Butein Ameliorates Experimental Anti-Glomerular Basement Membrane (GBM) Antibody-Associated Glomerulonephritis in Rats (1)

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ABSTRACT—Effects of butein on crescentic-type anti-glomerular basement membrane (GBM) nephritis in rats were investigated. When rats were treated with butein from 1 day after i.v. injection of anti-GBM serum, it inhibited the elevation of protein excretion into urine. In the butein-treated rats, cholesterol content in plasma was lower than that of the nephritic control rats. Histological observation demonstrated that this agent suppressed the incidence of crescent formation, adhesion of capillary wall to Bowman’s capsule and fibrinoid necrosis in the glomeruli. Furthermore, butein suppressed the accumulation of leukocytes, including CD4-positive cells and CD8-positive cells in the glomeruli. However, butein failed to suppress the production of the antibody against rabbit γ-globulin and the deposition of rat-IgG on the GBM. These results suggest that butein may be a useful medicine against rapidly progressive glomerulonephritis, which is characterized by severe glomerular lesions with diffuse crescents.

Keywords: Anti-GBM nephritis (crescentic type), Butein, CD4-positive cell, CD8-positive cell

It has been mentioned that the immune response participates in the development of nephritis (1). To put it concretely, glomerular injury is mediated by immune complex deposition in the glomeruli, followed by the immune-inflammatory reaction, including complement activation (2), migration of leukocytes into glomeruli by leukotriene B4 (LTB4) (3, 4), complement components (4) and various cytokines (5–7) and production of superoxide anion in activated leukocytes (8). Moreover, much attention has been paid to the contribution of cell-mediated immune response in the development and progression of glomerulonephritis (9, 10). Neild et al. (11) reported that suppression of T cell function by cyclosporin A blocked the subsequent development of glomerular lesions in acute sickness nephritis. We reported that azathioprine (2), cyclosporin A (12, 13) and methylprednisolone (14), which have immunosuppressive action, showed a salutary effect on anti-glomerular basement membrane (GBM) nephritis. Although these immunosuppressive agents exert antinephritic action, it is difficult to use them in clinical trial because of their side effects (15). Therefore, natural products and plant components, which generally have less side effects than immunosuppressive agents, are widely noticed as potential starting materials for developing new drugs and antinephritic agents that can be tested clinically.

Butein, a chalcone derivative, is a plant component extracted from the flower of Butea frondosa and includes caffeic acid in its structure. Recent investigations have demonstrated that chalcone derivants have anti-proliferative action against human tumor cells (16, 17), inhibitory actions on 5-lipoxygenase (18), 12-lipoxygenase (19) and cyclooxygenase activity (19), an anti-oxidant effect (20) and anti-ulcerous activity (21).

We previously reported that acteoside, a component of Stachys sieboldii MIQ (2, 13), which contains a caffeic acid structure, inhibited the accumulation of leukocytes in nephritic glomeruli and markedly inhibited the development of experimental nephritis. Moreover, it has been reported that a 5-lipoxygenase inhibitor attenuates the development of glomerulonephritis in rats (22), and anti-oxidant agents block cultured mesangial killing by leukocytes (8).

The purpose of the present study was to elucidate the anti-nephritic effect of butein on crescentic-type anti-GBM nephritis in rats.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley strain rats, weighing 150–170 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 butein (3,4,2',4'-tetrahydroxy-chalcone) (Dainippon Ink and Chemicals, Inc., Tokyo) is shown in Fig. 1. Butein and dipyridamole (Sigma, St. Louis, MO, USA) were suspended in 1% gum arabic. Cyclosporin A (Sandoz Co., Ltd., Tokyo) was dissolved in 5% ethanol in olive oil.

Induction of anti-GBM nephritis and treatment of test drug

Crescentic-type anti-GBM nephritis was inducted by immunizing rats, which had received a nephritogenic dose of rabbit anti-rat GBM serum, with rabbit γ-globulin according to a slight modification of the previously reported method (2). In this experiment, rats weighing 150-170 g were administered 0.6 ml/animal of anti-GBM serum into the tail vein. In the experiments, 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 a similar level. Four groups were orally given 2, 20 or 200 mg/kg/day of butein or 200 mg/kg/day of dipyridamole, respectively, in a volume of 1 ml per 100 g of body weight, daily from 1 day after i.v. injection of anti-GBM serum to 15 or 40 days after. 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.

Original-type anti-GBM nephritis was inducted in rats by injecting a nephritogenic dose of anti-rat GBM serum into their tail veins, as described previously (2). Two groups were orally given 200 mg/kg/day of butein or 20 mg/kg/day of cyclosporin A, respectively, in a volume of 1 ml per 100 g of body weight, at 6 hr before i.v. injection of anti-GBM serum (n = 10).

Urine and blood collections

The 24-hr urine samples were obtained by keeping each animal in an individual metabolic cage for 24 hr on days 1, 5, 10, 18, 30 and 40 for crescentic-type anti-GBM nephritis and day 1 for original-type anti-GBM 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 810 × g for 15 min at 4 °C, and the supernatant was used for determination of protein. On the final day of the experiment on crescentic-type anti-GBM nephritis and at 3 hr after the induction of original-type anti-GBM nephritis, 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 heparin. The blood was centrifuged at 2,250 × g to obtain plasma for the determination of parameters.

Determinations of urinary protein and plasma cholesterol content

The urinary protein was determined by the method of Kingsbury et al. (23) and the results are expressed as mg/24-hr urine. The cholesterol content was determined with a commercial assay kit (Determina TC-5; Kyowa Medex Co., Ltd., Tokyo) (24) and the results are expressed as mg/dl plasma.

Measurement of plasma antibody level against rabbit IgG and complement CH50 level

The measurement of plasma antibody level against rabbit IgG was performed by enzyme-linked immunosorbent assay (25). The plasma complement CH50 level was determined by the method of Mayer (26).

Assessment of histopathological parameters in crescentic-type anti-GBM nephritis

For the light microscopic study, kidneys were isolated from rats anesthetized with pentobarbital on day 40 and then fixed in 10% formalin in 0.01 M phosphate-buffered saline (PBS) and dehydrated 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 anti-GBM nephritis, the sections were stained with Masson’s trichrome. 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 a random sampling method. Fifty glomeruli in each sampled cross section were examined by light microscopy to evaluate crescent formation, adhesion of capillary wall to Bowman’s capsule (adhesion) and fibrinoid necrosis. These histological parameters and the index of glomeru-
lar lesion were calculated by the previously described method (12). The evaluation was performed by a different person in blind fashion.

**Quantification of rat-IgG on glomerulus**

In tissues for immunoenzymatic staining of rat-IgG, the paraffin sections were cut as described above; and then the sections were treated with 0.1% protease in 0.05 M tris-HCl buffer for 7 min, followed by washing in chilled 0.01 M PBS, pH 7.4. The sections were then incubated with anti-rat IgG mouse monoclonal antibody (mAb) (Cappel, West Chester, PA, USA) at a dilution of 1:100 for 90 min. The sections were washed again with 0.01 M PBS, treated with 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.

The total area of immunoreactive rat-IgG in the glomerulus was measured in 30 glomeruli per section using an image analyzer (Toyobo Image Analyzer V1; Toyobo Co., Ltd., Tokyo) and the results are presented as mm²/glomerular cross section (G.C.S.).

**Analysis of glomerular leukocyte accumulation**

Kidney removed at 3 hr or 15 days after i.v. injection of anti-GBM serum was used for the analysis of glomerular leukocyte accumulation. Renal tissue samples were fixed in 10% formalin in 0.01 M PBS for immunoenzymatic staining. Immunocytochemical studies were performed on paraffin sections (2-3 μm). Nonspecific binding was blocked by treating the section with normal rabbit serum for 30 min. The sections were then sequentially incubated with a mAb to CD45 (leukocyte common antigen; total leukocyte, OX-1), ED-1 (most macrophages and some dendritic cells), CD4 (helper/inducer T cells, W3/25) or CD8 (cytotoxic/suppressor T cells, OX-8) (Serotec, Oxford, England), rabbit anti-mouse immunoglobulin G, and horseradish peroxidase-avidin biotin complex. The color reaction was development with DAB. Each tissue section stained with each mAb was analyzed with an image analyzer with regards to the number of total leukocytes and leukocyte subset, and the results are expressed as the number of cells per G.C.S.

**Statistical analyses**

The data represent means±S.D., and the results were statistically evaluated by using the Yums Statistic Library (ASB, Tokyo). When these results were parametric, they were statistically evaluated by the Duncan multiple-range test. When the results were non-parametric, they were statistically evaluated by the Kruskal-Wallis test.

**RESULTS**

**Urinary protein excretion and plasma cholesterol content in crescentic-type anti-GBM nephritis (Fig. 2)**

When the treatment with butein was started from the day after the anti-GBM serum injection, butein at 200 mg/kg significantly suppressed the urinary protein excretion by 34% or 55% at 18 or 40 days, respectively. In addi-

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**Fig. 2.** Effects of butein on urinary protein and plasma cholesterol in crescentic-type anti-GBM nephritis in rats. Test drugs were given p.o. daily during the period from 1 day after i.v. injection of anti-GBM serum to 40 days. ○ and a: normal (n=5), ● and b: nephritic control (n=8), △ and c: nephritis+butein (2 mg/kg/day, p.o.) (n=8), ▲ and d: nephritis+butein (20 mg/kg/day, p.o.) (n=8), ■ and e: nephritis+butein (200 mg/kg/day, p.o.) (n=8), and f: nephritis+dipyridamole (100 mg/kg/day, p.o.) (n=8). Each plot and column denotes the mean±S.D. of mg/day and mg/dl, respectively. *P<0.05, compared to the nephritic control.
tion, at 40 days, butein at 200 mg/kg had inhibited the increase of plasma cholesterol content by 49%.

**Histological observation in crescentic-type anti-GBM nephritis (Figs. 3 and 4)**

Light microscopic examination of the nephritic glomeruli revealed lesions characterized by severe crescent formation, adhesion and fibrinoid necrosis. Histological observation demonstrated that butein inhibited the incidence of crescent formation and adhesion in the glomeruli at 40 days. The crescent formations of dipyridamole-treated nephritic rats were also less than those of the nephritic control rats.

**Plasma antibody level against rabbit-IgG and deposition of rat-IgG in crescentic-type anti-GBM nephritis (Table 1)**

Nephritic rats have been shown to have markedly accelerated antibody production. The accelerated antibody production was not affected by butein and dipyridamole. It was possible to observe rat-IgG deposits on the GBM in

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**Fig. 3.** Light micrographs of glomeruli from rats of the normal group (a), nephritic control group (b), group given butein (200 mg/kg/day, p.o.) (c). Butein was given from 1 day after i.v. injection of anti-GBM serum. The rats were examined at 40 days after i.v. injection of anti-GBM serum (Masson's trichrome stain × 400). Note that crescent formation is markedly less in the group treated with butein than in the nephritic control group.
nephritic control rats; however, they were not present in normal rats. Butein and dipyridamole did not affect the rat-IgG deposition.

**Effect of butein on the accumulation of leukocytes in nephritic glomeruli in crescentic-type anti-GBM nephritis (Figs. 5 and 6)**

On day 15, the number of total leukocytes and the numbers of each leukocyte subpopulation in the glomeruli were markedly greater in the nephritic control rats than in the normal rats. Butein at 200 mg/kg markedly suppressed the increase in total leukocytes, CD4-positive cells and CD8-positive cells by 22%, 43% and 41%, respectively. However, Butein failed to suppress the accumulation of ED-1-positive cells in the nephritic glomeruli.

**Effect of butein on complement activity and the intraglomerular accumulation of leukocytes in original-type anti-GBM nephritis (Fig. 7)**

Original-type anti-GBM nephritis was induced in rats by the injection of 0.6 ml of anti-GBM serum into their tail veins, as described previously (2). Nephritic rats were pretreated (at 6 hr before i.v. injection of anti-GBM serum) with butein at 200 mg/kg or cyclosporin A at 20 mg/kg. Butein and cyclosporin A treatments reduced urinary protein excretion with an inhibitory percentage of 34% and 41%, respectively. The CH50 in nephritic control rats was markedly lower than that in the normal rats at 3 hr after nephritis induction. Butein or cyclosporin A did not inhibit the decreasing CH50 in nephritic control rats. On the other hand, butein and cyclosporin A inhibited...
the accumulation of leukocytes in the nephritic glomeruli at 3 hr by 44% and 33%, respectively.

DISCUSSION

Rapidly progressive glomerulonephritis and kidney disease in Goodpasture's syndrome are malignant diseases that lapse into renal failure in 2 or 3 months after the development of the diseases (27). Characteristics of this nephritis include hypercellularity, marked infiltration of neutrophils and monocytes, and crescent formation in the glomeruli. Cocktail therapy with immunosuppressive agents, anti-platelet drugs and steroids has been mainly applied to this disease. On the other hand, although cyclosporin A is a surpassing immunosuppressant and exerts a beneficial effect on experimental nephritis (11–13), it has been reported that cyclosporin A renders kidney dysfunction and causes irreversible histological alteration in the glomeruli when cyclosporin A treatment is carried out on a long term basis or a high concentration (15). Methylprednisolone causes many side effects. Furthermore, the rebound phenomenon and withdrawal syndrome after the long term treatment with this medicine are one of the problems. Therefore, a new immunosuppressive agent for the treatment of nephritis that has less side effects must be developed and natural products and plant components, which are the starting point for the development of drugs, have gained much attention.
Antinephritic Effect of Butein

Fig. 6. Effects of butein on the accumulation of leukocytes in the glomeruli with crescentic-type anti-GBM nephritis. Total leukocytes and leukocyte subsets were analyzed at 15 days after the anti-GBM serum injection. a: normal, b: nephritic control, c: nephritis + butein (200 mg/kg/day, p.o.). G.C.S.: glomerular cross section. Each column denotes the mean ± S.D. of 5 rats. *P < 0.05, **P < 0.01, compared to the nephritic control.

Fig. 7. Effect of butein on urinary protein excretion, plasma complement level (CH50) and the intraglomerular infiltration of leukocytes in original-type anti-GBM nephritis in rats. Butein (200 mg/kg, p.o.) or cyclosporin A (20 mg/kg, p.o.) were administered at 6 hr before anti-GBM serum injection (n=10). Urine was collected at 24 hr after the anti-GBM serum injection (n=5). a: normal, b: nephritic control, c: nephritis + butein, d: nephritis + cyclosporin A. G.C.S.: glomerular cross section. Each column denotes the mean ± S.D. of 5 rats. *P < 0.05, **P < 0.01, compared to the nephritic control.
This experimental model, crescentic-type anti-GBM nephritis, shows histologically and pathologically similar alterations to the rapidly progressive glomerulonephritis and kidney disease of Goodpasture's syndrome (28). The development and progression of this nephritis is 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 LTB4- or complement components-dependent accumulation of polymorphonuclear granulocytes (3, 4). The late phase, the so-called autologous phase, is developed by the binding of autologous antibody deposited along the GBM and the influx of monocytes/macrophages and T lymphocytes to the glomeruli following autologous antibody deposition (29, 30). This nephritis is characterized by the presence of biphasic urinary protein, crescent formation and fibrinoid necrosis (31). Crescent formation is thought to be mediated by migrated macrophages and proliferated epithelial cells (32).

Butein treatments suppressed the development of anti-GBM glomerulonephritis as assessed by the reduction of proteinuria and plasma cholesterol and the prevention of progressive histological changes including the development of glomerular crescents. Moreover, butein treated animals did not show the reduction of weight and the increase in plasma creatine content that was observed in nephritic rats treated with cyclosporin A (20 mg/kg, p.o.) (data not shown). However, butein failed to inhibit the reduction of CH50 in nephritic control rats (Fig. 7), to inhibit the production of the antibody against rabbit IgG, and to reduce the amount of rat-IgG deposition on GBM (Table 1). Therefore, although butein inhibits the development of renal injury, it is unlikely that the antinephritic action of butein is due to the suppression of complement activation and antibody formation.

On the other hand, the present study demonstrates that butein suppresses the accumulation of CD8-positive cells in the glomeruli with crescentic-type anti-GBM nephritis on day 15. The role of leukocytes has been well-established in the progression of anti-GBM nephritis in rats (21). The inhibition of progression of glomerulonephritis by the administration of anti-macrophage sera (33) or anti-CD8 mAb (10, 34) suggests that monocytes or CD8-positive cells may be one of main factors in the progression of glomerular disease. We previously reported that azathioprine (2), cyclosporin A (12, 13), and acteoside, a component of Stachys sieboldii MIQ (2, 13), inhibited the accumulation of leukocytes in the nephritic glomeruli and markedly inhibited the development of experimental nephritis. These results suggest that the antinephritic action of butein is, at least in part, due to the suppression of the influx of leukocytes into the glomeruli.

It has been reported that LTB4 is an important inflammatory mediator in the early stage of acute glomerulonephritis (35, 36). Brady et al. (37) demonstrated that LTB4 provokes monocyte adhesion to human mesangial cells and mesangial cell injury. Moreover, it has been reported that LTB4 induces in vitro a transient state of hyperadhesiveness in cultured endothelial cells for neutrophils (38, 39), and anti-CD54 (intercellular adhesion molecule-1, ICAM-1) mAb and anti-CD18 mAb suppresses the LTB4-induced hyperadhesiveness (40). Recently, with respect to the development of experimental and human nephritis, it has been reported that the up-regulation of ICAM-1 expression was observed in the glomeruli (41, 42), and antibody against ICAM-1 suppressed the urinary protein by reducing the glomerular influx of neutrophils (43). On the other hand, it has been reported that renal injury can be significantly reduced by treatment of animals with an inhibitor of leukotriene biosynthesis (22). Sogawa et al. (18) reported that various hydroxychalcones suppressed 5-lipoxygenase activity. In this study, butein suppressed the accumulation of leukocytes in the nephritic glomeruli at 3 hr after the induction of original-type anti-GBM nephritis. These above results suggest that suppression of the influx of leukocytes into the glomeruli is, at least in part, due to the inhibition of LTB4 production.

Butein includes caffeic acid in its structure. Caffeic acid derivatives inhibited lymphocyte tyrosine kinase in an in vitro study (44). Moreover, it is reported that there are various protein kinase-mediated pathways in the intracellular signal transduction for TNF-α-induced ICAM-1 expression (45). Therefore, it is speculated that butein inhibits the accumulation of leukocytes in nephritic glomeruli by suppressing ICAM-1 expression through the inhibition of inflammatory cytokines-mediated signal transduction.

In conclusion, we have shown that butein is a potent and effective anti-nephritic agent and inhibits the intraglomerular leukocyte infiltration. The antinephritic mechanisms of butein remain unclear. Therefore, in a further study, we are going to investigate the effect of butein on the LTB4 production and expression of adhesion molecules in the nephritic glomeruli.

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