Rubus croceacanthus Leveille Inhibits Mast Cell-Mediated Anaphylactic-Like Reaction and Tumor Necrosis Factor- α Secretion

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This work aims at examining the effect of the concentrated methanol extract of *Rubus croceacanthus* Leveille (RCL) on mast cell-mediated anaphylactic-like reaction in a murine model. RCL inhibited compound 48/80-induced systemic anaphylactic-like reaction. When RCL was given as pre-treatment at concentrations ranging from 0.01 to 1 mg/ml, the histamine release from rat peritoneal mast cells induced by compound 48/80 or anti-dinitrophenyl (DNP) immunoglobulin E (IgE) was reduced in a dose-dependent manner. RCL also inhibited passive cutaneous anaphylaxis activated by anti-DNP IgE. In addition, RCL inhibited phorbol 12-myristate 13-acetate and A23187-induced tumor necrosis factor- α secretion from human mast cell line HMC-1 cells. These results indicate that RCL may possess a strong anti-anaphylactic activity.

Key words *Rubus croceacanthus* Leveille; systemic anaphylactic-like reaction; histamine; passive cutaneous anaphylaxis; tumor necrosis factor- α

Strawberries have long been associated with good health. And they contained only 30 calories and had absolutely no saturated fat, cholesterol, or sodium. Strawberries were also high in folate, overflowing with vitamin C, a good amount of dietary fiber and potassium, and traces of calcium and iron. Until this century, however, it was not known that strawberries contain ellagic acid. Ellagic acid, a natural organic compound, has a beneficial health effect.¹⁾ More recently, some studies have shown that ellagic acid has a fatty acid synthase (FAS) inhibitory activity and an anti-oxidant activity.^{2,3)} *Rubus croceacanthus* Leveille (Fig. 1) also contains ellagic acid that has various effects previously described. Here we investigated the effect of the concentrated methanol extract of *Rubus croceacanthus* Leveille (RCL) on allergic reactions in experimental models, as part of our continuing search for biologically active anti-allergic agents from the wild plants.

In general, mast cell-mediated allergic reaction is elicited by various chemical mediators.⁴⁾ Among the preformed and newly synthesized inflammatory substances released on degranulation of mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity.^{5,6)} Compound 48/80, well-known histamine releaser, was discovered in the search for hypotensive agents. Its hypotensive effect was

shown by Paton and Webster to be the result of histamine release.⁷⁾

The secretory responses of mast cells can be induced by aggregation of their cell surface-specific receptors for immunoglobulin E (IgE) by the corresponding antigen.^{8–10)} It has been established that the anti-IgE antibody induces passive cutaneous anaphylaxis (PCA) as a typical *in vivo* model for immediate hypersensitivity in anaphylactic reactions. Rat's skins are useful sites for studying PCA.¹¹⁾

Activated mast cells can produce histamine as well as a wide variety of other inflammatory mediators and several pro-inflammatory and chemotactic cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1, IL-4, IL-6, IL-8, and IL-13.^{12–15)} Modulation of these cytokines secretion from mast cells can provide us with a useful therapeutic strategy for allergic inflammatory disease. Recent studies have demonstrated that TNF- α contributes to the development of allergic rhinitis in mice.¹⁶⁾

We have evaluated the effect of RCL on the compound 48/80-induced systemic anaphylactic-like reaction, histamine release from rat peritoneal mast cells (RPMCs), and anti-dinitrophenyl (DNP) IgE antibody-induced PCA. We also investigated the effect of RCL on phorbol 12-myristate 13-acetate (PMA) and A23187-induced TNF- α secretion from

Fig. 1. Photographs of RCL

RCL grows in the mountains as well as in the plains. The leaves are alternate and consist of 5–7 small leaves. The blossoms are 3–3.5 cm in diameter and the ripe berries are red.³²⁾

human mast cell line HMC-1 cells.

MATERIALS AND METHODS

Materials Compound 48/80, DSCG (disodium cromoglycate), anti-DNP IgE, DNP-human serum albumin (HSA), metrizamide, PMA, A23187, *o*-phthalaldehyde (OPA), and Evans blue were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The α -minimal essential medium was purchased from Flow Laboratories (Irvine, U.K.). Fetal bovine serum was purchased from Life Sciences (Grand Island, NY, U.S.A.). Anti-human TNF- α antibody, biotinylated anti-human TNF- α antibody, and recombinant human TNF- α were purchased from R&D systems Inc., U.S.A.

Animals The original stock of male ICR mice (4 week old) and male Wistar rats (7 week old) were purchased from the Dae-Han Experimental Animal Center (Eumsung, Chungbuk, South Korea), and the animals were maintained in the College of Pharmacy, Wonkwang University. The rats were housed five to ten per cage in a laminar air-flow room maintained at a temperature of $22 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 10\%$ throughout the study. No animal was used more than once. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH publication #85—23, revised in 1985).

Preparation of RCL The plant material, *Rubus croceacanthus* Leveille, was collected in Jeju-Do, the Republic of Korea. The fresh whole plant samples were extracted with methanol at normal temperature for 48 h. The extracts were filtered through gauze and concentrated with vacuum dryer. For the bioassay test, the samples were dissolved in phosphate-buffered solution (PBS) and then filtered through 0.45- μm syringe filter.

Compound 48/80-Induced Systemic Anaphylactic-Like Reaction Mice ($n=4$) were given an intraperitoneal injection of the mast cell degranulator compound 48/80 (8 mg/kg). RCL or DSCG were dissolved in saline and administered orally with sonde 1 h before the injection of compound 48/80. Mortality was monitored for 23 min after induction of anaphylactic-like reaction.

Preparation of Rat Peritoneal Mast Cells (RPMCs) RPMCs were isolated as previously described.¹⁷⁾ In brief, rats were anesthetized by ether, and injected with 20 ml of Tyrode buffer B (NaCl, glucose, NaHCO_3 , KCl, NaH_2PO_4) containing 0.1% gelatin (Sigma) into the peritoneal cavity; the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated by Pasteur pipette. Then the peritoneal cells were sedimented at $150 \times g$ for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells (*i.e.* macrophages and small lymphocytes) according to the method described by Yurt *et al.*¹⁸⁾ In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B were layered onto 2 ml of 0.225 g/ml metrizamide (density 1.120 g/ml; Sigma) and centrifuged at room temperature for 15 min at $400 \times g$. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 ,

5.6 mM glucose, 0.1% bovine serum albumin) containing calcium. Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Histamine Assay Purified RPMCs were resuspended in Tyrode buffer A containing calcium for the treatment with compound 48/80. RPMC suspensions (2×10^5 cells/ml) were preincubated for 10 min at 37°C before the addition of compound 48/80 for stabilization. The cells were preincubated with the RCL or DSCG for 20 min, and then incubated for 15 min with compound 48/80 (6 $\mu\text{g/ml}$). RPMCs (3×10^5 cells/ml) were sensitized with 0.1 $\mu\text{g/ml}$ anti-DNP IgE for 24 h and preincubated with RCL or DSCG at 37°C for 10 min prior to challenge with DNP-HSA (1 $\mu\text{g/ml}$). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at $400 \times g$ for 5 min at 4°C . Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at $400 \times g$ for 5 min at 4°C . The histamine content was measured by the *o*-phthalaldehyde spectrofluorometric procedure of Shore *et al.*¹⁹⁾ The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

The inhibition percentage of histamine release was calculated using the following equation:

$$\% \text{ inhibition} = (A - B) \times 100 / A$$

where A is histamine release without RCL and B is histamine release with RCL.

PCA Reaction IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mice tail vein. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The mice were injected intradermally with 100 ng of anti-DNP IgE into each of three dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later, each mouse received an injection of 200 μl of the 1 : 1 mixture of 1 mg/ml DNP-HSA in PBS and 4% Evans blue *via* the tail vein. One hour before this injection, RCL or DSCG was administered orally. The mice were sacrificed 40 min after the intravenous challenge. The dorsal skin of the mouse was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 0.5 ml of 1.0 mol/l KOH and 4.5 ml of a mixture of acetone and phosphoric acid (with the ratio of 5 : 13), based on the method of Katayama *et al.*²⁰⁾ The absorbent intensity of the extraction was measured at 620 nm in a spectrofluorometer, and the amount of dye was calculated with the Evans blue measuring-line.

Assay of Cytokine A modified enzyme-linked immunosorbent assay (ELISA), as described in ref. 21, was used for measuring the cytokine. The ELISA was performed by coating 96-well plates with 6.25 ng/well of anti-human TNF- α antibody having specificity for TNF- α . Before use of and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and, in addition, washed twice with PBS alone. All the reagents and all the coated wells used in this assay were incubated for 1 h at room temperature. The standard curve was generated from known concentrations of cytokine, as pro-

vided by the manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies (TNF- α) by using avidin peroxidase. Optical-density readings were made within 10 min of the addition of the ABTS substrate solution on a Titertek Multiscan (Flow Laboratories) with a 405 nm filter. Appropriate specificity controls were included, and all samples were run in duplicate.

Statistical Analysis The results were expressed as mean $\pm$ S.E.M. for the number of experiments. Statistical evaluation of the results was performed by ANOVA with Tukey *post hoc* test. Mann-Whitney's *U* test was used on compound 48/80-induced histamine assay, because the results of histamine assay did not have normality. Results with $p < 0.05$ were considered statistically significant.

RESULTS

Effect of RCL on Compound 48/80-Induced Systemic Anaphylactic-Like Reaction To assess the contribution of RCL in anaphylactic-like reaction, we first used the *in vivo* model of systemic anaphylactic-like reaction. As a non-immunologic stimulator, compound 48/80 (8 mg/kg) was used. After the injection of compound 48/80, the mice were monitored for 23 min, after which the mortality rate was determined. The period for observation of mortality was based on the control mice that had died in 23 min by compound 48/80. As shown in Table 1, an oral administration of saline as a control induced a fatal reaction in 100% of each group. When the RCL was orally administered at concentrations of 0.01–1 g/kg 1 h before compound 48/80 injection, the mortality was reduced (Table 1). In addition, when the DSCG was orally administered at concentrations of 0.01–0.1 g/kg 1 h before compound 48/80 injection, the mortality was 30% and 0%, respectively.

Effect of RCL on Histamine Release from RPMCs The inhibitory effect of RCL on compound 48/80-induced histamine release from RPMCs is shown in Fig. 2. RCL dose dependently inhibited compound 48/80-induced histamine release at concentrations of 0.01–1 mg/ml. In particular, RCL significantly inhibited the compound 48/80-induced histamine release at concentrations of 0.1–1 mg/ml. The histamine intensity at the doses of 0.1–1 mg/ml was 18.13 ± 7.94 , 12.55 ± 4.81 , and 6.49 ± 2.35 , respectively. Control and spontaneous values were 18.13 ± 4.84 and 2.31 ± 0.51 . The histamine intensity of DSCG, a reference drug, was 3.63 ± 2.45 .

The inhibitory effect of RCL on IgE-mediated histamine release from RPMCs is shown in Fig. 3. RCL dose dependently inhibited IgE-mediated histamine release at concentrations of 0.1–1 mg/ml. Especially, RCL significantly inhibited the IgE-mediated histamine release at concentrations of 0.01–0.1 mg/ml. The histamine intensity at the doses of 0.1–1 mg/ml was 6.48 ± 0.24 , 6.38 ± 0.24 , and 5.83 ± 0.35 , respectively. Control and spontaneous values were 7.90 ± 0.86 and 0.63 ± 0.32 . The histamine intensity of a reference drug (DSCG) was 5.30 ± 0.25 .

Effect of RCL on PCA PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reactions.²²⁾ Local injection of anti-DNP IgE followed by an intravenous antigenic challenge was performed. Anti-DNP IgE

Table 1. Effect of RCL and DSCG on Compound 48/80-Induced Systemic Anaphylactic-Like Reaction in Mice

Treatment	Dose (g/kg) ^{a)}	Compound 48/80 (8 mg/kg) ^{b)}	Mortality (%) ^{c)}
None (saline)	—	+	100.0
RCL	0.01	+	50.0
	0.1	+	83.3
	1	+	83.3
	1	—	0.0
DSCG	0.01	+	30.0
	0.1	+	0.0

a) The groups of mice were orally pretreated with 200 μ l of saline or drugs (RCL or DSCG). The drugs were given at various doses 1 h before ($n=4$ /group) the compound 48/80 injection. b) The compound 48/80 solution was intraperitoneally given to the groups of mice. c) Mortality (%) is presented as the 'No. of dead mice $\times$ 100/total no. of experimental mice'. Each value was presented as the mean of three independent experiments.

Fig. 2. Effect of RCL and DSCG on Compound 48/80-Induced Histamine Release from RPMCs

RPMCs (2×10^5 cells) were pre-incubated with various concentrations of RCL at 37 °C for 10 min prior to incubation with compound 48/80. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. * $p < 0.05$; significantly different from the control value.

Fig. 3. Effect of RCL and DSCG on IgE-Mediated Histamine Release from RPMCs

RPMCs (3×10^5 cells) were pre-incubated with various concentrations of RCL at 37 °C for 10 min prior to incubation with compound 48/80. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. * $p < 0.05$; significantly different from the control value.

was injected into dorsal skin sites. After 48 h, all animals were injected intravenously with DNP-HSA containing Evans blue dye. The cutaneous anaphylactic reaction was best visualized by the extravasation of dye. When RCL was orally administered to the mouse, the PCA reaction was in-

Fig. 4. Effect of RCL and DSCG on PCA

RCL was administered orally 1 h ($n=3$ /group) prior to the challenge with antigen (DNP-HSA). Each datum represents the mean $\pm$ S.E.M. of three independent experiments. * $p<0.05$; significantly different from the control value.

Fig. 5. Effect of RCL and DSCG on PMA and A23187-Induced TNF- α Secretion from Mast Cells

TNF- α levels in supernatant were measured using ELISA method. Each datum represents the mean $\pm$ S.E.M. of three independent experiments. * $p<0.05$; significantly different from the control value.

hibited in a dose-dependent manner (Fig. 4). Especially, at the dose of 1 g/kg, the effect of RCL was the greatest. Amount of dye at the dose of 0.01–1 g/kg was 0.81 ± 0.13 , 0.63 ± 0.09 , and 0.59 ± 0.08 , respectively. The amount of dye in control mine was 1.02 ± 0.25 , the value of a reference drug (DSCG) was 0.30 ± 0.07 .

Effect of RCL on TNF- α Secretion from HMC-1 Cells

Human mast cell line HMC-1 cells were stimulated with PMA and calcium ionophore A23187. To assess the effect of RCL in PMA and A23187-induced TNF- α secretion, the cells were pretreated with various concentrations of RCL for 30 min prior to stimulators. Our results showed that pre-treatment of the cells with RCL resulted in inhibition of TNF- α secretion. Inhibitory effect of RCL was significant ($p<0.05$) at the doses of 0.1–1 mg/ml (Fig. 5). TNF- α production value at the doses of 0.1–1 mg/ml was 0.26 ± 0.01 , 0.20 ± 0.01 , and 0.10 ± 0.01 , respectively. Control and reference drug (DSCG) values were 0.27 ± 0.04 and 0.12 ± 0.03 .

DISCUSSION

The present study showed that RCL pre-treatment profoundly affected compound 48/80-induced systemic anaphylactic-like reaction and histamine release. And RCL inhibited anti-DNP IgE-induced histamine release and PCA reaction.

RCL also inhibited TNF- α secretion induced by PMA and A23187. RCL inhibited compound 48/80-induced fatal reaction, histamine release and anti-DNP IgE-induced histamine release, local allergic reaction up to 50%, 82.2%, 26%, and 45.4%, respectively. And 63.8% inhibition of TNF- α secretion was obtained by RCL. These results indicate that mast cell-mediated immediate-type allergic reactions are inhibited by RCL.

It is believed that stimulation of mast cells with compound 48/80 initiates the activation of a signal transduction pathway, which leads to histamine release. There have been some reports that compound 48/80 is able to activate G proteins.^{23–25} Recently, Chadi *et al.* announced that compound 48/80 activates mast cell phospholipase D (PLD) via heterotrimeric GTP-binding proteins. They identified recombinant $G_{\beta 2\gamma 2}$ subunit markedly synergized PLD activation by compound 48/80 in permeabilized RBL-2H3 cells.²⁶ Murine mast cells are a good experimental model for the study on compound 48/80-induced histamine release.²⁷ The report that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane indicates that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells.²⁸ So, it is possible to hypothesize that RCL might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80.

The RCL doses of 0.1 and 1 g/kg were ineffective for the shock, although effective in other experiments. Optimal dose of RCL for the shock might be lower than the dose range we studied. However, at the present moment the reason of the difference is remained unclear. We hope the reason can be revealed in the future study.

The RCL-administered mouse is protected from IgE-mediated local allergic reaction. The mechanism of the protection against anti-DNP IgE, while not clear at present, may be suggested only in some particular conditions. It is conceivable that RCL inhibits the initial phase of immediate type allergic reactions, probably through interference with the degranulation system.

HMC-1 cell line was a useful cell for studying cytokine activation pathways.²⁹ Our data demonstrated that RCL inhibited PMA and A23187-induced TNF- α secretion from HMC-1 cells. The effect of RCL on mast cell cytokine secretion *in vivo* and the relative importance of mast cell as a source of TNF- α during inflammatory and immune response are important areas for future studies. The amounts of RCL that have been used (*in vivo* or *in vitro*) in this study are large, raising the possibility that the active agents in the extract represent a small component of the total mass.

In this study, we investigated the effects of RCL extract on mast cell-mediated immediate-type allergic reactions. However, ellagic acid has very strong anti-oxidant properties, anti-carcinogenic activity and the ability to inhibit mutations within a cell's DNA.^{3,30} Ellagic acid is also considered to be a cancer inhibitor which has the ability to cause apoptosis or normal cell death in cancer cells.³¹ Therefore, further investigation is necessary to confirm the effect of ellagic acid on mast cell-mediated immediate-type allergic reactions.

In conclusion, the results obtained in the present study provide evidence that RCL inhibited the mast cell-dependent allergic reactions and inflammatory cytokines secretion.

Therefore, we can suggest that RCL has an anti-allergic effect. We believe that administration of RCL may have clinical applicability to the allergic disorders.

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