Studies on Antinephritic Effects of Plant Components (3):
Effect of Pachyman, a Main Component of *Porzia Cocos* Wolf on Original-Type Anti-GBM Nephritis in Rats and Its Mechanisms

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ABSTRACT — The antinephritic effect of pachyman on original-type anti-GBM nephritis in rats was investigated. Pachyman was given to original-type anti-GBM nephritic rats for 10 days from the day of anti-GBM serum injection. Pachyman prevented urinary protein excretion and the elevation of serum cholesterol content. Histopathological observations of the glomeruli indicated that although the number of nuclei and adhesion to capillary walls of Bowman’s capsule in nephritic control rats were significantly increased, pachyman reduced the degree of histopathological changes such as hypercellularity and adhesion as compared to the control group. Although the serum complement CH50 ratio in control group was significantly lower than that in the normal group, the decrease in serum complement CH50 was inhibited by pachyman, and rat C3 deposition in the glomeruli in the pachyman-treated group was significantly reduced. These results suggest that pachyman was effective against original-type anti-GBM nephritis in rats and that the antinephritic mechanisms of pachyman may be partly due to the inhibitory action of this agent on C3 deposition in the glomeruli.

Keywords: Anti-GBM nephritis, Pachyman, Bowman’s capsule, Serum complement CH50, C3 Deposition

It is well-known that *Hoelen* (*Bukuryou* in Japanese) is a crude drug, which is extracted from *Porzia cocos* Wolf (Polyaceae) and is included in many Japanese herbal medicines. Pachyman, consisting of a β-(1-3)-linked glucan with a small amount of β-(1-6)-linked branching glucan, makes up about 90% of the contents of *Hoelen* (1). Chihara et al. reported that pachyman may exert an antitumour action (1). Moreover, it has been hypothesized that polysaccharides having antitumor action or anti-complement action may potentiate the disposal actions of the circulating immune complex or the immune complex deposits in some organs (2). These findings suggest that pachyman, one of these polysaccharides, may be effective against autoimmune diseases.

On the other hand, it is believed that the immune complex deposition in the glomeruli is an essential determinant of the development of glomerulonephritis (3, 4). It has been reported that the in situ immune complex formation in subendothelial cells in the glomeruli is associated with the development of anti-GBM nephritis in rats (5). Moreover, we previously reported that the immunosuppressive agents mizoribin and azathioprine marked-down inhibited urinary protein excretion in experimental glomerulonephritis in rats (6). Recently, we demonstrated that TJ-8014 and Chai-Ling-Tang (*Saireito* in Japanese), two Japanese herbal medicines, were effective in immune-mediated experimental glomerulonephritis (7–13), and that *Hoelen* extract, one of the crude drugs which constitutes TJ-8014 and Chai-Ling-Tang, also improved urinary protein excretion as well as histopathological alteration in the glomeruli in original-type anti-GBM nephritis in rats (7). The overall purpose of the present study was to elucidate the antinephritic effect of pachyman, the main component of *Hoelen*, on original-type anti-GBM nephritis in rats. Furthermore, we tried to confirm the antinephritic mechanisms of pachyman. For these pur-
poses, we investigated the effects of pachyman on serum complement CH50, rat C3 and rabbit IgG deposition in glomeruli by immunohistochemical methods.

MATERIALS AND METHODS

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

Drugs
The chemical structure of pachyman (Calbiochem Co., La Jolla, CA) is shown in Fig. 1. This authentic component obtained from Poria cocus Wolf (Bukuryou in Japanese), has a molecular weight of 50,000–270,000 according to light scattering methods and a purity of 99%.

Induction of original-type anti-GBM nephritis
Original-type anti-GBM nephritis was induced in rats by injecting 0.75 ml of rabbit anti-rat GBM serum (anti-GBM serum) into their tail veins, as described previously (14).

Evaluation of antinephritic effects of the drug
Rats were divided into 5 groups, so that the average body weight of all groups was the same (Normal: 155.4 ± 9.3 g; Control: 155.0 ± 2.2 g; Pachyman, 5 mg/kg: 154.9 ± 4.0 g, 10 mg/kg: 155.4 ± 6.0 g, 20 mg/kg: 160.0 ± 6.4 g). Test drugs were given to each group i.p. daily in a volume of 0.25 ml/100 g of body weight from 2 hr after i.v.-injection of anti-GBM serum (the 0 day) to the 10th day. One group of nephritic rats served as the nephritic control and was given i.p. only the vehicle (saline). In addition, a non-treated (normal) group of 8 rats was used for comparison to the nephritic groups. On the 11th day, blood was drawn and the kidneys were taken. Evaluation of the antinephritic effect of test drugs was done by comparing biochemical parameters such as urinary protein excretion and plasma cholesterol content and histopathological parameters in the kidneys of the pachyman-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 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 10 min at 4°C, and the supernatant was used for the determination of protein. Immediately after the urine collection, 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 μmol of EDTA-2Na. The blood was centrifuged at 5,000 rpm at 4°C to obtain plasma for the determination of serum cholesterol.

Determinations of urinary protein and serum cholesterol contents
The urinary protein excretion was determined by the method of Kingsbury et al. (15) and expressed as mg/24 hr urine. The cholesterol content was determined in accordance with the method of Allain et al. (16) and expressed as mg/dl serum.

Assessment of histopathological parameters
For light microscopic study, kidneys were dehydrated and fixed by immersing the tissues stepwise into various concentrations of ethyl alcohol from low to high. The tissues were then embedded in paraffin and sectioned into 2- to 3-μm-thick slices. The sections were stained with hematoxylin and eosin and Masson's trichrome. The number of nuclei (hypercellularity) and adhesion to Bowman's capsule of capillary walls (adhesion) in the glomeruli were observed under light microscopy (Fig. 2). For assessing hypercellularity, an equatorial cross section was selected by random sampling methods. The number of nuclei (including nuclei from glomerular cells and exudate leukocytes) was counted and expressed as the mean number per equatorial cross section in 10 glomeruli/animal. For assessing adhesion (an index in hyperproliferation of glomerular cells), 25 glomeruli per section were observed, and the appearance rate of adhesion was expressed as the percentage of glomeruli exhibiting adhesion per 25 glomeruli. All the above experiments were performed "blindly" on the coded section (1).
Measurements of serum complement CH$_{50}$ level and deposition of rabbit IgG and rat C$_3$ in glomeruli

To evaluate the in vivo effects of pachyman, the agent was given i.p. daily to groups of 5 rats after antisem injection. The control group of 5 rats was given i.p. the vehicle (saline) instead of test agents. The animals in the pachyman-treated and control groups had blood taken from the renal vein and left kidney on the 1st, 3rd and 5th days, after anti-GBM serum injection.

Plasma complement CH$_{50}$ level and immunoperoxidase technique

The plasma complement CH$_{50}$ level was determined by the method of Mayer (17). The paraffin sections were cut as described above, and the sections were washed in chilled 0.01 M phosphate buffered saline (PBS) pH 7.4. Immunohistochemistry was performed using a Vectastain ABC kit (Vector Burlingame CA). The specimens were then incubated with anti-rat polyclonal C$_3$ mouse antibody and anti-rabbit IgG mouse polyclonal antibody (Cappel West Chester PA) at a dilution of 1:100 for 90 min. The sections were washed again with PBS and incubated with avidin-conjugated anti-mouse IgG antibody and then incubated with biotinated-alkaline phosphatase or peroxidase after washing with PBS. After washing with PBS, the final reaction was achieved by incubating the sections with vector red (Vector Burlingame CA) or 3,3'-diaminobenzidine and substrate (0.5 mg/ml Tris-HCl buffer, pH 7.6, containing 0.01% H$_2$O$_2$). The total area of immunoreactive cat C$_3$ and rabbit IgG per glomeruli equatorial cross section was measured for 25 glomeruli per section using Image analysis (Toyobo V-1, Tokyo) and presented as pixel/cross section. At the same time, we observed the hypercellularity and adhesion in the glomeruli as histopathological alterations.

Statistical analysis

The data represent the mean ± S.D., and the results were statistically evaluated by ANOVA, Student's t-test and Mann-Whitney's U-test. Inhibitory percentage was calculated as follows:

\[
\text{Inhibitory percentage (\%)} = \frac{\text{Control} - \text{Test drugs}}{\text{Control} - \text{Normal}} \times 100
\]
RESULTS

Effect of pachyman on original-type anti-GBM nephritis

Urinary protein excretion (Fig. 3): The urinary protein excretion in the control group was approx. 250 mg/day throughout the experimental period. Pachyman at 20 mg/kg/day, i.p. reduced the urinary protein excretion by the 5th day and the 10th day by 52% and 50%, respectively. Pachyman at 10 mg/kg/day, i.p. also reduced the urinary protein excretion by the 10th day.

Serum cholesterol content (Fig. 4): By the 11th day, serum cholesterol content in the control was markedly increased, as compared to the normal rats. In contrast, pachyman at 10 and 20 mg/kg/day, i.p. significantly inhibited the elevation in serum cholesterol content by the 11th day by 42% and 49%, respectively.

Histopathological observation (Fig. 5): Histopathological observations of the glomeruli on the 11th day indicated that the number of nuclei and the incidence of adhesion in glomeruli had significantly increased in the control group. Hypercellularity and adhesion were suppressed in the pachyman-treated group by 49% to 124%, as compared to the control.

Effect of pachyman on complement activity and histopathological changes in original-type anti-GBM nephritis

Serum complement activity (CH50) and deposition of rat C3 in glomeruli (Fig. 6): The serum complement CH50 in the nephritic control was markedly lower than that of the normal rats through the experimental periods. The decreased serum complement CH50 rate by the 1st and 3rd days was inhibited in the case of pachyman at 10 and 20 mg/kg/day, i.p. by 67% to 105%. We employed immunohistochemical methods to

![Fig. 3. Effect of pachyman on urinary protein excretion in original-type anti-GBM nephritis in rats. Test drugs were given i.p. daily during the period from the day (0 day) of anti-GBM serum injection to the 11th day. Each plot denotes the mean ± S.D. of 8 rats. The number in parentheses indicates inhibitory percentage. Normal: ○; control: ●; pachyman, 5 mg/kg: ■; pachyman, 10 mg/kg: ▲; pachyman, 20 mg/kg: □. ** and *** indicate a significant difference from the control at P < 0.05 and 0.01, respectively.](image)

![Fig. 4. Effect of pachyman on serum cholesterol content in original-type anti-GBM nephritis in rats. The number in parentheses indicates inhibitory percentage. A: Normal; B: Control; C: pachyman, 5 mg/kg; D: pachyman, 10 mg/kg; E: pachyman, 20 mg/kg. * indicates a significant difference from the control at P < 0.05. For other details, see the legend to Fig. 3.](image)
Fig. 5. Effects of pachyman on histopathological changes in the glomeruli in original-type anti-GBM nephritis in rats. The value for the adhesion of the normal group is almost 0. The number in parentheses indicates inhibitory percentage. A: Normal; B: Control; C: pachyman, 5 mg/kg; D: pachyman, 10 mg/kg; E: pachyman, 20 mg/kg. *** indicates a significant difference from the control at $P < 0.001$. For other details, see the legend to Fig. 3.

Fig. 6. Effect of pachyman on serum complement activity (CH50) and deposition of C3 in the glomeruli in original-type anti-GBM nephritis in rats. Test drugs were given i.p. daily during the period from the day (the 0 day) of anti-GBM serum injection to the 5th day. Each plot and column denote the mean ± S.D. of 5 rats. The number in parentheses indicates inhibitory percentage. Normal: (A); control: (B); pachyman, 5 mg/kg: (C); pachyman, 10 mg/kg: (D); pachyman, 20 mg/kg: (E). *, ** and *** indicate a significant difference from the control at $P < 0.05$, 0.01 and 0.001, respectively.

Observe the effect of pachyman on rat complement C3 deposition in the glomeruli (Fig. 6). The rat C3 deposits in the glomeruli were already observed on the 1st day and were still present on the 10th day after i.v. injection of anti-GBM serum (Fig. 7). In contrast, pachyman at 20 mg/kg/day, i.p. prevented the deposition of rat C3 in the glomeruli by the 3rd day.

Time course study of histopathological parameters in the glomeruli (Fig. 8): Pachyman at 5, 10 and 20 mg/kg/day, i.p. markedly inhibited the incidence of adhesion throughout the 1st day to the 5th day by 40% to 70%. However, hypercellularity in the glomeruli was inhibited in the pachyman (20 mg/kg)-treated group by 93%, as well as rabbit IgG deposition in the glomeruli by 27% to 50% by the 5th day.

DISCUSSION

The glomerular lesions of anti-GBM nephritis induced by injection of anti-GBM antibody are caused by acute inflammation with immediate deposition of the injected antibody together with complement along the GBM, followed by accumulation of polymorphonuclear granulocytes in the capillary vessels. Recently, there has been considerable evidences that the complement system is an important mediator of immune-mediated
Fig. 7. Micrographs determined by immunohistochemical method for rat C₃ deposition in glomeruli of normal rats, control rats and rats treated with pachyman at 10 and 20 mg/kg/day, i.p. on the day 3 after antiserum injection.

Fig. 8. Effect of pachyman on histopathological parameters in the glomeruli in original-type anti-GBM nephritis in rats. The value for the adhesion and deposition of rabbit IgG in the glomeruli of the normal group is 0. The number in parentheses indicates inhibition percentage. A: Normal; B: Control; C: pachyman, 5 mg/kg; D: pachyman, 10 mg/kg; E: pachyman, 20 mg/kg. * and ** indicate a significant difference from the control at P < 0.05 and 0.01, respectively. For other references, see the legend to Fig. 5.
renal disease (18–21). Studies carried out primarily on models of anti-GBM antibody induced glomerulonephritis have elucidated an indirect role for the complement system which acts through chemotaxis (C5a) or immune adherence (C3b) mechanisms to attract circulating inflammatory cells that have been shown to be the principal mediators of tissue injury and proteinuria in these models. Moreover, Perkison et al. (19) demonstrated that the assembly of the complement membrane attack complex (MAC) also involves the pathogenesis of glomerular injury. The MAC has been shown to be a potent stimulus for production of several potential inflammatory mediators, including reactive oxygen species (22), prostaglandins (23), and interleukin-1-like cytokines (24), as well as collagen synthesis (25). It is believed that these mediators are associated with the increased permeability of GBM and hyperproliferation in the mesangial cell or extracellular matrix that is characteristic of original-type anti-GBM nephritis.

The present study demonstrated that the polysaccharide pachymann markedly reduced the level of urinary protein excretion and serum cholesterol content, as well as histopathological alterations in the glomeruli in original-type anti-GBM nephritis in rats. Furthermore, the present study demonstrated that the serum complement activity of nephritic rats was remarkably decreased immediately after the anti-GBM antibody injection, and rat complement C3 linear deposits along the GBM were clearly observed in the glomeruli by the 1st day after i.v.-injection of antiserum. In contrast, pachymann treatment prevented the decrease in serum complement activation (CH50), and deposition of rat C3 in the glomeruli before the urinary protein excretion was inhibited, although the rabbit IgG deposition and hypercellularity were inhibited by the 5th day. These results indicate that the antinephritic effect of pachymann on original-type anti-GBM nephritis may be due to the inhibitory effects on complement activation within the glomeruli by this agent. Furthermore, our data also showed that pachymann prevented the adhesion to capillary wall of Bawman’s capsule by day 1, suggesting that pachymann may inhibit renal injury by preventing overproliferation of the extracellular matrix mediated by complement activation in the glomeruli.

The detailed mechanisms of the anticomplement action of pachymann remain unclear. However, when pachymann was given to normal rats, we found no alterations of biochemical and histological parameters in the pachymann-treated normal group. Moreover, pachymann at 20 mg/kg did not alter the serum complement CH50 rate in the normal rats; and in the in vitro assay pachymann had very little effect on serum CH50 rate (less than 1 mg/ml) (unpublished data by T. Hattori et al.). These results indicate that some step of complement consumption, which is induced by anti-GBM antibody treatment, may be inhibited by pachymann. In addition, when lipopolysaccharide (LPS), bacterial endotoxin polysaccharide, at the dose of 250 µg/kg, i.p. was administered to anti-GBM nephritic rats, we observed that in the LPS-treated group, urinary protein excretion significantly increased and the number of nuclei within the glomeruli markedly increased, to a level higher than that in non-treated nephritic rats. It is well-known that among the polysaccharides, LPS inhibits the desposal action of the reticulo-endothelial system (2) and its active site is “lipid A”, which is bound to the polysaccharide. Accordingly, it is considered that the structure of the polysaccharide has an important role in its physiological action. The present results indicated that the deposition of rat C3 was not inhibited by the 5th day in the pachymann (20 mg/kg/day)-treated group. The other preliminary test demonstrated that oxygen-radical species-scavenging enzymes activities of isolated glomeruli were observed with pachymann treatment. These findings suggest that other mechanisms for the antinephritic action of pachymann may also exist.

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