Studies on the Antinephritic Effects of Plant Components (6): Antinephritic Effects and Mechanisms of Phellodendrine (OB-5) on Crescentic-Type Anti-GBM Nephritis in Rats (2)

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ABSTRACT—Effects of phellodendrine (OB-5) on crescentic-type anti-GBM nephritis in rats and the cell number of the various leukocyte subpopulations in the glomeruli of the nephritic rats were investigated. OB-5 at 25, 50 and 100 mg/kg/day, p.o. prevented the urinary protein excretion by the 19th day after i.v.-injection of anti-GBM serum. In the OB-5-treated rats, plasma cholesterol and creatinine contents were lower than those of the control rats throughout the 40-day experimental period. Histopathological observations demonstrated that OB-5 inhibited the incidence of crescent formation, adhesion and fibrinoid necrosis in the glomeruli by the 41st day. OB-5 did not affect the plasma antibody titer against rabbit gamma globulin. The increases in total leukocytes, macrophages, cytotoxic/suppressor T cells, Ia positive cells, and IL-2 receptor positive cells in the glomeruli in OB-5, 100 mg/kg-treated rats as well as those of the animals treated with azathioprine or cyclosporin A were lower than those of the anti-GBM nephritic control. These results indicate that OB-5 was effective in crescentic-type anti-GBM nephritis and the antinephritic mechanisms of this agent may be due to its ability to inhibit the proliferation or the migration of macrophages and cytotoxic T lymphocytes in the glomeruli.

Keywords: Phellodendrine, Crescent formation

Of the crude drugs, Phellodendri Cortex obtained from Phellodendron amurense Rupr (Rutaceae) has been prescribed in many Japanese herbal medicines, and it has been widely accepted that Phellodendri Cortex exerts stomachic effects. Recent investigations have demonstrated the innovative actions of components, which are included in Phellodendri Cortex. It has been demonstrated that components isolated from Phellodendri Cortex could exhibit the anti-inflammatory action against carrageenin-induced paw edema in rats (1).

Moreover, Koda (2) documented in mice that of the components, phellodendrine (OB-5) markedly inhibited the local graft versus host reaction (GvHR). It has been considered that its action of OB-5 may be mainly restricted to effector cells. These findings suggest that phellodendrine may have inhibitory effects against chronic inflammatory or delayed type hypersensitivity.

It has been generally accepted that the induction and development of glomerulonephritis is mediated by immunological events. In addition, the infiltration of various leukocyte subpopulations into the glomeruli has been observed, and human and animal studies have suggested that these cells are involved in the glomerular damages (3–5). Accordingly, there is much evidence indicating that local cellular immune activation may be associated with the immunopathogenesis of glomerulonephritis (3–6).

Original-type anti-GBM nephritis is characterized by moderate proteinuria and mild proliferation of glomerular cells. A previous study (7) demonstrated that OB-5 inhibited the urinary protein excretion in original-type anti-GBM nephritis. However, the antinephritic mechanisms of OB-5 on glomerulonephritis are still unclear.

To elucidate the antinephritic actions and mechanisms of OB-5 on glomerulonephritis, we investigated the effects of OB-5 on crescentic-type anti-GBM nephritis, which has more severe proteinuria and proliferation of glomerular cells than those of the original-type model (8). In addition, we undertook studies utilizing monoclonal antibody against total leukocytes (OX-1), macrophages (ED-1), cytotoxic/suppressor T cell (OX-8),
Ia positive cells (RT1B) and IL-2 positive cells (OX-39) to detect the effect of OB-5 on the number of these cells in the glomeruli of nephritic rats.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley strain rats, weighing approx. 160 g (Nihon SLC), were used for all experiments. They were housed in groups of four to five under a 12 hr light-dark cycle with food and water freely available.

Drugs
The chemical structure of phellodendrine (OB-5) (Tsumura Co., Ltd., Tokyo) is shown in Fig. 1. This component was synthesized by Tsumura Co., Ltd. The purity of OB-5 was verified by HPLC (Column: YMC-PACK A-312 S-5 120A ODS 150 mm; Mobile phase: CH3CN/PIC B-7/THF, 90:300:2; Detect Ex 292 nm; Em 622 Range 8) at Tsumura Co., Ltd. The OB-5 employed in these experiments had a purity of greater than 99%. OB-5 was dissolved in distilled water. Cyclosporin A (Sandoz, Germany) and azathioprine (Sigma, St. Louis, MO) were also used. Cyclosporin A was dissolved in olive oil with 1% ethanol. Azathioprine was suspended in 1% gum arabic.

Fig. 1. Chemical structure of phellodendrin.

Induction of crescentic-type anti-GBM nephritis
Crescentic-type anti-GBM nephritis was inducted by immunizing rats that had received a nephritogenic dose of rabbit anti-rat GBM (anti-GBM) serum with rabbit y-globulin (rgG) according to a slight modification of the previously reported method (8). Fifty rats weighing approx. 160 g were administered 0.75 ml/animal of anti-GBM serum into the tail vein. On the day after the injection of anti-GBM serum, 24-hr urine samples were collected, and the rats were then divided into 5 groups of 8 rats, so that the average protein content in the 24-hr urine in each group was at the same level. After grouping, these animals, were injected with 6.5 mg of rgG in 0.25 ml of Freund's complete adjuvant (FCA) into the hind foot pads.

Evaluation of antinephritic effects of test drugs
Three groups were orally given 25, 15 and 100 mg/kg of OB-5, respectively in a volume of 1 ml per 100 mg of body weight, daily from the day after anti-serum injection (the 1st day) to the 41st day. The remaining group was orally given the vehicle (distilled water) instead of test drugs and served as the control. In addition, a non-treated (normal) group was used for comparison with the nephritic groups.

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 and the supernatant was used for determination of protein. Immediately after the urine collection, 0.4 ml of blood was drawn from tail vein of each conscious animal with a disposable syringe and put into a tube containing 0.025 ml of heparin. The blood was centrifuged at 5,000 rpm to obtain plasma for the determination of cholesterol and creatinine contents.

Determination of urinary protein, plasma cholesterol and creatinine contents
The urinary protein excretion was determined by the method of Kingsbury et al. (9) and expressed as mg/day. The cholesterol content was determined in accordance with the method of Zurkowski (10) and expressed as mg/dl plasma. The creatinine content was determined by a creatinine determination kit (CRE-EN, Kainos, Inc., Tokyo).

Measurement of plasma antibody titer against rgG
The plasma antibody titer against rgG was determined by indirect hemagglutination using sensitized sheep red blood cells (11).

Assessment of histopathological parameters
For light microscopic study, kidneys were isolated from rats anesthetized with pentobarbital, then 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. In the studies of
crescentic-type nephritis, the sections were stained with hematoxylin and eosin and Masson's trichrome. The crescent formation, adhesion of Bowman's capsule to capillary walls (adhesion) and fibrinoid necrosis in glomeruli was observed under a light microscope. For assessing these parameters, an equatorial cross section was selected by random sampling methods. Fifty glomeruli per section were observed, and the appearance rate of crescent formation, adhesion and fibrinoid necrosis was expressed as the percentages of glomeruli (incidence) having these morphological alterations. All the above observations were performed blindly on coded sections.

Evaluation of the effects of OB-5 on the changes in the number of leukocyte-subsets in glomeruli of crescentic-type anti-GBM nephritis

To evaluate the effects of OB-5, azathioprine and cyclosporin A on the number of leukocyte-subsets in glomeruli of crescentic-type anti-GBM nephritis, these drugs were given p.o. daily to groups of rats after anti-GBM serum injection. The control rats were given p.o. the vehicles instead of test drugs. The blood and kidneys of animals in the drug-treated and control groups were taken 2.0 hr after the treatment with the respective drugs, under pentobarbital anesthesia, on the 1st, 5th and 15th days after anti-GBM serum injection. Tissues for immunoenzymatic staining were frozen in liquid nitrogen as described below.

Immunohistochemistry

Tissues for immunoenzymatic staining were fixed in paraformaldehyde-lysine-periodate (12). Serial cryostat sections were adhered to microscope slides, air dried and preincubated with 1–2% normal horse serum in phosphate-buffered saline (PBS) for 20 min. The sections were then incubated with mouse anti-rat monoclonal antibodies for 90 min, washed twice in PBS, treated with 0.3% hydrogen peroxide in methanol for 40 min to block endogenous peroxidase, and incubated with biotinated affinity purified anti-Mouse IgG and avidinated horseradish peroxidase with diaminobenzidine tetrahydrochloride (Vecta stain ABC Kit, Vector Burlingame, CA). All steps were carried out at room temperature.

Monoclonal antibodies

Monoclonal antibodies used in this study were as follows: OX-1 (leukocyte common antigen), W3/25 (helper/helper inducer T cells), ED-1 (most macrophages and some dendritic cells), OX-8 (cytotoxic/suppressor T cell), OX-39 (IL-2 receptor positive cells) and OX-18 (Ia positive cells).

Quantification of leukocytes on tissue sections

Labelled cells within each glomerulus were counted with an image analyzer (TOYOBIO VI, Toyobo Co., Ltd., Tokyo), and the results were expressed as the number of cells per glomerular cross section (13). Cell counts were performed three times to reduce counting errors.

Statistical analyses

The data represent the mean ± S.D., and the results were statistically evaluated by one way analysis of variance with Student's t-test. The inhibitory percentage was calculated as follows:

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

Effects of OB-5 on crescentic-type anti-GBM nephritis

General condition and body weight (data not shown): No rats given OB-5 died during the 40-day experimental period. A similar amount of rat chow was consumed by both the control and OB-5 groups. In addition, none of the rats given OB-5 exhibited any abnormal behavior or diarrhea. The control and OB-5 groups had lower body weights than that of the normal group throughout the experimental periods. Both control and OB-5-treated rats showed a similar course of weight gain.

Urinary protein excretion (Fig. 2): From the 10th day after i.v.-injection of anti-GBM serum, the urinary protein excretion of control rats markedly increased. OB-5 at 100 mg/kg/day, p.o. had inhibited urinary protein excretion by the 19th day after the anti-GBM serum injection. In addition, OB-5 at dose of 25 and 50 mg/kg/day, p.o. had also inhibited the excretion by the 29th day. These effects were maintained until the end of the experimental schedule.

Plasma cholesterol and creatinine contents (Figs. 3 and 4): The plasma cholesterol and creatinine contents were determined on the 26th, 34th and 40th days. The plasma cholesterol contents in the control rats were markedly elevated from the 26th to the 40th day (Fig. 3). In contrast, OB-5 at all doses inhibited the elevation of the cholesterol content with an inhibitory percentage of 68% and 87% on the 34th and the 40th days, respectively. The plasma creatinine contents in the control rats were markedly elevated from the 26th to the 40th day (Fig. 3). In contrast, OB-5 at all doses inhibited the elevation of the creatinine content with an inhibitory percentage of 68% and 87% on the 34th and the 40th days, respectively. The plasma creatinine contents in the control rats were also elevated from the 26th to the 40th days (Fig. 4). On the other hand, in the OB-5, 100 mg/kg/day-treated rats, the plasma creatinine content was similar to that of normal rats on the 40th day.

Plasma antibody titer against rgG (data not shown): The plasma antibody titer against rgG was not affected
by OB-5 during the experimental period.

**Histological observation (Fig. 5):** Light microscopic examination of the glomeruli of anti-GBM serum-injected rats revealed lesions characterized by severe crescent formation, adhesion and mesangioproliferative form. The incidence of crescent formation and adhesion in the glomeruli of control rats was approx. 35%, respectively. In contrast, the lesion of OB-5, 50
mg/kg/day or 100 mg/kg/day-treated rats was less than those of the nephritic control rats. The incidence of crescent formation in OB-5-treated rats had been reduced with an inhibitory percentage of approx. 70%; adhesion, approx. 20% to 50%; and fibrinoid, approx. 30% to 80%.

**Effects of OB-5 on the cell number of the leukocyte subpopulation in the glomeruli (Fig. 6)**

The experiments were performed using cyclosporin A and azathioprine as positive control drugs. When the effects of azathioprine and cyclosporin A on crescentic-type anti-GBM nephritis were measured on the 20th day after the injection of anti-GBM serum, both drugs significantly reduced urinary protein excretion (Normal: 5.9 ± 2.9 mg/day; Control: 249.6 ± 68.3 mg/day; Azathioprine: 168.6 ± 77.6 mg/kg, P < 0.01 vs. Control; cyclosporin A: 50.5 ± 35.7 mg/kg, P < 0.01 vs. Control). The number of total leukocytes in the control had been significantly increased by the 15th day after the in-
Injection of anti-GBM serum. In contrast, OB-5 at 100 mg/kg/day, azathioprine at 20 mg/kg day, and cyclosporin A at 20 mg/kg/day inhibited the increase in the number of total leukocytes in glomeruli with an inhibitory percentage of approx. 80% on the 5th day. On the 15th day, the number of total leukocytes in the glomeruli was reduced to the normal level by the treatment with these drugs. Macrophages, helper T cells and cytotoxic T cells in the glomeruli of the control rats had time-dependently increased by the 15th day. All three

Fig. 6. Effect of cyclosporin A, OB-5 and azathioprine on leukocyte subpopulation in glomeruli of crescentic-type anti-GBM nephritic rats. Each column denotes the mean ± S.D. of 5 rats. * and ** indicate a significant difference from the normal at P < 0.05 and P < 0.01, respectively. * and ** indicate a significant difference from the control at P < 0.05 and P < 0.01, respectively.
drugs extremely suppressed the accumulation of macrophages and cytotoxic T cells in the glomeruli. While cyclosporin A and azathioprine reduced the number of glomerular helper T cells more than 80% as an inhibitory percentage, OB-5 had not exhibited any effect on it by the 15th day. The number of Ia-positive cells in the control glomeruli increased time-dependently as well as macrophages and cytotoxic T cells. In the OB-5-, cyclosporin A-, or azathioprine-treated groups, however, the numbers of these cells did not increase during the experimental period, and their levels were normal on the 15th day. The glomeruli in control rats showed a significantly increased number of IL-2 receptor-positive cells on the 5th and the 15th days after the injection of anti-GBM serum. OB-5- or cyclosporin A-treated rats showed a markedly suppressed number of the cells on both the 5th and the 15th days compared with the control rats. Azathioprine had not prevented the increase of the number of the cells by the 5th day.

DISCUSSION

In the previous paper, we demonstrated the antinephritic effect of OB-5 at 50 mg/kg, but not 10 and 20 mg/kg by intraperitoneal administration (7). We undertook further experiments to elucidate the antinephritic effect of OB-5 by oral administration and to obtain the dose-dependent effect of OB-5 using 25, 50, and 100 mg/kg. Furthermore, to elucidate the mechanisms of the antinephritic effect of OB-5 in detail, we investigated the effect of OB-5 on various subsets of mononuclear cells in nephritic glomeruli. Although the present study has demonstrated that OB-5 significantly prevents the development of crescentic-type anti-GBM nephritis by oral administration, the effects of OB-5 were not dose-dependent. OB-5 itself is detected in vivo at a very low level compared to the metabolite of OB-5 (personal communication from Tsumura Co.). Therefore, there may be a limit to the uptake of OB-5 in vivo.

In addition, we have also shown that daily OB-5 treatment decreases the number of leukocyte subpopulations in the glomeruli of the nephritic rats. The cell-mediated immunity may be induced by localized reactions to organisms, usually intracellular pathogens, mediated by lymphocytes and phagocytes rather than by antibody. Recently, it has been demonstrated that the infiltration of activated mononuclear cells into the glomeruli or cortical tubulointerstitium arise and delayed hypersensitive reactions may be associated with renal injury in glomerulonephritis (3–5, 14). A cell transfer study (15) demonstrated that proliferative glomerulonephritis developed with a significant macrophage accumulation and proteinuria, when peritoneal mononuclear inflammatory cells were injected intravenously to leukocyte-depleted rabbits that had already received anti-GBM serum. Recently, Li et al. (16) have reported that IL-2 receptor-positive cells and macrophages in the glomeruli are closely associated with decreased renal function in IgA nephritis with crescent formation. Moreover, Schreiner et al. (17) demonstrated that enhanced Ia expression in nephrotoxic nephritis was seen during proteinuria, and decompimentation prior to anti-GBM serum administration abrogates both the proteinuria and the increased Ia expression, although an increase in cells expressing leukocyte common antigen still occurred. These findings suggest that activated macrophages and T lymphocytes in glomeruli may be directly associated with function in proliferative glomerulonephritis.

The present study indicated that the number of macrophages, Ia-positive cells and cytotoxic T cells in the glomeruli of nephritic control rats were markedly increased on the 15th day after i.v.-injection of antisera. The plasma antibody titer against rG in control rats was also elevated on the same day (unpublished data of T. Hattori). These results suggest that the cell-mediated immunity system in addition to the humoral immunity system also may be partly associated with the progression of crescentic-type nephritis. Furthermore, we performed a time-course experiment on the glomerular mononuclear leukocyte subset both in original type- and crescentic-type anti-GBM nephritis (unpublished data of T. Hattori et al.). Consequently, we found that the accumulation of mononuclear cells in nephritic glomeruli reached the maximum on the 15th day after the injection of anti-GBM serum, then gradually diminished. We considered that the difference in severity of glomerulonephritis between original- and crescentic-type anti-GBM nephritis can be attributed to the difference of the accumulation of mononuclear leukocytes into the glomeruli. Additionally, OB-5 revealed its antinephritic effect on crescentic-type anti-GBM nephritis from the 19th day after the injection of anti-GBM serum. Therefore, we considered that the experiments on glomerular leukocytes had to be performed before the 19th day following the anti-GBM serum injection to elucidate whether the suppressive effects of OB-5 on accumulation of glomerular leukocytes were the cause or the results for the antinephritic effect of OB-5 on crescentic-type anti-GBM nephritis.

IL-2 receptor expression is widely accepted marker of immune activation, and it can be anticipated that such cells are functionally active within the tissue. It has been reported that IL-2 receptors are expressed not only the surface of helper T cells, but also macrophages
and cytotoxic T cells (18, 19). In addition, IL-2 induces not only the proliferation of helper T cells but also both the proliferation and the differentiation of effector cell precursors to the cytolytic state. Eventually, activated cytotoxic T cells and macrophages with IL-2 receptors can induce lysis of target cells (18, 19). It has been well-demonstrated (20) that cyclosporin A inhibits the proliferation and the function of T lymphocytes by inhibiting IL-2 production and then suppresses the antibody production and the activation of effector cells. While azathioprine widely inhibits the proliferation of mononuclear cells during the immunoreaction. Accordingly, it is reasonable to consider that the specific inhibitory action of cyclosporin A against activated mononuclear cells such as IL-2-positive cells is greater than that of azathioprine. In the present study, the increase in the number of IL-2 receptor-positive cells in the glomeruli had been reduced by OB-5 as well as cyclosporin A, by the 5th day after the injection of anti-GBM serum, while the increase in the number of cytotoxic T cells, macrophages and Ia-positive cells in the OB-5 treated-group had been reduced by the 15th day. However, OB-5 did not reduce the increase in the number of helper T cells in the glomeruli; in contrast, cyclosporin A markedly inhibited it. In addition, OB-5 did not affect the antibody production against rGG through-out the experimental period, although the number of IL-2 receptor-positive cells, macrophages and Ia-positive cells was reduced by the treatment with OB-5. These results suggest that OB-5 may directly inhibit only the proliferation of IL-2-positive effector cells or IL-2 receptor expression on the effector cells, such as cytotoxic T cells and macrophages, leading to the reduced cytolytic activity in the glomeruli. It is considered that the inhibitory effect of OB-5 on the immunoreaction may be quite different from those of cyclosporin A and azathioprine. Moreover, we found that the antinephritic effect of OB-5 on accelerated passive Heymann nephritis (APHN) was also less than that on crescentic-type nephritis (unpublished data of T. Hattori et al.). It has been believed that the induction and development of APHN are not mediated by leukocyte infiltration into glomeruli, and it may depend on the antibody formation against heterologous antigens (21, 22). In addition, we also found that the numbers of cytotoxic T cells, macrophages, Ia antigen and IL-2 receptor-positive cells in the glomeruli of APHN rats was not higher than those of normal rats. These results further support our speculation regarding the mechanisms of the antinephritic effect of OB-5 on crescentic-type anti-GBM nephritis.

REFERENCES

17 Schreiner, G.F., Cotran, R.S. and Unanue, E.R.: Modulation of Ia and leukocyte common antigen expression in rat glomeruli during the course of glomerulonephritis and amino-

18 Hancock, W.W., Muller, W.A. and Corran, R.S.: Interleukin-2 receptors are expressed by alveolar macrophages during pulmonary sarcoidosis are inducible by lymphokine treatment of normal human lung macrophages, blood monocytes and monocyte cell lines. J. Immunol. 138, 185–191 (1987)


