IMMUNOPHARMACOLOGICAL STUDIES OF SODIUM COPPER CHLOROPHYLLIN (SCC)

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Abstract—Effect of sodium copper chlorophyllin (SCC) on experimental allergic reaction was investigated. IgE antibody mediated reactions, homologous passive cutaneous anaphylaxis (PCA) in rats and the release of anaphylactic mediators (histamine and/or slow reacting substance of anaphylaxis (SRS-A)) from sensitized guinea pig lung tissues or rat peritoneal mast cells classified as a Type I reaction were clearly inhibited by SCC at a similar potency as N-(3',4'-dimethoxy cinnamoyl) anthranilic acid (N-5'). The increase of vascular permeability in rat skin caused by autacoids or enzymes that participate in the Type I reaction was also inhibited by SCC. Type II or III, complement dependent, reactions including reversed cutaneous anaphylaxis (RCA) in rats and Forssman cutaneous vasculitis (FCV) in guinea pigs were inhibited by SCC. Prednisolone inhibited RCA in rats, but did not inhibit FCV in guinea pigs. Two experimental types of glomerulonephritis, nephrotoxic serum (NTS) nephritis in rats and immune complex nephritis in (NZW×NZB) F₁, mice, in which Type II and III reactions might participate in the onset and the development of the disease, were slightly inhibited by SCC in terms of the biochemical changes of blood and urine parameters and histopathological scores. A moderate remission of the onset and development of these two experimental types of nephritis was recognized by the administration of prednisolone. Delayed hypersensitivity reaction as a Type IV reaction caused by sheep red blood cells (SRBC) in sensitized mouse footpad was not affected by SCC. Prednisolone clearly inhibited the SRBC induced footpad reaction in mice. IgM antibody production in mice and IgE antibody production in rats were not influenced by daily injection of SCC.

Sodium copper chlorophyllin (SCC) is one of the water soluble derivatives of chlorophyll. Chlorophyll has been used orally to reduce odors and topically to promote the healing of skin lesions. Pharmacological actions of SCC so far reported are mainly anti-microbial (1), anti-oxidant (2, 3), anti-mutagenic (4) and anti-complement (5–8) activities. In connection with anti-complement action, the inhibition of anaphylactic shock was reported (6–8). It is therefore considered of interest to investigate the effect of SCC on allergic reactions in experimental animals.

Materials and Methods

Animals: Male ddY mice weighing 18 to 20 g, male Wistar rats weighing 120 to 180 g and male Hartley guinea pigs weighing 350 to 400 g were used. (NZW×NZB) F₁ mice were kindly supplied by the Nippon Shinyaku Co. Ltd., Kyoto, Japan.

Drugs: SCC was kindly provided by Eisai Pharmaceutical Co. Ltd., Tokyo, Japan. N(3',4'-dimethoxy cinnamoyl) anthranilic acid (N-5') was kindly provided by Takeda Chemical Industries Ltd., Osaka. Prednisolone was kindly provided by Eli Lilly and Co., Indianapolis, Indiana.
4'-dimethoxycinnamoyl) anthranilic acid (N-5') was given by Kissei Pharmaceutical Co. Ltd., Matsumoto, Japan. Prednisolone acetate was purchased from Shionogi Co. Ltd., Osaka, Japan.

**Antiserum:** Rat homocytotropic antibody against dinitrophenylated ascaris (DNP-As) was obtained from rats that had been immunized with DNP-As mixed with killed *Bordetella Pertussis* according to Tada and Okumura (9). Guinea pig homocytotropic antibody was made by the method of Levine et al. (10) using benzylpenicilloyl bovine γ-globulin (BPO-BGG) mixed with alum as the antigen and adjuvant, respectively. Rabbit antibody to rat serum was obtained from rabbits which had been immunized by injecting 1.0 ml of rat serum i.v. every other day for a total of 10 doses. Hemolysin (Forssman antibody) was prepared by the same procedure as the antibody to rat serum by injecting $10^9$ sheep red blood cells (SRBC) instead of rat serum. Nephrotoxic serum was obtained from rabbits immunized by injecting the glomerular basement membrane rich sediment of rat kidney emulsified with complete Freund's adjuvant. Antiserum was extensively absorbed with homologous erythrocytes after heating at 56°C for 30 min.

**Type I reactions:** Homologous passive cutaneous anaphylaxis (PCA) reaction was induced in rats by injecting 0.1 ml of diluted anti-DNP-As antibody intradermally 48 hr before intravenous injection of 2.0 mg of DNP-As and 1.0 ml of 0.25% Evans blue solution as described before (11). After 30 min, the reaction was evaluated by measuring the amount of extravasated dye. The amount of dye was extracted from skin with 1.0 N KOH and a mixture of acetone and phosphoric acid by the method of Katayama et al. (12). Antigen induced histamine release from mast cells was caused by the addition of antigen (finally $10^{-5}$ g/ml) to a suspension of $10^5$ sensitized peritoneal mast cells isolated from rats which were sensitized by the i.p. injection of 1.0 ml anti-DNP-As homocytotropic antibody 24 hr prior to harvesting the cells. After incubation of the cells with antigen for 20 min, the histamine released into the supernatant was quantitated by a biological assay with isolated guinea pig ileum. Histamine and the slow reacting substance of anaphylaxis (SRS-A) release was measured by the methods previously described (13, 14). Guinea pigs were passively sensitized by intravenous injection of antiserum containing homocytotropic antibody against BPO-BGG. The reaction was caused by the addition of antigen (finally $10^{-4}$ g/ml) into a suspension of 500 mg chopped lung tissue in the presence or absence of drugs. The amount of histamine and SRS-A in the incubation medium was assayed by using isolated guinea pig ileum. The antagonistic effect of mediators was examined by measuring the effect of drugs on the increase of capillary permeability caused by injecting serotonin, histamine, phospholipase A$_2$, hyaluronidase and trypsin into rat shaved back skin. The reaction was evaluated by measuring the amount of extravasated dye as was measured in PCA.

**Types II and III reactions:** Reversed cutaneous anaphylaxis (RCA) in rats and Forssman cutaneous vasculitis (FCV) were done according to a previously described method (11, 13). RCA was caused by injecting 14% rabbit antiserum against rat serum into shaved rat skin. Two hours later, the weight of a skin disc, which was cut out using a leather punch (12 mm in diameter), was measured. FCV was caused by injecting 0.1 ml of 10% Forssman antibody into guinea pig shaved back skin, followed by an i.v. injection of 1.0 ml of 0.1% Evans blue. The blueing spot was measured by the same method as was used for PCA. As another experimental model for Types II and III reactions, two kinds of experimental
glomerulonephritis were studied. Nephrotoxic serum nephritis was caused by the single i.v. injection of 1.0 ml NTS into rats as described before (15). 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% sulfosalicycylate according to the method of Kingsbury et al. (16). Serum complement level was measured by an ultramicro titration method according to the method of Irie et al. (17). Pathological changes in the kidney were assessed in a semiquantitative fashion after staining with hematoxylin and eosin or periodic acid-Schiff according to the method of Litwin et al. (18). The scoring was done on the findings of crescent formation, deposition of fibrin or fibrinoid substance in capillary, adhesion of glomerular tufts and hypercellularity in glomerus. Another experimental glomerulonephritis was immune complex nephritis in (NZW×NZB) F1 mice. Fifteen week-old female (NZW×NZB) F1 mice with less than 30 mg/dl proteinuria were employed for testing the effect of drugs. The severity of symptoms was measured in terms of the amount of urinary protein, serum BUN and percent incidence of survivals. Protein in the urine was measured by using a test paper containing tetrabromophenol blue (Combi sticks II, Miles Lab.). Blood urea nitrogen (BUN) was measured by the urease-indophenol method as described by Saito et al. (19). With relation to Types II and III reactions, effects of drugs on the hemolytic activity of complement and the generation of superoxide anion from leukocytes were examined according to the previously described method (11). In brief, 5×10⁶/ml SRBC sensitized with hemolysin were added to the mixture of 5.5 ml gelatin veronal buffer and 1.0 ml guinea pig complement, adjusted as 1 unit of CH50, and incubated at 37°C for 1 hr. The reaction was stopped by immersing the test tubes into ice water. The mixture was centrifuged, and the supernatant was assayed colorimetrically at 541 nm. The inhibition percent was calculated by the ratio of hemolytic activity in the presence or absence of drug. For the experiments of superoxide anion generation, the procedure described by Nakagawara et al. was followed (20). In brief, 5×10⁵ rat peritoneal leukocytes were preincubated with cytochalasin E (5 μg/ml) and cytochrome c (80 μM) at 37°C for 10 min. Wheat germ agglutinin (100 μg/ml) was then added and incubated for 5 min. Cytochrome c reduction was determined at 550–540 nm by a Hitachi 556 double beam spectrophotometer.

Type IV reaction: Delayed type footpad reaction was caused by the method of Lagrange et al. (21). In brief, mice were immunized by injecting 10⁷ SRBC in a volume of 40 μl into one hind footpad. Five days later, hypersensitivity was measured as the increase in footpad thickness 24 hr after injecting an eliciting dose of 10⁸ SRBC in a volume of 40 μl into the other hind footpad.

Antibody production: IgM antibody production in mice was examined by counting the hemolytic plaque forming cells (HPFC) in spleen by the method of Cunningham (22). The production of IgE antibody was tested by the measuring homocytotropic antibody titre in the serum which was obtained from the immunized rats by injection of DNP-As and B. pertussis as described before (23).

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

Results

Type I reaction: The amount of extravasated dye in the PCA site was calculated by subtracting that in the normal site. As shown in Table 1, SCC inhibited the rat homologous
PCA reaction in a dose dependent manner. N-5' also inhibited the reaction. The inhibitory activity of SCC is less than that of N-5'. Antigen induced histamine release from guinea pig lung tissues or rat peritoneal mast cells were significantly decreased by SCC (Table 2). A similar inhibition was observed by the treatment with N-5'. The release of SRS-A from guinea pig lung tissue was inhibited by SCC, but not inhibited by N-5'. No release of SRS-A was indicated by the addition of antigen to sensitized rat peritoneal mast cells. The increase of vascular permeability caused by serotonin, histamine, phospholipase A_2, hyaluronidase or trypsin in rat skin was inhibited by SCC in a dose response manner (Table 3).

**Types II and III reactions:** The swelling caused by RCA and dye leakage caused by FCV were inhibited by administration of SCC (Table 4). Prednisolone inhibited RCA, but did not affect FCV. In the studies of NTS nephritis in rats, the increases of proteinuria and white blood cell in nephritic rats were inhibited by the administration of SCC. (Fig. 1). The changes of complement, however, were not influenced. The histological scores between control and SCC administered

### Table 1. Effect of SCC and N-5' on 48 hr homologous PCA in rats

| Dose (mg/kg) | N   | Amount of dye (μg/site) |
|--------------|-----|-------------------------|
| Control      | 17  | 10.6±1.12               |
| 50           | 16  | 7.4±0.54†               |
| SCC          | 100 | 7.0±0.58†               |
| 200          | 14  | 4.3±0.44†               |
| Control      | 6   | 9.9±1.46                |
| N-5'         | 25  | 6.7±0.38*               |
| 50           | 6   | 4.8±0.47†               |

Each value represents the mean±S.E. SCC was administered i.p. 2 hr prior to challenge. N-5' was administered i.p. 1 hr prior to challenge. †: P<0.05, *: P<0.01.

### Table 2. Effect of SCC and N-5' on histamine and SRS-A release from guinea pig lung tissues and rat peritoneal mast cells

| Concentration (g/ml) | Histamine a (ng) | SRS-A b (μ) |
|----------------------|------------------|-------------|
| Control              | 120.6±6.35       | 23.7±1.93   |
| SCC 10^{-7}          | 88.1±4.61*       | 11.1±0.60†  |
| A SCC 10^{-6}        | 78.4±2.44†       | 6.6±0.22*   |
| 10^{-2}              | 68.2±2.89*       | 6.5±1.71†   |
| N-5' 10^{-4}         | 89.8±8.51†       | 17.2±2.71†  |
| Control              | 58.4±3.58        | ND          |
| 10^{-7}              | 49.5±2.20        | ND          |
| B SCC 10^{-8}        | 39.5±0.87†       | ND          |
| 10^{-5}              | 31.5±2.18†       | ND          |
| N-5' 10^{-4}         | 33.1±3.33†       | ND          |

A: Guinea pig lung tissues, B: Rat peritoneal mast cells. Each value represents the mean±S.E. of 4 experiments. SCC and N-5' were incubated with tissues at 37°C for 20 min in each case. a: The amount of released histamine was expressed ng/100 mg lung tissues or ng/5×10^5 rat peritoneal mast cells. b: A SRS-A unit was calculated from a standard curve with 5×10^{-9} g/ml histamine as 1 unit. ND: not done. †: P<0.05, *: P<0.01.
Table 3. Effect of SCC on dye leakage by the capillary permeability factor in rats

| Dose (mg/kg) | Serotonin | Histamine | Amount of dye (µg/site) | Hydase | Trypsin |
|-------------|-----------|-----------|-------------------------|--------|---------|
| Control     | 4.4±0.47  | 4.1±0.38  | 6.3±0.44                | 7.4±0.70 | 4.5±0.32 |
| 50          | 4.0±0.38  | 3.0±0.31* | 4.0±0.30†               | 5.4±0.44† | 3.4±0.25† |
| SCC         | 100       | 3.2±0.43* | 3.1±0.21*               | 3.6±0.40† | 6.2±0.42 | 2.6±0.30† |
| 200         | 2.6±0.51† | 2.6±0.25† | 2.5±0.37†               | 4.7±0.34† | 2.3±0.27† |

Each value represents the mean±S.E. of 12 to 14 animals. SCC was given in doses of 50, 100 and 200 mg/kg i.p. 2 hr prior to the s.c. injection of factors. Each animal was given intradermally the following substances in 0.1 ml saline at different sites on the back: serotonin, 10 µg/ml; histamine, 50 µg/ml; phospholipase A₂, 10 U/ml; hyaluronidase, 250 TRU/ml; trypsin, 50 µg/ml. Statistical significance was calculated by the t-test: *: P<0.05, †: P<0.01.

Table 4. Effect of SCC and prednisolone on RCA in rats and FCV in guinea pigs

| Dose (mg/kg) | N | RCA (% of edema) | N | FCV (µg/site) |
|-------------|---|------------------|---|--------------|
| Control     | 7 | 88.6±9.97        | 8 | 4.4±6.53     |
| 50          | 7 | 58.6±3.82*       | 8 | 3.5±0.45     |
| SCC         | 100 | 65.1±5.45*     | 4 | 2.6±0.50*    |
| 200         | 6 | 64.5±4.75*       | 8 | 2.1±0.27†    |

Each value represents the mean±S.E. SCC and prednisolone were administered i.p. 2 hr prior to challenge. *: P<0.05, †: P<0.01.

Table 5. Effect of SCC and prednisolone on hemolytic activity of guinea pig complement and generation of superoxide anion in rat neutrophil

| Concentration (g/ml) | Complement (% to CH₅₀) | O₂⁻ (%) to control |
|----------------------|------------------------|--------------------|
| Control              | 100                    | 100                |
| SCC                  | 10⁻⁷                   | 92.2±0.70          | 125.0±32.5        |
|                      | 10⁻⁴                   | 82.3±2.37          | 75.0±22.5         |
|                      | 10⁻⁵                   | 62.8±1.99          | 37.5±13.3         |
|                      | 10⁻⁴                   | 17.5±0.76          | 0                 |
| Pred                 | 10⁻⁶                   | 99.5±5.92          | 82.0±3.63         |
|                      | 10⁻⁴                   | 74.3±15.07         | 93.3±7.81         |

Each value represents the mean±S.E. of 4 experiments. Complement or neutrophil was incubated with SCC at 20°C for 20 min prior to testing.

groups were not significantly different. Prednisolone at doses of 5 and 10 mg/kg showed a clear remission of the development of nephritis (Fig. 2). The histological score in prednisolone is lower than that of the control. In (NZW×NZB) F₁ mice, increases of each parameter were slightly inhibited by the administration of SCC in a dose of 50 mg/kg, but not 10 mg/kg (Fig. 3). The histological score of the SCC administered group was not significantly different from that of the control group. Prednisolone showed a slight
Fig. 1. Effect of SCC on NTS nephritis in rats. SCC was administered i.p. for 12 days after the injection of antiserum. Results represent the mean of 6 to 8 animals. The standard error is not shown for clarity, but it is less than 38.5% of the mean value in all points. *: P<0.05, #: P<0.01. ○: Control, ●: 10 mg/kg SCC, △: 50 mg/kg SCC.

Fig. 2. Effect of prednisolone on NTS nephritis in rats. Prednisolone was administered i.p. for 12 days after the injection of antiserum. Results represent mean of 6 to 10 animals. The standard error is not shown for clarity, but it is less than 32.8% of the mean value in all points. *: P<0.05, #: P<0.01. ○: Control, ●: 5 mg/kg prednisolone, △: 10 mg/kg prednisolone.

Table 6. Effect of SCC and prednisolone on delayed type hypersensitivity in mice

| Dose (mg/kg) | Administration before challenge | Administration after challenge |
|-------------|--------------------------------|-------------------------------|
|             | N | Footpad swelling (×10⁻² cm) | N | Footpad swelling (×10⁻² cm) |
| Control     | 6 | 6.2±0.43                    | 9 | 6.5±0.66                    |
| 50          | 8 | 6.2±0.54                    | 8 | 7.5±0.60                    |
| SCC         | 100 | 7.2±0.33                  | 7 | 8.5±0.83                    |
| 200         | 8 | 6.0±0.44                    | 8 | 6.0±0.54                    |
| Control     | 9 | 4.8±0.54                    | 9 | 3.8±0.66                    |
| 1           | 9 | 1.7±0.40†                   | 9 | 3.1±0.79                    |
| Prednisolone| 5 | 2.1±0.58†                   | 9 | 2.0±0.83†                   |
| 10          | 9 | 2.1±0.31†                   | 9 | 1.5±0.31†                   |

Each value represents the mean±S.E. †: Drugs were administered by daily i.p. injection for 5 days after immunization. ‡: Drugs were administered by i.p. injection at 0 and 18 hr after challenge. *: P<0.01.

remission of the development of disease. There is no significant difference between the histological score of the control group and that of the prednisolone administered group. In in vitro experiments, the pre-incubation of complement with SCC resulted in dose-dependent inhibition of immunological hemolysis (Table 5). The production of superoxide anion from rat neutrophils was significantly decreased by the coexistence of SCC in the experimental medium. Prednisolone had no effect on both
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Fig. 3. Effect of SCC on the nephritis in (NZW × NZB) F₁ mice. SCC was administered i.p. twice a week for 12 months. Results in proteinuria and BUN represent the means of 4 to 16 animals. Results in survivals are expressed as a percentage incidence. The standard error is not shown for clarity, but it is less than 43.9% of the mean value in all points. *: P<0.05, #: P<0.01. ○: Control, ●: 10 mg/kg SCC, △: 50 mg/kg SCC.

Fig. 4. Effect of prednisolone on the nephritis in (NZW × NZB) F₁ mice. Prednisolone was administered i.p. twice a week for 12 months. Results in proteinuria and BUN represent the means of 4 to 10 animals. Results in survivals are expressed as a percentage incidence. The standard error is not shown for clarity, but it is less than 36.6% of the mean value in all points. *: P<0.05, #: P<0.01. ○: Control, ●: 1 mg/kg, △: 10 mg/kg.

reactions.

Type IV reaction: SCC was administered before or after the injection of challenging antigen. Neither administration affected the swelling of footpad by the delayed type hypersensitivity reaction with SRBC (Table 6). Contrary to SCC, prednisolone inhibited the reaction in both cases.

Antibody production: The number of HPFC (IgM forming cell) per spleen or per 10⁶ spleen cells were not affected by SCC (Table 7). The production of IgE antibody was not influenced. Prednisolone inhibited both HPFC production and IgE antibody formation.

Discussion

In the present study, SCC showed an obvious inhibitory effect on the humoral antibody mediated hypersensitivity reaction, but not on the cell mediated hypersensitivity reaction. As for the inhibitory mechanisms of SCC, there are some reports that indicate the connection of anti-anaphylactic action and anti-complement activity (6–8). The present results indicated the possibility of other mechanisms in the anti-anaphylactic action of SCC because SCC showed clear inhibition of IgE antibody mediated, complement independent, reactions. The other mechanisms are related to the inhibition of mediator release and antagonisms to
Table 7. Effect of SCC and prednisolone on antibody production

|        | Dose (mg/kg) | 10^3/spleen | HPFC /10^6 cells | HTA PCA titre (log_2) |
|--------|--------------|-------------|-----------------|----------------------|
| Control| 50           | 140±27.1    | 732±11.43       | 7.7±0.47             |
| SCC    | 100          | 179±24.9    | 903±76.0        | 7.6±0.27             |
| SCC    | 200          | 154±39.4    | 834±219.9       | 7.3±0.48             |
| Prednisolone | 20   | 136±15.4    | 835±130.1       | 7.5±0.32             |
|        |              | 103±18.2*   | 575±82.5        | 6.7±0.21             |

Each value represents the mean±S.E. of 6 animals. Hemolytic plaque forming cells (HPFC) in mice spleen were counted 5 days after the immunization. Homocytotropic antibody (HTA) titres were assayed in the sera which were obtained from immunized rats on the 8th day after the immunization. Drugs were administered i.p. for 5 days after the immunization in both cases.

mediators. This mode of action of the anti-anaphylactic effect is similar to some other anti-allergic agents such as ketotifen, tiaramide and others (24-26), and the potency of the inhibition of the Type I reaction is nearly equivalent to that of N-5'-by i.p. administration. However, little inhibition of homologous PCA was observed by p.o. administration of SCC (unpublished data). These evidences suggest the possibility of the use of SCC as a remedy for the diseases caused by Type I mechanisms, but its use in therapy is restricted to an administration route other than the oral one.

In regards to Type II and III reactions, SCC inhibited RCA and FCV reactions, complement activity and generation of super oxide anion. These characteristics of the agent suggest the possibility for an application of the agent to the treatment of glomerulonephritis since the participation of Type II or III mechanisms, especially complement, are reported for the onset and development of glomerulonephritis by many workers (27-29). From the results of experimental glomerulonephritis in the present study, however, little efficacy of SCC was recognized. The results concerning the efficacy of an anti-complement agent on experimental glomerulonephritis in the present and previous studies are inconsistent (15).

Potent complement inhibitors, cobra venom factor which was investigated previously (15), and SCC were found to have little effect. The aqueous extract of *Cinnamonomum Cassia*, a moderate complement inhibitor, however, indicated a clear remission of the disease. These evidences suggest that more experiments are required to discuss the meaning of anti-complement agents as a remedy for glomerulonephritis.

In conclusion, SCC showed an inhibitory action against antibody mediated allergic reaction in experimental animals. In addition, SCC did not affect the cell mediated hypersensitivity reaction and antibody production. The mode of action for the anti-allergic action of SCC is related to the inhibition of anaphylactic mediator release, antagonisms to mediators, anti-complement activity and the inhibition of the generation of superoxide anion. Further work will be done to investigate the molecular mechanisms of these inhibitions, and the possibility for the application of SCC to allergic diseases.

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