (Fx1A antigen) following immunization of rats with rabbit γ-globulin in Freund’s complete adjuvant (FCA) (4). This model is
more similar to human idiopathic MN, compared to passive Heymann nephritis (PHN) induced in rats only by i.v. injection of rabbit antiserum against Fx1A antigen.

The purpose of the present investigation was to evaluate the antinephritic effect of TJ-8014, as compared to dipyridamole, an antiplatelet agent, on APHN in rats. The second aim of the present investigation was to clarify the mechanisms of the antinephritic effect of this herbal medicine. For this purpose, we investigated the effects of TJ-8014 on plasma antibody titer against rabbit \( \gamma \)-globulin, plasma complement level, the deposition of rat IgG and rat C3 in the GBM and renal blood flow.

**Materials and Methods**

**Animals:** Male Sprague-Dawley strain SPF rats (Nihon SLC, Shizuoka), weighing approx. 160 g, were used in the experiments. These animals were housed in an air-conditioned room at 23±1 °C during the experimental periods.

**Drugs:** Drugs used were TJ-8014 [a lyophilized extract] (Tsumura Co., Ltd., Tokyo) and dipyridamole (Boehringer Ingelheim West Germany). These drugs were suspended in 1% gum arabic.

**Induction of APHN:** APHN was induced in rats by injecting them with 1.0 ml of rabbit antiserum directed to Fx1A antigen (antiserum) into the tail vein once a day for 2 days starting from the day after injection of 6.5 mg rabbit \( \gamma \)-globulin in FCA into the hind foot pads in accordance with the method reported previously (4).

In this experiment, the effects of test drugs were examined by administering from the heterologous (the day of the antiserum injection) and autologous (the 22nd day after the antiserum injection) phases. In the experiments in which the drugs were administered from the heterologous phase, the animals were then divided into 6 groups (n=8), so that the average body weight of each group was at approx. the same level. After grouping, the animals of 5 groups were immunized with rabbit \( \gamma \)-G in FCA, following the antiserum injection. Four groups were given 0.5, 2.0 and 4.0 g/kg of TJ-8014 and 0.4 g/kg of dipyridamole, respectively, in a volume of 1 ml per 100 g of body weight, orally, daily from the day of antiserum injection (the 1st day) to the 39th day. The remaining group was orally given the vehicle (1% gum arabic) instead of test drugs and served as the control. In addition, a non-treated (normal) group (n=8) was used for comparison with nephritic groups.

In the experiments in which the drugs were administered from the autologous phase, the nephritic rats were divided into 4 groups (n=8), so that the average urinary protein excretion of each group was at same level. Three groups were orally given 2.0 and 4.0 g/kg of TJ-8014 and 0.4 g/kg of dipyridamole, respectively, daily from the 22nd day to the 53rd day. The remaining group was given only the vehicle as the control. In addition, a non-treated (normal) group (n=8) was used in the experiment. Evaluation of the antinephritic effect of test drugs was done by comparing biochemical parameters such as urinary protein, plasma cholesterol and protein contents and histopathological parameters in the glomeruli of the test drug-treated group with those of the control group.

**Urine and blood collections:** The 24 hr-urine samples were obtained by keeping each animal in an individual metabolic cage for 24 hr at various intervals after the induction of nephritis. At the beginning of the urine collection, each animal received 8 ml of distilled water orally without feeding. The urine was then centrifuged at 3,000 rpm for 15 min at 4°C, and the supernatant was used for the determination of protein. Blood samples were also obtained at various intervals after the induction of nephritis. In this case, each 0.4 ml of blood was drawn from the tail vein of conscious animals with a disposable microsyringe and put into a tube containing 4.5 \( \mu \)mol of EDTA·2Na. The blood was centrifuged at 5,000 rpm at 4°C to obtain the plasma for the determination of cholesterol, urea nitrogen and antibody titer against rabbit \( \gamma \)-G. Immediately after the last collection of urine samples, blood was also taken from the renal vein for the measurement of platelet aggregation.

**Measurements of urinary protein, and plasma cholesterol and plasma protein concentration:** The urinary protein content was
determined by the method of Kingsbury et al. (5) and expressed as mg/24 hr urine. The plasma cholesterol and protein concentration were determined in accordance with the methods of Zurkowski (6) and Kingsbury et al. (5), respectively, and expressed as mg/dl plasma.

Assessment of histopathological parameters in glomeruli: For light microscopic study, kidneys were fixed in alcohol and then the tissues embedded in paraffin were cut into 2-3-μm thick sections. The sections were stained with periodic acid-methenamine silver for observing the thickening of GBM and the spike formation in the GBM. For assessing each histopathological parameter, the degree of thickening and spike formation was scored as 1 (mild), 2 (moderate), and 3 (severe) in accordance with typical examples of the degree as shown in Fig. 1. The number of glomeruli corresponding to each score was represented as n₁, n₂ and n₃. The thickening (TI) and the spike formations index (SI) were calculated by the following formula: TI and SI=1×n₁+2×n₂+3×n₃. All the above experiments were performed "blindly" on coded sections (4).

Measurements of plasma antibody titer, plasma complement CH₅₀ level and deposition of rat IgG and rat C₃ in glomeruli: The plasma antibody titer against rabbit γ-G was determined by indirect hemagglutination using sensitized sheep red blood cells (7). The plasma complement CH₅₀ level was determined by the method of Mayer (8).

Immunofluorescent (FI) studies: The kidneys were flash frozen in liquid nitrogen and stored at -70°C until processed. Cryostat sections were cut, stained and studied with the fluorescein-conjugated IgG fractions of monospecific antiserum to rat IgG and rat C₃ (Cappel Laboratories, Inc., Cochraneville, Pennsylvania U.S.A.). The intensity of fluorescence was evaluated semiquantitatively by the following scoring system: 0, indistinguishable from normal; 1+, present but faint; 2+, definite; 3+, strongly positive; 4+, maximally positive. (9)

Measurements of serum and adrenal corticosterone levels: The corticosterone levels in serum and adrenal glands were measured as reported previously (10).

Measurement of renal blood flow: The renal blood flow was determined by hydrogen gas clearance methods (Biomedical Science Co., Ltd., Model RBF-2).

Statistical analysis: The data represent the mean±S.D., and the results were statistically evaluated by analysis of variance, Student’s t-test and Mann-Whitney’s U-test.

Results
1. The antinephritic effect of TJ-8014 administered from the heterologous phase
Fig. 2. Effects of TJ-8014 and dipyridamole administered from the heterologous (A) and autologous (B) phases on urinary protein excretion in APHN in rats. Each plot denotes the mean±S.D. of 8 rats. The number in parentheses indicates a percent inhibition which is derived from the following formula: 
\[
\frac{C-T}{C-N} \times 100 \quad (C: \text{Control}, \ T: \text{Test drug}, \ N: \text{Normal}).
\]
* indicates a significant difference from the control at \(P<0.05\).

Fig. 3. Effects of TJ-8014 and dipyridamole administered from the heterologous (A) and autologous (B) phases on serum cholesterol content in APHN in rats. Each number denotes the mean±S.D. of 8 rats. The number in parentheses indicates a percent inhibition which is derived from the following formula: 
\[
\frac{C-T}{C-N} \times 100 \quad (C: \text{Control}, \ T: \text{Test drug}, \ N: \text{Normal}).
\]
* indicates a significant difference from the control at \(P<0.05\).
Urinary protein excretion (Fig. 2A): When test drugs were given from the day of the antiserum injection (the 1st day) to the 40th day, TJ-8014 at 4.0 g/kg/day, p.o. inhibited the urinary protein excretion by 63–70% through the 21st day to the 40th day. However, TJ-8014 at lower doses (0.5 and 2.0 g/kg/day, p.o.) had only a tendency to inhibit the protein excretion. Dipyridamole at 0.4 g/kg/day, p.o. had no apparent effect.

Plasma cholesterol (Fig. 3A) and protein contents: On the 29th and 43rd days, TJ-8014 at 4.0 g/kg/day, p.o. inhibited the elevation in the plasma cholesterol level by 83–110%. On the 40th day, the decrease in the plasma protein content was significantly inhibited by 2.0 and 4.0 g/kg/day, p.o. of TJ-8014 (Normal: 7.6±1.8 mg/dl; Control: 6.7±1.3 mg/dl; TJ-8014, 2.0 g/kg/day, p.o.: 8.7±0.8 mg/dl; TJ-8014, 4.0 g/kg/day, p.o.: 8.8±1.3 mg/dl; P<0.05, TJ-treated group vs. control). TJ-8014 at 0.5 g/kg/day, p.o. and

\[
\frac{C-T}{C} \times 100 \quad (C: \text{Control}, \ T: \text{Test drug}).
\]

* indicates a significant difference from the control at P<0.05.
dipyridamole at 0.4 g/kg/day, p.o. were ineffective in inhibiting the decrease in the protein content.

Histopathological parameters in glomeruli (Fig. 4A): Histopathological observations of the glomeruli on the 41st day indicated that TJ-8014 at 4.0 g/kg/day, p.o. markedly reduced the SI and the TI by 42% and 36%, respectively. In addition, at 2.0 g/kg/day, p.o., the drug also inhibited the SI by 29%.

2. The antinephritic effect of TJ-8014 administered from the autologous phase

Urinary protein excretion (Fig. 2B): When test drugs were given from the 22nd day to the 45th day, TJ-8014 at 4.0 g/kg/day, p.o. significantly inhibited the urinary protein excretion by 85% on the 32nd day.

Plasma cholesterol content (Fig. 3B): TJ-8014 given at 4.0 g/kg/day, p.o. significantly inhibited the elevation of the plasma cholesterol level by 80% on the 27th day.

Histopathological parameters in glomeruli (Fig. 4B): On the 53rd day, TJ-8014 at 4.0 g/kg/day, p.o. reduced only the SI, by 30%.

3. The mechanisms of the antinephritic actions of TJ-8014

Serum and adrenal corticosterone levels (Fig. 5): The serum corticosterone level of normal rats was 0.4–0.5 μg/ml through the 5th day to the 35th day. However, the level of nephritic control rats was significantly lower (60–80%) than that of normal rats for the same period. TJ-8014 at 4.0 g/kg/day, p.o. inhibited the decrease in the corticosterone level by 20–40%.

The adrenal corticosterone level of the nephritic control was approx. 10%–80% lower than the normal level throughout the experimental periods of the 5th to 35th day. The decrease in the adrenal corticosterone level was inhibited by TJ-8014 at 4.0 g/kg/day, p.o. by 50–120%. On the other hand, the corticosterone level in the serum and adrenal glands were decreased to no detectable level by the treatment with dexamethasone (0.3 mg/kg/day, p.o.).

Renal blood flow (Fig. 6): The renal blood flow (RBF) of the nephritic control rats on the 43rd day after antiserum injection decreased to 60% that of normal rats. TJ-8014 at 4.0 g/kg, p.o. significantly inhibited the decrease in the RBF by 57%. Dipyridamole at 0.4 g/kg, p.o. did not affect the RBF.

Plasma antibody titer against rabbit γ-G and plasma complement CH50 level (data not shown): The plasma antibody titer against rabbit γ-G determined on the 41st day after antiserum injection, was 9.8±0.92 to the Nth
power in nephritic control rats, although the antibody titer could not be detected in the plasma of the normal rats. On the other hand, the plasma complement CH50 levels were 48% lower than that of the corresponding normal rats, when the levels were determined on the

Fig. 6. Effects of TJ-8014, dipyridamole and corticosterone on renal blood flow of rats with APHN. The renal blood flow of nephritic rats on the 43rd day after the antiserum injection was determined 2 hr after each test drug was given. Each column denotes the mean±S.D. of 6 to 8 rats. * indicate a significant difference from the control at P<0.05.

Fig. 7. Effects of TJ-8014 and dipyridamole administered from the heterologous phase on deposition of rat IgG and rat C3 in APHN in rats. Each column denotes the mean of 8 rats. The intensity indicates the intensity of fluorescence for rat IgG and rat C3. ** and *** indicate a significant difference from the control at P<0.01 and 0.001, respectively.
35th day. Neither the antibody titer nor the complement CH50 levels were affected by any doses of TJ-8014.

Deposition of rat IgG and rat C3 in glomeruli (Fig. 7): Although in the sections of the normal rats, no fluorescent staining rat IgG and rat C3 could be detected on any day after the antiserum injection, in those taken from nephritic control rats, granular deposits of rat IgG and rat C3 were observed along the GBM from the 15th day onwards. The deposition of rat IgG and rat C3 was significantly weaker in the sections from TJ-8014 (4.0 g/kg/day, p.o.)-treated-rats than in those from control rats. Representative immunofluorescent micrographs for rat IgG and rat C3 in the glomeruli from TJ-8014-treated and control rats are given in Fig. 8. Dipyridamole (0.4 g/kg/day, p.o.) did not affect the deposition of both immunopathological parameters.

Platelet aggregation in vivo (data not shown): TJ-8014 (4.0 g/kg/day, p.o.) and dipyridamole (0.4 g/kg/day, p.o.) significantly inhibited the increase in the platelet aggregation on the 35th day (normal, 7.5±2.9 %; control, 11.8±3.2 %; 4.0 g/kg/day TJ-8014, 4.7±3.3 %; 0.4 g/kg/day dipyridamole, 6.8±1.8 %).

![Rat IgG](A) ![Rat C3](C)

![Rat IgG](B) ![Rat C3](D)

**Fig. 8.** Immunofluorescent micrographs for rat IgG and rat C3 in glomeruli of rats treated with TJ-8014 at 4.0 g/kg/day, p.o. (B, D) and control (A, C) rats on the 35th day after antiserum injection.
Discussion

In the present study, the glomerular injury of APHN in rats used as an experimental animal model for the idiopathic MN has been considered to be initiated by immunological reactions consisting of two phases (1). Namely, immediately after the injection of rabbit antiserum against FxIA antigen (antiserum), the reaction in the heterologous phase is caused by immediate fixation of the injected heterologous antibody to glomerular epithelial cell antigen, that is, \textit{in situ} immune complex formation. From approx. 10 days after the first \textit{in situ} immune complex formation in the heterologous phase, the reaction in the antologous phase is caused by the interaction of the host antibody against the injected heterologous antibody with prefixing heterologous \(\gamma\)-globulin on the surface of the epithelial cells, that is, the second \textit{in situ} immune complex formation. In the APHN model, there was no apparent proteinuria in the heterologous phase in contrast with the anti-GBM nephritic model, which induces proteinuria from the day after the anti-GBM serum injection and the proteinuria appeared in the autologous phase.

The present study demonstrated that TJ-8014 (4.0 g/kg/day, p.o.) given from the heterologous phase (from the day of the antiserum injection) prior to the onset of proteinuria was markedly effective in preventing the development of proteinuria and hypercholesterolemia as well as histopathological changes such as the thickening of GBM and spike formation. According to Feenstra et al. (11), immunosuppressive agents (i.e., azathioprine), which have been widely used for the treatment of glomerulonephritis with steroids, had a clear effect on PHN in rats, only when administered before or simultaneously with the onset of the heterologous phase. However, at present, even when the treatment was started from the autologous phase (from the 22nd day after the antiserum injection) after proteinuria had been fully developed, TJ-8014 (4.0 g/kg/day, p.o.) was also effective in remitting the disease. Dipyridamole (0.4 g/kg/day, p.o.), an antiplatelet agent, showed no beneficial effect by administrating either from the heterologous or autologous phase.

Recently, the terminal C5b-9 membrane attack complex (MAC) of complement has received a great deal of attention as a direct mediator of glomerular injury of PHN in rats and idiopathic MN in humans (12, 13). Namely, in the PHN, the \textit{in situ} immune complex formation on the subepithelial cells in the autologous phase may cause the activation of complement with the MAC formation which mediates the increase in glomerular permeability. The present experiment on the mechanisms of the antinephritic action of TJ-8014 indicated that this medicine given from the heterologous phase reduced granular deposits of rat IgG and rat C\(_3\) along the GBM on the 15th and 35th days after the antiserum injection. Therefore, the antinephritic action of TJ-8014 on rat APHN may be related to the reduction of deposits of host antibody and MAC of complements on the membrane of glomerular epithelial cells during the autologous phase. However, TJ-8014 affected neither the plasma antibody titer against rabbit \(\gamma\)-globulin on the 41st day nor the plasma complement level (CH\(_{50}\)) on the 15th and 35th days. Therefore, the decrease in glomerular rat IgG and rat C\(_3\) deposits by TJ-8014 does not seem to be due to the inhibition of the host antibody formation against rabbit \(\gamma\)-globulin during the autologous phase.

The renal blood flow (RBF) of rats with APHN on the 43rd day after the antiserum injection decreased to 60\% that of the normal rats. The formation of the C5b-9 in rat PHN has been shown to stimulate the production of arachidonic acid, prostaglandin F\(_{2\alpha}\) (PGF\(_{2\alpha}\)) and thromboxane A\(_2\) (TXA\(_2\)) (14). The reduction of the RBF of rats with APHN is probably due to the increase in glomerular synthesis of prostanoids such as PGF\(_{2\alpha}\) and TXA\(_2\) that induce vasoconstriction (15, 16). At the present time, TJ-8014, given as a single p.o.-dose of 4.0 g/kg, inhibited the decrease in the RBF by 57\%. The increase in the reduced RBF by TJ-8014 may contribute to the elimination of glomerular rat IgG and rat C\(_3\) deposits. In rat APHN, a marked reduction of serum and adrenal corticosterone levels was observed through the experimental period of the 5th to the 35th day. TJ-8014 (4.0 g/kg/day, p.o.) given from the heterologous phase markedly prevented the reduc-
tion of the serum and adrenal corticosterone levels. In addition, corticosterone (10 mg/kg, p.o.), like TJ-8014, prevented the decrease in the RBF in rats with APHN. In this connection, chronic administration of pharmacological doses of glucocorticoids such as cortisone, hydrocortisone and prednisolone has been indicated to increase glomerular filtration rate in dog, rat and man (17–19). It is postulated from these results that TJ-8014 may exert its antinephritic action by eliminating the glomerular subepithelial immune deposits and MAC through the increase in the RBF mediated by endogenous corticosterone.

In the present study, dipyridamole, an antiplatelet agent, was ineffective on rat APHN, although it was found to have a beneficial effect on the original-type and crescentic-type anti-GBM nephritis in rats (9). Accordingly, intraglomerular platelet aggregation does not seem to play an important role in the initiation and progression of the APHN in contrast to the original-type and crescentic-type anti-GBM nephritis.

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