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Action of *Salvia miltiorrhiza* on anaphylaxis by anal therapy

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SUMMARY

The root of *Salvia miltiorrhiza* B_{GE}. (Labiatae) (SM), has a bitter and a slightly "cold" property, and is nontoxic. The effect of SM on mast cell-dependent anaphylaxis by anal therapy was investigated. SM inhibited compound 48/80-induced systemic anaphylaxis 100% with the dose of 0.1 g/kg by anal treatment. However, SM showed no significant inhibitory effect on the same reaction by oral treatment. SM also inhibited by 79.8% local cutaneous anaphylaxis activated by anti-dinitrophenyl (DNP) IgE by anal treatment. SM dose-dependently inhibited the histamine release and tumor necrosis factor-α (TNF-α) production from rat peritoneal mast cells (RPMCs) by anti-DNP IgE. These results indicate that anal therapy of SM may be beneficial in the treatment of systemic and local mast cell-dependent anaphylaxis.

Key words: *Salvia miltiorrhiza*, Anaphylaxis, Anal therapy, Compound 48/80, Anti-dinitrophenyl IgE, Histamine, Tumor necrosis factor-α

INTRODUCTION

An aqueous extract of *Salvia miltiorrhiza* B_{GE} (Labiatae) (SM) is commonly used as an antiallergic drug in South Korea. However, it is still unclear how the herb prevents allergic diseases.

In general, immediate allergic reaction, which involves urticaria, allergic rhinitis and asthma, is mediated by various chemical mediators released from mast cells (Wasserman and Marquardt, 1988). Among the preformed and newly synthesized inflammatory substances, released on the degranulation of mast cells, histamine is the best characterized and most potent vasoactive mediator implicated in the anaphylaxis (Petersen *et al.*, 1996). Mast cell degranulation can also be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells (Lagunof *et al.*, 1983). Compound 48/80 is one of the most potent secretagogues of mast cells (Ennis *et al.*, 1980). The secretory response of mast cells can be induced by aggregation of their cell surface-specific receptors for IgE by the corresponding antigen (Segal *et al.*, 1977; Metzger *et al.*, 1986; Alber *et al.*, 1991). Passive cutaneous anaphylaxis (PCA) is one of the most important in vivo model of anaphylaxis in local allergic reactions (Wershil *et al.*, 1987). The skin of rat or guinea pig is a useful site for studying PCA (Saito and Nomura, 1989). Although mast cells also store small amounts of cytokines in their granules (Gordon and Galli, 1990), these cells dramatically increase the production of tumor necrosis factor-α (TNF-α), IL-6, and other cytokines within 30 min after their surface FceRI are cross-linked with specific antigen (Plaut *et al.*, 1989; Wodnar-filipowicz *et al.*, 1989; Burd *et al.*, 1989; Gurish *et al.*, 1991). It was previously reported that SM inhibited the IgE-mediated allergic reaction (Kim *et al.*, 1999). This paper deals with an evaluation of the effect of SM on compound 48/80-induced systemic anaphylaxis and anti-IgE antibody-induced PCA by anal therapy and histamine release from rat peritoneal mast cells (RPMCs). We also investigated the influence of SM on TNF-α production of RPMCs.

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MATERIALS AND METHODS

Materials

SM was obtained from College of Oriental Medicine, Wonkwang University (Chinju, Korea). Anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), compound 48/80, and metrizamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The α-minimal essential medium (α-MEM) was purchased from Flow Laboratories (Irvine, UK). Fetal calf serum (FCS) was purchased from Life Technologies (Grand Island, NY, USA). Recombinant tumor necrosis factor-α (rTNF-α) (1×10^5 U/ml), and rabbit anti-murine TNF-α antibody were purchased from Genzyme (Munchen, Germany). Phosphatase-labeled anti-rabbit IgG was purchased from Serotec (Oxford, England). The original stock of ICR mice and Wistar rats were purchased from Dae-Han Experimental Animal Center (Eumsung, Chungbuk, Republic of Korea), and the animals were kept at the College of Pharmacy, Wonkwang University. The animals were housed five to ten per cage in a laminar air flow room maintained under a temperature of 22±1°C and relative humidity of 55±10% throughout the study.

Preparation of SM

SM was identified by Goo Moon, Professor of College of Oriental Medicine, Wonkwang University (Iksan, Korea), and their voucher specimens (number 6-97-27) have been deposited at the Herbarium at the Wonkwnag University. The plant sample was extracted with distilled water at 70°C for 5 hr. The extract was filtered through a 0.45 μm filter and the filtrate was lyophilized. The dried extract was dissolved in saline or 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) just before use.

Compound 48/80-induced systemic anaphylaxis

Mice were given an intraperitoneal injection of 0.006 g/kg of compound 48/80. The dried SM was dissolved in saline and administered anally and orally ranging from 0.001 to 1.0 g/kg 1 hr before the injection of compound 48/80. Mortality was monitored for 1 hr after induction of anaphylactic shock.

PCA

An IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 hr later with an injection of DNP-HSA into the rat's tail vein. DNP-HSA was diluted in PBS. Rats were injected intradermally with 0.5 μg of anti-DNP IgE into each of 4 dorsal skin sites that had been shaved 48 hr earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later, each rat received an injection of 1 μg of DNP-HSA in PBS containing 4% Evans blue (1:4) via the tail vein. The SM (0.001 to 1.0 g/kg) was anally and orally administered 1 hr before the challenge. Thirty minutes after the challenge, the rats were sacrificed and the dorsal skin was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 1 ml of 1.0 N KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13), based on the method of Katayama *et al.* (1978). The absorbant intensity of the extraction was measured at 620 nm using a spectrophotometer, and the amount of dye was calculated with the Evans blue measuring-line.

Preparation of RPMCs

RPMCs were isolated as previously described (Shin *et al.*, 1997). In brief, rats were anesthetized by ether, and injected with 20 ml of Tyrode buffer B (150 mM NaCl, 0.1% glucose, 3 mM $NaHCO_3$, 3.7 mM KCl, 3.5 mM NaH_2PO_4) containing 0.1% gelatin (Sigma Chemical Co.), into the peritoneal cavity, and the abdomen was gently massaged for about 90 sec. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated by a Pasteur pipette. Thereafter, the peritoneal cells were sedimented at 150×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.* (1977). In brief, peritoneal cells suspended in 1 ml Tyrode buffer B were layered on 2 ml of 22.5% w/v metrizamide (density, 1.120 g/ml, Sigma Chemical Co.) and centrifuged at room temperature for 15 min at 400×g. The cells remaining at the buffer-metrizamide

interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1ml Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1mM $MgCl_2$, 5.6 mM glucose) containing 0.1% bovine serum albumin (Sigma Chemical Co.). 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.

Inhibition of histamine release

Purified mast cells were resuspended in Tyrode buffer A containing 0.1% bovine serum albumin. Mast cells (1×10^6 cells/ml) were sensitized with 10 mg/ml anti-DNP IgE for 2 hr and preincubated with dried SM at 37°C for 10 min prior to the challenge with DNP-HSA (1 μg/ml). Mast cell suspensions (1×10^6 cells/ml) were preincubated at 37°C for 10 min before the addition of compound 48/80 (5 μg/ml). The cells were preincubated with SM preparations, and then incubated (10 min) with compound 48/80. The reaction was stopped by cooling the tubes on ice. The cells were separated from the released histamine by centrifugation at 400×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×g for 5 min at 4°C.

Histamine determination

Histamine content was measured by the *o*-phthalaldehyde spectroflurometric procedure of Shore *et al.* (1959). The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer.

Assay of histamine release

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

$$\% \text{ Inhibition} = \frac{\text{Histamine release without SM- Histamine release with SM}}{\text{Histamine release without SM}} \times 100$$

Assay of TNF-α production

TNF-α secretion was measured by a modified enzyme linked immunosorbent assay (ELISA) as described (Scuderi *et al.*, 1986). The ELISA was sensitive to TNF concentrations in the medium above 40 pg/ml. The ELISA was performed by coating 96-well plates with 6.25 ng/well of murine monoclonal antibody with specificity for murine TNF-α. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 (PBS-tween) and twice with PBS alone. All reagents used in this assay and the coated wells were incubated for 1 hr at room temperature. For the standard curve, rTNF-α was added to serum previously determined to be negative for endogenous TNF-α. After exposure to the medium, the assay plates were sequentially exposed to rabbit anti-TNF-α, phosphatase-conjugated goat anti-rabbit IgG, and *p*-nitro phenyl phosphate. Optical density readings were made within 10 min of the addition of the substrate on a Titertek Multiscan (Flow Laboratories) with a 405 nm filter. Appropriate specificity controls were included.

Statistical analysis

The results obtained were expressed as mean ±SE for the number of experiments. The ANOVA with Dunnett's test was used to make a statistical comparison between the groups. Results with $P<0.05$ were considered statistically significant.

RESULTS

Compound 48/80-induced systemic anaphylaxis

To determine the effect of SM by anal therapy in systemic allergic reaction, we used compound 48/80 (0.006 g/kg) as a fatal anaphylaxis inducer. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 hr, after which the mortality rate was determined. As shown in Table 1, an intraperitoneal injection of 200 ml saline as a control induced fatal shock in 100% of each group. When the SM was anally administered at a concentration ranging from 0.001 to 1.0 g/kg for 1 hr, the mortality with compound 48/80 was significantly reduced (n=10/group). Of special note, SM inhibited compound 48/80-induced mortality 100% with the dose of 0.1 g/kg (Table 1). However, in the case of oral administration, the mortality was not significantly changed. In addition, the mortality

Table 1. Effect of SM on compound 48/80-induced systemic anaphylaxis

SM addition (g/kg)	Compound 48/80 (0.006g/kg)	Mortality (%)	
		Anal	Oral
None (Saline)	+	100	100
0.001	+	40±10*	100
0.01	+	40±20*	100*
0.1	+	0*	90±10
1	+	20±10*	90±10
1	-	0	0

Mortality (%) within 1 hr following the compound 48/80 injection is presented as the No. of dead mice ×100/total No. of experimental mice. Data are presented as the means ±SE of three independent experiments. *P<0.05; significantly different from the saline value.

Table 2. Time-dependent effect of SM on compound 48/80-induced systemic anaphylaxis

SM addition (g/kg)	Time (min)	Compound 48/80 (0.006g/kg)	Mortality (%)	
			Anal	Oral
None (Saline)	0	+	100	100
0.1	0	+	0*	90±10
	5	+	20±10*	100
	10	+	60±20*	100
	20	+	90±10	100

Mortality (%) within 1 hr following the compound 48/80 injection is presented as the No. of dead mice ×100/total No. of experimental mice. Data are presented as the means ±SE of three independent experiments. *P<0.05; significantly different from the saline value.

of mice administered anally with SM (0.1 g/kg) 5 min, 10 min, and 20 min after compound 48/80 injection increased time-dependently (Table 2). Treatment with SM (1.0 g/kg) caused no apparent side effects.

PCA

Local extravasation is induced by a local injection of anti-DNP IgE followed by an intravenous antigenic challenge. Anti-DNP IgE was injected into the right dorsal skin sites. As a control, the left dorsal skin site of these rats was injected with saline alone. After 48 hr, all animals were injected intravenously with DNP-HSA plus Evans blue dye. SM was administered anally and orally 1 hr prior to the challenge with antigen. As shown in Table 3, SM significantly inhibited PCA in the both conditions. Especially SM (0.1 g/kg) by anal therapy showed a marked inhibition (79.8%) in PCA reaction.

Histamine release from RPMCs

The inhibitory effects of SM on IgE-mediated

Table 3. Effect of SM on the 48-hr PCA in rats

SM addition (g/kg)	Amount of dye (g/site)		Inhibition (%)	
	Anal	Oral	Anal	Oral
None (Saline)	44.26±4.56	41.85±2.24	-	-
0.001	36.31±3.66*	38.64±1.55	18.0	7.3
0.01	15.40±2.87*	17.25±1.83*	65.2	58.8
0.1	8.92±5.24*	11.43±0.96*	79.8	72.7
1	12.38±2.23*	15.10±1.26*	72.0	63.9

Data are presented as the means ±SE of three independent experiments. *P<0.05; significantly different from the saline value.

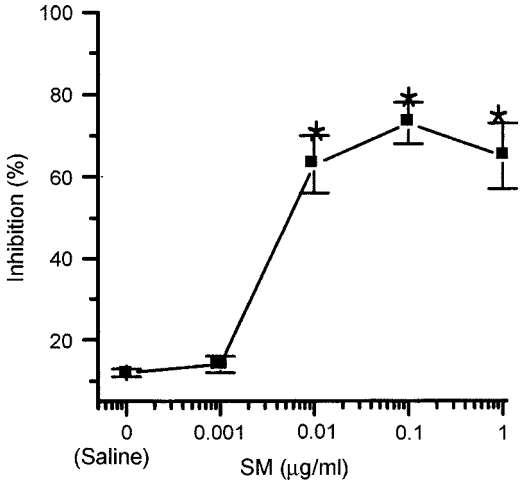

Fig. 1. Effect of SM on IgE-mediated histamine release from RPMCs. Data are presented as the mean±SE of four independent experiments. *P<0.05; significantly different from the saline value.

histamine release from PRMC are shown in Fig. 1. RPMCs (2×10^5 cells/ml) were preincubated with the drug at 37°C for 10 min prior to the challenge with DNP-HSA. SM dose-dependently inhibited IgE-mediated histamine release at concentrations of 0.001 to 1.0 μg/ml (Fig. 1). However, SM showed no apparent inhibitory effect on the compound 48/80-induced histamine release from RPMCs (data not shown).

TNF-α production from RPMCs

Finally, we determined the amount of TNF-α production from RPMCs. RPMCs (2×10^6 cells/ml) were sensitized with anti-DNP IgE (1.0 μg/ml) and incubated for 4 hr in the absence or presence of SM before stimulation with DNP-HSA (0.1 μg/ml). SM (0.1 μg/ml) significantly inhibited IgE-mediated TNF-α production (Table 4).

Table 4. Effect of SM IgE-mediated TNF-α production from RPMCs

SM addition (μg/ml)	Anti-DNP IgE+ DNP-HSA	TNF-α production (pg/ml)
None (Saline)	-	16±12
None (Saline)	+	78±19
0.1	+	35±13*

Data are presented as the means ±SE of five independent experiments. *P<0.05; significantly different from the saline value.

DISCUSSION

The present study showed that SM pretreatment by anal therapy profoundly affected compound 48/80- or anti-DNP IgE-induced anaphylaxis. However, SM by oral administration had no significant inhibitory effect on compound 48/80-induced systemic anaphylaxis. These results indicate that SM may possess anti-anaphylactic action and also suggest that the differential activity following administration routes may be caused by difference of bioavailability. However, the exact reason for this differential effects is at present unclear.

In spite of the increasing evidence of the role of several other mediators (Rimmer and Church, 1990; Rafferty and Holgate, 1989), histamine is still regarded as the principal mediator of antigen-induced skin reactions. In addition, intradermal and intranasal application of chemical mediators and chemical mediator releasers increase vascular permeability in a manner similar to that of allergic models (Inagaki *et al.*, 1989; Inagaki *et al.*, 1990). The SM-administered rats in both anal and oral therapy are protected from IgE-mediated local anaphylaxis. It suggests that SM might be useful in the treatment of allergic skin reactions. Our data demonstrated that SM inhibited anti-DNP IgE-induced TNF-α production from RPMCs (Table 4). The effect of SM on mast cell cytokine production *in vivo* and the relative importance of mast cells as a source of TNF-α during inflammatory and immune responses are important areas for future studies.

In conclusion, the results obtained suggest that SM may contain compounds with actions that inhibit mast cell-mediated anaphylaxis. Therefore, further investigation is necessary to clarify unknown antianaphylactic constituents which may be more active than SM extract itself. The studies on the isolation and characterization of the active chemical constituents are in progress.

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