Acteoside, a Component of *Stachys Sieboldii* MIQ, May Be a Promising Antinephritic Agent: Effect of Acteoside on Crescentic-Type Anti-GBM Nephritis in Rats

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ABSTRACT—Effects of acteoside (ACT) on crescentic-type anti-GBM nephritis in rats were investigated. When rats were treated with ACT from the 1st day after i.v. injection of anti-GBM serum, ACT inhibited the elevation of protein excretion into urine. In the ACT-treated rats, cholesterol and creatinine contents and antibody production against rabbit γ-globulin in the plasmas were lower than those of the nephritic control rats. Histological observation demonstrated that this agent suppressed hypercellularity and the incidence of crescent formation, adhesion of capillary wall to Bowman's capsule and fibrinoid necrosis in the glomeruli. Furthermore, rat-IgG and C3 deposits on the GBM were significantly less in the ACT-treated group than in the control nephritic group. When the treatment was started from the 20th day after i.v. injection of anti-GBM serum, by which the disease had been established, ACT resulted in a similar effect on the nephritic rats as stated above. These results suggest that ACT may be a useful medicine against rapidly progressive glomerulonephritis, which is characterized by severe glomerular lesions with diffuse crescents.

**Keywords:** Crescentic-type anti-GBM nephritis, Acteoside, Rat-IgG, Rat-C3

Chyorogi *Stachys sieboldii* MIQ (Labiatae) is prescribed for many sicknesses, and this tuber is used as food by Chinese, Russian and Japanese. Recent investigations have demonstrated that chyorogi has anti-anoxia action (1), inhibitory action on hyarulonidase activity (2) and immunosuppressive action (3).

On the other hand, it has been mentioned that the immune response participates in the development of nephritis. To put it more concretely, glomerular injury is mediated by immune complex deposition in the glomeruli, followed by immune-inflammatory reaction, including complement activation and the releases of other inflammatory mediators. In recent studies, much attention has been paid to the contribution of the cell-mediated immune response in the development of glomerulonephritis (4, 5). Moreover, Neild et al. (6) reported that suppression of T cell function by cyclosporin A (CyA) blocked the subsequent development of glomerular lesions in acute sickness nephritis. We reported that mizoribin (7), azathioprine (7), CyA (8), methylprednisolone (9) and some plant components (10) that have immunosuppressive action showed a salutary effect on anti-GBM nephritis. Although these immunosuppressive agents exert antinephritic action, it is difficult to use them in clinical trials because of their side effects (11). Therefore, a new immunosuppressive agent for the treatment for nephritis in the clinical stage is expected to be developed.

The purpose of the present study was to elucidate the anti-nephritic effect of acteoside, a component of chyorogi, on crescentic-type anti-GBM nephritis in rats.

**MATERIALS AND METHODS**

**Animals**

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

**Drugs**

The chemical structure of acteoside (ACT) (Tsumura Co., Ltd., Tokyo) is shown in Fig. 1. This component was extracted from the aerial part of chyorogi (*Stachys sieboldii* MIQ). The purity of ACT was verified by HPLC (Column: TSK gel ODS-80TM (4.0 id × 250 mm); Mobile phase: 20% CH3CN/H2O, containing 1% AcOH; Flow
rate: 0.7 ml/min; Temp.: room temperature; Detect: UV 254 nm) at Tsumura Co., Ltd. ACT employed in these experiments had a purity of greater than 99%. ACT was dissolved in distilled water. Dipyridamole (Dip) (Boehringer Ingelheim, Germany) and azathioprine (Aza) (Sigma, St. Louis, MO, USA) were also used; These drugs were suspended in 1% gum arabic.

**Induction of crescentic-type anti-GBM nephritis**

Crescentic-type anti-GBM nephritis was induced by immunizing the rats that had received a nephritogenic dose of rabbit anti-rat GBM (anti-GBM) serum with rabbit γ-globulin (γ-G) according to a slight modification of the previously reported method (12). In this experiment, rats weighing approx. 160 g were administered 0.6 ml/animal of anti-GBM serum into the tail vein.

The effect of test drugs was estimated by administrating them from the 1st day after the anti-GBM serum injection (heterologous phase) or from the 20th day after the anti-GBM serum injection (autologous phases). In the experiments, 24-hr urine samples were collected, and the rats were then divided into 5 or 6 groups of 8 rats, so that the average protein content in the 24-hr urine in each group was at the similar level.

**Evaluation of antinephritic effect of the test drugs**

In the experiment of drug treatment from the heterologous phase, four groups were orally given 3, 10 or 30 mg/kg/day of ACT or 100 mg/kg/day of Dip, respectively, in a volume of 1 ml per 100 g of body weight, daily from the 1st day or the day after i.v. injection of anti-GBM serum to the 40th day. In the experiments of drug treatment from the autologous phase, three or four groups were orally given 3, 10 or 30 mg/kg/day of ACT (A) or 30 mg/kg/day of ACT, 100 mg/kg/day of Dip or 50 mg/kg/day of Aza (B), respectively, in a volume of 1 ml per 100 g of body weight, daily from the 20th day after i.v. injection of anti-GBM serum to the 40th (A) or the 45th (B) day. The remaining group was orally given the vehicle (distilled water) instead of test drugs and served as the nephritic control. In addition, a nontreated (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 15 min at 4°C and the supernatant was used for determination of protein. On the final day of the experiment, 2.0 ml of blood was drawn from the renal vein of each anesthetized rat with a disposable syringe and put into a tube containing 0.125 ml of heparin. The blood was centrifuged at 5,000 rpm to obtain plasma for the determination of some parameters.

**Determinations of urinary protein and plasma cholesterol and creatinine contents**

The urinary protein excretion was determined by the method of Kingsbury et al. (13) and expressed as mg/24-hr urine. The cholesterol content was determined with a commercial assay kit (Determina TC-5; Kyouwa Medix Co., Ltd, Tokyo) (14) and expressed as mg/dl plasma. The creatinine content was determined by using a creatinine determination kit (CRE-EN; Kainos, Inc., Tokyo) and expressed as mg/dl plasma/100 g body weight.

**Measurement of plasma antibody titer against γ-G**

The plasma antibody titer against γ-G was determined by indirect hemagglutination using sensitized sheep red blood cells (15).

**Measurement of plasma complement CH50 level**

The plasma complement CH50 level was determined by the method of Mayer (16).

**Assessment of histopathological parameters**

For light microscopic study, kidneys were isolated from rats anesthetized with pentobarbital, then dehydrated and fixed by perfusing 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 anti-GBM nephritis, the sections were stained with hematoxylin and eosin and Masson’s trichrome. The number of
nuclei (hypercellularity), crescent formation, adhesion of Bowman's capsule to capillary wall (adhesion) and fibrinoid necrosis in the glomeruli were observed under a light microscope. For assessing these parameters, an equatorial cross section was selected by random sampling methods. Fifty glomeruli/cross section were observed, and the appearance rate of crescent formation, adhesion and fibrinoid necrosis was expressed as the percentages of the glomeruli (incidence) having these morphological alterations as previously described (17). The evaluation was performed by a different person who had no knowledge of the identity of each sample. For assessing hypercellularity, an equatorial cross section was selected by a random sampling method. The number of nuclei (including nuclei of glomerular cells and exudative leukocytes) was counted and expressed as the mean number per glomerular cross section in 10 glomeruli/section.

Immunohistochemistry

In tissues for immunoenzymatic staining of rat-IgG, the paraffin sections were cut as described above, and the sections were treated with 0.1% protease in 0.05 M tris-HCl buffer for 7 min and then washed in chilled 0.01 M phosphate buffered saline (PBS), pH 7.4. The sections were then incubated with anti-rat IgG mouse monoclonal antibody (mAb) (Cappel, West Clester, PA, USA) at a dilution of 1:100 for 90 min. The sections were washed again with PBS, treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase, and incubated with biotinated affinity purified anti-mouse IgG and avidinated horseradish peroxidase with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Vecta stain ABC Kit; Vector institution, Burlingame, CA, USA). All steps were carried out at room temperature.

In tissues for immunoenzymatic staining of rat-C3, the cryostat sections were incubated with peroxidase-conjugated goat-IgG fraction to rat complement C3 with DAB (Cappel) after washing in PBS.

Tissues for immunoenzymatic staining of proliferating cell nuclear antigen (PCNA), which is a marker for cell proliferation, were fixed in 10% formalin in PBS, and the paraffin-embedded tissues were stained by the same procedure as that for rat-IgG except for the use of a mAb (19A2; Coulter Immunology, Hialeal, FL, USA) to the PCNA.

Quantification of rat-IgG, C3 and PCNA on tissue sections

The total area of immunoreactive rat-IgG and C3 in the glomerulus was measured in 30 glomeruli per section using an image analyzer (Toyobo Image analyzer V1; Toyobo Co., Ltd., Tokyo) and presented as mm²/glomerular cross section (G.C.S.). PCNA-positive cells in the glomerulus were counted with the image analyzer, and the results were expressed as the number of cells/G.C.S.

Statistical analyses

The data represent the mean ± S.D., and the results were statistically evaluated by ANOVA. When these results were parametric, these were statistically evaluated by Duncan's test. When the results were non-parametric, they were statistically evaluated by the Kruskal-Wallis test. Inhibitory percentage was calculated as follows:

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

RESULTS

Effect of ACT in crescentic-type anti-GBM nephritis

Urinary protein excretion (Figs. 2 and 3): When the treatment with ACT was started from the day after the anti-GBM serum injection (heterologous phase), the first significant suppression on urinary protein was observed on the 5th day at 30 mg/kg, p.o.; on the 20th day at 3 and 10 mg/kg, p.o.; and on the 40th day with Dip at 100 mg/kg, p.o.

When ACT was given from the 20th day after the anti-GBM serum injection (autologous phase), ACT at 10 and 30 mg/day inhibited the elevation of the protein excretion into urine by the 30th day. On the other hand, Aza at 50 mg/kg was weaker than ACT at 30 mg/kg, although not significantly, and Dip had no effect.
Plasma cholesterol and creatinine contents (Table 1): The plasma cholesterol and creatinine contents were determined on the 40th or 45th days. The plasma cholesterol contents in the nephritic control rats were markedly elevated. In contrast, the elevation of the cholesterol content was reduced with ACT (30 mg/kg) from the heterologous phase by 60% of the control level and with ACT (30 mg/kg) from the autologous phase by 62% of the control level. The plasma creatinine contents in the nephritic rats were also elevated. On the other hand, in the ACT (30 mg/kg) from both phase-treated rats, the plasma creatinine content was similar to that of the normal rats.

Fig. 3. Effect of acteoside on urinary protein excretion in crescentic-type anti-GBM nephritis in rats. Acteoside was given p.o. daily during the period from the 20th day after i.v. injection of anti-GBM serum to the 40th day (A). (): normal, (●): nephritic control, (▲): nephritis + acteoside (3 mg/kg/day, p.o.), (△): nephritis + acteoside (10 mg/kg/day, p.o.), (□): nephritis + acteoside (30 mg/kg/day, p.o.). Test drugs were given p.o. daily during the period from the 20th day after i.v. injection of anti-GBM serum to the 45th day (B). (): normal, (●): nephritic control, (□): nephritis + acteoside (30 mg/kg/day, p.o.), (▲): nephritis + dipyridamole (100 mg/kg/day, p.o.), (○): nephritis + azathioprine (50 mg/kg/day, p.o.). Each plot denotes the mean ± S.D. of mg/day. *P < 0.05, **P < 0.01, relative to control rats.

Plasma antibody titer against γ-G: Nephritic rats had shown markedly accelerated antibody production. The accelerated antibody production was suppressed to 72% of the control level by the treatment with ACT (10 and 30 mg/kg) from the heterologous phase by 60% of the control level and with ACT (30 mg/kg) from the autologous phase by 62% of the control level. The plasma creatinine contents in the nephritic rats were also elevated. On the other hand, in the ACT (30 mg/kg) from both phase-treated rats, the plasma creatinine content was similar to that of the normal rats.

Histological observation (Table 3, Figs. 4 and 5): Light microscopic examination of the nephritic glomeruli revealed lesions characterized by severe crescent formation, adhesion, fibrinoid necrosis and proliferation of mesangi-

| Table 1. Effect of acteoside on plasma cholesterol and creatinine content in crescentic-type anti-GBM nephritis in rats |
|---------------------------------------------------------------|
| Heterologous$^{1)}$                                      | Autologous$^{2)}$                                      |
| cholesterol       | creatinine       | cholesterol       | creatinine       |
| Normal            | 49.5 ± 9.4       | 0.18 ± 0.03       | 49.3 ± 9.2       | 0.14 ± 0.02       |
| Control           | 184.4 ± 75.4     | 0.32 ± 0.19       | 180.5 ± 64.6     | 0.18 ± 0.02       |
| Acteoside 3 mg/kg | 129.1 ± 39.6     | 0.27 ± 0.05*      | 150.2 ± 76.8     | 0.16 ± 0.02       |
| 10 mg/kg          | 162.8 ± 30.1     | 0.35 ± 0.09       | 95.2 ± 20.2**    | 0.16 ± 0.03       |
| 30 mg/kg          | 111.7 ± 18.9*    | 0.23 ± 0.03**     | 93.6 ± 22.5**    | 0.15 ± 0.01*      |
| Dipyridamole 100 mg/kg | 138.1 ± 51.5 | 0.25 ± 0.04**    | 141.1 ± 40.3    | 0.15 ± 0.03*       |
| Azathioprine 50 mg/kg | 207.4 ± 88.2   | 0.20 ± 0.03      |                  |                  |

The cholesterol content was expressed as mg/dl. The creatinine content was expressed as mg/dl/100 g body weight. Values are expressed as the mean ± S.D. $^{1)}$ Test drugs were given from the 1st day after i.v. injection of anti-GBM serum. $^{2)}$ Test drugs were given from the 20th day after i.v. injection of anti-GBM serum. Blood was taken at the 40th or the 45th days. *P < 0.05, **P < 0.01, relative to control rats.
al cells. Histological observation demonstrated that ACT (from both the heterologous and autologous phase) inhibited hypercellularity and the incidence of crescent formation, adhesion and fibrinoid necrosis in the glomeruli by the 40th or 45th day. The lesions of Dip and Aza-treated nephritic rats were also less than those of the nephritic control rats. However, when nephritic rats were treated with Dip from the autologous phase, histological changes were little affected by Dip (data not shown).

Glomerular cell proliferation, namely the increase in PCNA-positive cells in the glomeruli, was observed in

| Table 2. Effect of acteoside on antibody titer and rat-IgG and C3 deposition on the GBM in crescentic-type anti-GBM nephritis in rats |
|---------------------------------------------------------------|
| Antibody titer | Rat-IgG deposition | Rat-C3 deposition |
| Control | 6.1 ± 2.0 | 1.59 ± 0.21 | 3.23 ± 0.58 |
| Acteoside 3 mg/kg | 5.0 ± 0.6 | 1.41 ± 0.36 | 2.09 ± 0.24** |
| 10 mg/kg | 4.7 ± 1.4* | 1.36 ± 0.12 | 2.06 ± 0.32** |
| 30 mg/kg | 4.7 ± 0.8* | 1.14 ± 0.22** | 2.27 ± 0.25* |
| Dipyridamole 100 mg/kg | 5.8 ± 1.4 | 1.65 ± 0.32 | 3.27 ± 0.12 |

Table 3. Effect of acteoside on total glomerular cellularity and proliferating (PCNA*) cells in crescentic-type anti-GBM nephritis in rats

| Total cells | Proliferating (PCNA*) cells |
|------------|-----------------------------|
| Normal | 32.5 ± 3.8 | 0.43 ± 0.09 |
| Control | 48.3 ± 1.1 | 2.50 ± 0.47 |
| Acteoside 30 mg/kg | 39.6 ± 2.8** | 1.22 ± 0.37** |

For assessing these parameters, an equatorial cross section was selected by a random sampling method. The number of nuclei (including nuclei of glomerular cells and exudate leukocytes) was counted and expressed as the mean number per glomerular cross section (G.C.S.) in 10 glomeruli/section. PCNA* cells in the glomerulus were counted with the image analyzer, and the results were expressed as the number of cells/G.C.S in 30 glomeruli/section. The values are expressed as the mean number ± S.D. of cells/G.C.S. Acteoside was given at 30 mg/kg/day, p.o. from the 1st day after i.v. injection of anti-GBM serum. The kidney was taken at the 30th day. **P<0.01, relative to control rats.

Fig. 4. Light micrographs of glomeruli from rats of the normal group (a, d); nephritic control group (b, e); group given acteoside, 30 mg/kg/day, p.o. (c, f). Acteoside was given from the 1st day after i.v. injection of anti-GBM serum. The rats were examined on the 40th day after i.v. injection of anti-GBM serum (Masson’s trichrome stain ×400) (a, b, c). Note that crescent formation is markedly less in the group treated with acteoside than in the nephritic control group. The rats were examined on the 30th day after i.v. injection of anti-GBM serum (immunohistochemical glomerular staining for PCNA, ×400) (d, e, f).
Fig. 5. Effect of acteoside on histopathological parameters in crescentic-type anti-GBM nephritis in rats. Test drugs were given p.o. daily during the period from the 1st day after i.v. injection of anti-GBM serum to the 40th day. (■): nephritic control, (□): nephritis + acteoside (3 mg/kg/day, p.o.), (△): nephritis + acteoside (10 mg/kg/day, p.o.), (▲): nephritis + acteoside (30 mg/kg/day, p.o.), (●): nephritis + dipyridamole (100 mg/kg/day, p.o.). Each column denotes the mean ± S.D. of % or index. **P < 0.01, relative to control rats.

Fig. 6. Photographs of glomeruli immunohistochemically stained. The kidney was taken at the 40th day after i.v. injection of anti-GBM serum. Acteoside was given at 30 mg/kg/day, p.o. from the 1st day after i.v. injection of anti-GBM serum to the 40th day. Linear staining of rat-IgG (a, b, c) and C3 (d, e, f) was observed on the GBM. Both rat-IgG and C3 on the GBM were negative in the normal rats (a, d) and were less in the acteoside-treated rats (c, f) than in the nephritic control rats (b, e), ×400.
nephritic rats. In contrast, at the 30th day, a 52% reduction in cell proliferation was observed, and this was associated with a significant reduction in glomerular cellularity as compared with the control (Table 3).

Deposition of immune reactant (Table 2 and Fig. 6): It was possible to observe rat-IgG and C3 deposits on the GBM in the nephritic control rats; however, they were not found in normal rats. ACT (30 mg/kg) from the heterologous phase reduced rat-IgG deposition on the GBM by 72% of the control level. Moreover, ACT (3, 10 and 30 mg/kg) from the heterologous phase reduced rat-C3 deposition on the GBM by 60 to 69% by the 40th day. Even when ACT was given from the autologous phase, inhibition of rat-IgG and C3 deposition were similar to the above results. Dip did not affect rat-IgG and C3 deposition. Aza, however, reduced rat-IgG and C3 deposition on the GBM by 67 and 68%, respectively (data not shown).

Effect of ACT on complement activity and C3 deposits on the GBM in original-type anti-GBM nephritis (Fig. 7)

Original-type anti-GBM nephritis was induced in rats by injection of 0.6 ml of anti-GBM serum into their tail veins, as described previously (18). Nephritic rats were pretreated and posttreated with ACT at 30 mg/kg, p.o. and cobra venom factor (CVF) (Sigma) at 10 μg/rat, i.p., three times, every 8 hr. ACT and CVF treatments reduced urinary protein excretion with an inhibitory percentage of approx. 35% and 71%, respectively. The CH50 in nephritic control rats was markedly lower than that in the normal rats by 24 hr after nephritis induction. ACT inhibited the decreased CH50 and increased C3 deposition on the GBM in nephritic control rats. CVF abolished the CH50 and C3 deposition. Furthermore, in the in vitro experiment, ACT inhibited the complement activation by 37%. When ACT was administered to normal rats, ACT reduced the complement activation in the ex vivo experiment (data not shown).

DISCUSSION

Rapidly progressive glomerulonephritis and kidney diseases in Goodpastur's syndrome are malignant disease that lapse into renal failure by 2 or 3 months after the development of the disease (19). Characteristics of this nephritis involved hypercellularity, marked infiltration of neutrophils and monocytes and crescent formation in the glomeruli. Cocktail therapy with immunosuppressive agents, anti-platelet drugs and steroids have been mainly applied to this disease. On the other hand, although CyA is a surpassing immunosuppressant that exerts a beneficial effect on experimental nephritis (6, 8), it has been reported that CyA causes kidney dysfunction and causes irreversible histological alteration in the glomeruli (11). Methylprednisolone possesses many side effects. Furthermore, the rebound phenomenon and withdrawal syndrome after a long treatment with this medicine are two of the problems. Therefore, a new immunosuppressive agent for the treatment of nephritis that has less side effects must be developed.

Crescentic-type anti-GBM nephritis is an experimental model that shows histological and pathological alterations similar to those in rapidly progressive glomerulonephritis and kidney disease of Goodpastur's syndrome (20). The development and progression of this nephritis consists of 2 phases that are mediated by immune responses. The early reaction, the so-called heterologous phase, is due to the deposition of anti-GBM antibody, followed by com-
plement-dependent accumulation of polymorphonuclear granulocytes (21, 22). The late phase (autologous phase) develops by the binding of autologous antibody deposited along the GBM and the influx of monocytes/macrophages to the glomeruli following this reaction (22, 23). This nephritis is characterized by biphasic urinary protein and hypercellularity that involves crescent formation and fibrinoid necrosis. Crescent formation is thought to be mediated by migrated macrophages and proliferated epithelial cells (22, 24).

We previously reported that mizoribin and Aza, an immunosuppressive agent (7), inhibited the elevation of plasma antibody against rabbit IgG and Pachymen, a main component of *Poria cocos* (18), decreased the degree of C3 deposition on the GBM, and both were markedly effective against anti-GBM nephritis.

ACT treatments suppressed the development of anti-GBM glomerulonephritis as assessed by reduction of proteinuria and plasma cholesterol, prevention of renal function impairment and prevention of progressive histological changes including the development of glomerular crescents and hypercellularity. On the 40th day, ACT at 30 mg/kg alone significantly reduced the amount of rat-IgG deposition on the GBM mediated by suppression of antibody production in this nephritic model. On the other hand, the amount of C3 deposits on the GBM were decreased by ACT at 3, 10 or 30 mg/kg. Moreover, ACT significantly inhibited complement activation in both in vitro and ex vivo experiments. The reduction of CH50 in nephritic control rats was inhibited by ACT. These above data suggest that ACT inhibits renal injury by suppressing complement activation, because complement membrane attack complex (MAC) is involved in the pathogenesis of glomerular injury (25), and the sublytic MAC has potential inflammatory mediators that could be associated with glomerular damage, mesangial cell proliferation and extracellular matrix diffusion, such as reactive oxygen species (26), protease (27), prostaglandins (28) and interleukin-1 like cytokines (29), as well as collagen (30).

It is believed that complement activation is closely related to leukocyte infiltration. In recent studies, it was reported that C3a provoked monocyte and neutrophil adhesion to mesangial cells and endothelial cells, respectively (31, 32). Therefore, in a further study, we are going to investigate the effect of ACT on the accumulation of leukocytes in the glomeruli.

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