IMMUNOPHARMACOLOGICAL STUDIES OF THE AQUEOUS EXTRACT OF CINNAMOMUM CASSIA (CCAq) II. EFFECT OF CCAq ON EXPERIMENTAL GLOMERULONEPHRITIS

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Abstract—Effect of the aqueous extract of Cinnamomum Cassia (CCAq) on experimental glomerulonephritis was studied and compared with that of cobra venom factor (CoVF). In rat nephrotoxic serum (NTS) nephritis, CCAq clearly inhibited the excretion of protein into the urine and the increase of peripheral leucocyte counts. The histological score in CCAq administered animals was significantly lower than that in control animals. However, CCAq did not inhibit or lower the serum complement level. Contrary to CCAq, hypocomplementation was observed by the administration of CoVF, and the excretion of protein into urine was inhibited in a high dose group. In immune complex (IC) and autologous IC nephritis in rats, CCAq clearly inhibited the excretion of protein into urine and the elevation of blood urea nitrogen (BUN). The administration of CoVF caused hypocomplementation, but little inhibition of the excretion of urinary protein was observed in both types of immune complex nephritis. The histological score was slightly inhibited by a low dose of CCAq and a high dose of CoVF. In the experiment employing NZB/NZW F1 mice, the proteinurea, the elevation of BUN level, and the production of antibodies were clearly inhibited by the administration of CCAq. Similar inhibition was observed by CoVF at a high dose. However, the histological changes of the kidney in NZB/NZW F1 mice were not prevented by the administration of CCAq or CoVF.

In a previous paper, we reported the inhibitory actions of the aqueous extract of Cinnamomum Cassia (CCAq) on complement dependent allergic reactions in vitro and in vivo (1). According to Johnson, anti-complement agents should be useful not only for treating transplantation rejection, but also for alleviating autoimmune disease or immune complex (IC) disease caused by the activation of complement system (2). In spite of the above proposal, there have been no experimental or clinical studies in which an attempted application of anti-complement drug for the treatment of the above diseases was successful.

There are many evidences that complement
participates in the production of tissue damage in glomerulonephritis (3, 4). Therefore, an application of an anti-complement agent to the treatment of glomerulonephritis was planned. The present paper describes the effect of CCAq on some type of experimental glomerulonephritis.

MATERIALS AND METHODS

Animals: Male Wistar rats weighing 150–200 g and female New Zealand Black (NZB) and New Zealand white (NZW) F₁ mice were used. (NZB×NZW) F₁ mice were kindly supplied by Dr. T. Nomura, Nippon Shinyaku Co. Ltd.

Drug: CCAq was prepared by the previously described method (1). In brief, 100 g of finely cut Cinnamomum Cassia was extracted with 1 l of distilled water by heating at 60°C for 4 hr. After filtration, the extract was concentrated by evaporation at a temperature under 30°C. The extract was kept at -20°C until use. Cobra venom factor (CoVF) was purchased from Cordis Laboratories, Fla., U.S.A.

Nephrotoxic serum (NTS) nephritis in rats: The preparation of NTS and production of NTS nephritis were done by the same method previously reported (1). NTS was obtained from rabbits immunized by injection of the glomerular basement membrane rich sediment of rat kidney emulsified with complete Freund’s adjuvant (CFA). Anti-serum was extensively absorbed with homologous erythrocytes after heating at 56°C for 30 min. NTS nephritis was caused by the single i.v. injection of 1.0 ml NTS into rats. In order to evaluate the severity of the symptoms, urine and blood samples were collected at 1, 3, 5, 8, 10, and 12 days after NTS injection. The amount of urinary protein was determined quantitatively by employing 3% sulfosalicylate according to the method of Kingsbury et al. (5). Serum complement level was measured by an ultramicro titration method according to the method of Irie et al. (6). Pathological changes of the kidney was assessed in a semiquantitative fashion as described by Litwin et al. (7).

IC nephritis in rats: IC nephritis caused by repeated injection of bovine serum albumin (BSA) was evoked by the method of Yamamoto et al. (8). Rats were immunized by injection of 2.5 mg BSA emulsified with 0.5 ml CFA into the back and hip muscle. Eight weeks later, 1 mg BSA dissolved in 1 ml saline was injected into the rats every day for 4 weeks. The amount of urinary protein, serum complement level, and leucocyte count were measured once a week. Light microscopical study was done 5 weeks after the daily injection of antigen.

Autologous IC nephritis in rats: Autologous IC nephritis was caused by a single footpad injection of renal tubular epithelial antigen suspended in CFA. Nephritogenic tubular epithelial antigen was prepared by the method of Spiro (9). Thin kidney slices from freshly killed rats were forced through 150 mesh stainless steel sieves. The material which emerged through the sieve was collected in phosphate buffered saline (pH 7.4) without Ca²⁺ and Mg²⁺ and centrifuged at 400 g at 4°C for 10 min. Supernatant was obtained as a tubular antigen after lyophylization. The severity of symptoms in nephritis was measured by collecting urine and blood samples once a week after the injection of antigen. The amounts of urinary protein and serum complement level were measured by the same method as described above. Cholesterol was measured using acetic acid anhydrate and sulfuric acid according to the method of Zurkowski (10), and blood urea nitrogen (BUN) was measured by urease-indophenol method as described by Saito et al. (11). Pathological studies was done as described above.

Spontaneous nephritis in (NZB×NZW) F₁ mice: Fifteen-week-old female (NZB×NZW)
F₁ mice with less than 30 mg/dl proteinuria were employed. The effect of drugs was assessed by measuring the amount of urinary protein, antinuclear antibody (ANA), serum BUN, and hematocrit value. The number of animals possessing ANA and a hematocrit below 30% was counted. Protein in the urine was measured by using a test paper containing tetrabromophenol blue (Combi sticks II, Miles Lab.). ANA was measured by the immunofluorescent antibody technique employing chicken red blood cells. BUN was measured by the same method as described before.

Statistics: Results were statistically evaluated using the Student’s t-test. In histopathological studies, statistical significance was tested by Wilcoxon’s U-test.

RESULTS

NTS nephritis in rats: The effects of CCAq and CoVF on the development of the disease process caused by NTS are indicated in Figs. 1 and 2. As evident in Fig. 1, the amount of urinary protein in the control rats increased on the 1st day, decreased on the 3rd day, and again increased gradually until the 12th day. A biphasic decrease in serum complement level and a gradual increase in peripheral leucocyte count were observed. By the administration of CCAq at doses of 10 and 50 mg/kg, the increase in urinary protein was inhibited. CCAq at both doses showed a tendency toward inhibition of the increase in leucocyte count. A decrease in complement level was significantly influenced by CCAq.

Fig. 1. Effect of CCAq on NTS nephritis in rats. CCAq was administered p.o. for 10 days after the injection of antiserum. Results represent the means of 6 to 8 animals. The standard error is not shown for clarity, but it is less than 33.4% of the mean value in all points. *: P<0.05 and †: P<0.01, significantly different from the control. ○: Control. ●: 10 mg/kg CCAq. △: 50 mg/kg CCAq.

Fig. 2. Effect of CoVF on NTS nephritis in rats. CoVF was administered i.p. for 10 days after the injection of antiserum. Results represent the means of 4 to 8 animals. The standard error is not shown for clarity, but it is less than 35.7% of the mean value in all points. *: P<0.05 and †: P<0.01, significantly different from the control. ○: Control. ●: 1 U/kg CoVF. △: 10 U/kg CoVF.
CoVF at a dose of 10 U/kg inhibited the increase in the urinary protein. The complement level was lowered below the control level by the administration of CoVF, but CoVF did not affect the changes in complement level by NTS. The histological scores in the drug administered groups, except the CoVF group, given 1 U/kg were significantly lower than that of the control group (P<0.05).

**IC nephritis in rats:** The effects of CCAq and CoVF on the development of IC nephritis are indicated in Figs. 3 and 4. In the control animals, the amount of urinary protein increased following the repeated injection of antigen and reached the maximum at 4 weeks later after the onset of repeated injection. The serum complement level decreased, and the peripheral leucocyte count increased biphaseically. The increase in urinary protein was clearly inhibited by the administration of CCAq and CoVF. However, the effects of drugs on the changes in complement level and leucocyte counts were inconsistent. In the histopathological studies of kidney, there were no significant difference between the control and each drug administered group.

**Autologous IC nephritis in rats:** The effects of CCAq and CoVF on the develop-

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**Fig. 3.** Effect of CCAq on immune complex nephritis in rats. CCAq was administered p.o. every other day for four weeks. Results represent the means of 5 to 8 animals. The standard error is not shown for clarity, but it is less than 48.2% of the mean value in all points. *: P<0.05 and †: P<0.01, significantly different from the control. ○: Control, ●: 10 mg/kg CCAq, △: 50 mg/kg CCAq.

**Fig. 4.** Effect of CoVF on immune complex nephritis in rats. CoVF was administered i.p. every other day for four weeks. Results represent the means of 5 to 8 animals. The standard error is not shown for clarity, but it is less than 46.7% of the mean value in all points. *: P<0.05 and †: P<0.01, significantly different from the control. ○: Control, ●: 10 mg/kg CCAq, △: 50 mg/kg CCAq.
ment of autologous IC nephritis in rats are shown in Figs. 5 and 6. In the control animals, the amount of urinary protein increased 6 weeks after the injection of antigen and reached the maximum 10 weeks later. Significant increase in BUN level was observed at 4, 8, and 10 weeks. Serum complement level gradually decreased and

Fig. 5. Effect of CCAq on autologous immune complex nephritis in rats. CCAq was administered p.o. every other day for 10 weeks. Results represent the means of 5 to 6 animals. The standard error is not shown for clarity, but it is less than 31.7% of the mean value in all points. *: P<0.05 and †: P<0.01, significantly different from the control. ○: Control, ●: 10 mg/kg CCAq, △: 50 mg/kg CCAq.

Fig. 6. Effect of CoVF on autologous immune complex nephritis in rats. CoVF was administered i.p. every day for 10 weeks. Results represent the means of 5 to 6 animals. The standard error is not shown for clarity, but it is less than 22.1% of the mean value in all points. *: P<0.05 and †: P<0.01, significantly different from the control. ○: Control, ●: 1 U/kg CoVF, △: 10 U/kg CoVF.
reached the minimum 8 weeks later. Cholesterol level increased first at 4 weeks and then gradually decreased. The increases in urinary protein and BUN level were significantly inhibited by CCAq. The decrease in complement level due to NTS injection was slightly inhibited. However, the change in complement level was not affected by CCAq. CoVF at a dose of 1 U/kg showed a slight inhibition of urinary protein and BUN augmentation, but did not affect the changes in complement and cholesterol levels. CoVF at a dose of 10 U/kg caused a greater fall in the complement level than in the control group. Histopathological scores in the groups given 10 mg/kg CCAq and 10 U/kg CoVF were significantly lower than that in the control (P<0.01). With the exception of the above two groups, there were no differences between the control and other groups.

Nephritis in (NZB x NZW) F1 mice: The effects of CCAq and CoVF on the development of glomerulonephritis in (NZB x NZW) F1 mice are shown in Figs. 7 and 8. In the control mice, the excretion of urinary protein increased in a biphasic fashion, and BUN increased gradually during the 4th. to 10th. month except for the 7th. month. The mice with hematocrit values below 30% were 10% of the animals at 6 months and then increased to 30%. The administration of CCAq in doses of 10 to 50 mg/kg twice a week for 4 to 10 months caused the inhibition of the increases in urinary protein, BUN level, and ANA production. CCAq in a dose of 50 mg/kg inhibited the increase in the number of animals with a hematocrit disorder. CoVF at a dose of 10 U/kg showed a slight inhibition of the increase in each of the parameters, but 1 U/kg CoVF had no influence on them. The histological scores in the control and drug administered groups were not significantly different.

**DISCUSSION**

Under normal conditions, the complement system is a potent natural defense mechanism of the host. However, in certain diseases,
glomerulonephritis or autoimmune diseases, this system is disadvantageous because of the generation of inflammatory fragments or the production of tissue damage. Therefore, whether a complement inhibitor can be used for a therapeutic agent or not was investigated. From the present results, the efficacy of CCAq on experimental glomerulonephritis is obvious. However, it is obscure whether the efficacy of CCAq is based upon its anti-complement activity or not. CCAq had no effect on the hemolytic complement activity in serum as well as no influence on the decrease in complement activity due to the development of the disease. There are many reports showing the correlation between serum hemolytic complement activity and the development of nephritis (12–15). In spite of many efforts, a clear conclusion as to the role of complement in nephritis has not yet been obtained. Unanue (14) has reported that the rats injected with antibodies to complement, heat-aggregated γ-globulin or zymosan, are temporarily depleted of complement and do not develop NTS nephritis. On the other hand, Rother et al. (16, 17) have reported that rabbits and mice deficient in the sixth and fifth components of complement, respectively, when injected with NTS, develop NTS nephritis as do normal animals. These contradictory findings indicate that measurement of the hemolytic activity of serum complement is not a useful method for determining the role of complement in the glomerulonephritis. For elucidating this problem, it is considered better to detect the complement or its component on the glomerulus by C1a fixation and use the transfer method or the fluorescence method (18). If the number of complement molecules can be detected by the above methods, the mechanisms of disease and drug action will be clarified more precisely. This should be done in further experiments.

The role of complement in IC nephritis caused by BSA or tubular antigen has

Fig. 8. Effect of CoVF on the nephritis in (NZB×NZW) F, mice. CoVF was administered i.p. twice a week for 12 months. Results in proteinuria and BUN represent the means of 4 to 15 animals. Results in ANA and HT are expressed as the percentage incidence. The standard error is not shown for clarity, but it is less than 29.3% in all points. *, P<0.05 and †, P<0.01, significantly different from the control. ○, Control. ●: 1 U/kg CoVF, △: 10 U/kg CoVF.
mainly been investigated by immunofluorescence antibody techniques. This may be one of the important evidences to support that the injurious process involves the complement system. In the present study, CoVF showed the decreases in serum complement level and remission of diseases. Therefore, the participation of the complement in the development of diseases is evident. The inhibitory action of CCAq on IC nephritis seems to be based upon the inhibition of complement activity.

In NZB/NZW mice, the existence of complement along the glomerular basement membrane has been demonstrated by immunofluorescence studies (19, 20). There have been some reports employing glucocorticoids or immunosuppressive drugs as a remedy for glomerulonephritis in NZB/NZW F1 mice (21). However, there are few reports in which the use of an anti-complement drug was attempted (22). The present data indicate the efficacy of CCAq on the glomerulonephritis in NZB/NZW F1 mice. It has a similar potency to glucocorticoids or immunosuppressive drugs. These findings may indicate the possibility for applying anti-complement drugs to SLE. However, because of the conflicting results on CoVF, more experiments employing various anti-complement drugs should be done before a definite conclusion can be reached.

In conclusion, the presented results suggest that CCAq may be useful for a treatment of glomerulonephritis. Further experiments will be done with effective substances isolated from CCAq.

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