

Inhibitory Effect of Yunbutang on Mast Cell-Dependent Allergic Reactions

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ABSTRACT

Yunbutang (YB) is an Oriental herbal prescription, which has been successfully applied for the treatment of allergic disorders, mainly atopic dermatitis in clinical medicine. However, its effect in experimental models remains unknown. Here, we report inhibitory effects of YB on mast cell-dependent immediate-type allergic reactions. YB inhibited systemic allergic reaction induced by compound 48/80. YB dose-dependently inhibited the histamine release from the mast cells activated by compound 48/80. YB also showed an inhibitory effect of anti-dinitrophenyl IgE antibody-induced passive cutaneous anaphylaxis reaction by oral administration. Furthermore, YB inhibited tumor necrosis factor- α secretion from stimulated mast cells. These findings provide evidence that YB inhibits the mast cell-dependent allergic reactions.

Key Words: Yunbutang; Mast cells; Histamine; Passive cutaneous anaphylaxis reaction; Tumor necrosis factor- α .

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INTRODUCTION

Mast cell is a tissue-based inflammatory cell of bone marrow origin that responds to danger signals of innate and acquired immunity with immediate and delayed release of inflammatory mediators.^[1] Mast cells have long been implicated in the mortality of anaphylaxis and other allergic disorders by virtue of their ability to be activated through FcεRI-bound antigen-specific IgE and IgE-independent stimuli.

Mast cell activation brings about the process of degranulation that results in the fusion of the cytoplasmic granule membranes with the plasma membrane. In IgE independent allergic disorders, intracellular calcium, cAMP, and histamine release in murine mast cells were effectively stimulated by positively charged substances such as compound 48/80.^[2] The synthetic compound 48/80 is known to be one of the most potent secretagogues.^[3] An appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of systemic anaphylactic reaction.^[4] Studies on the compound 48/80-induced ear swelling response and mast cell degranulation have been continuously performed on these theoretical bases by Kim et al.^[5–9] Among the preformed and newly synthesized inflammatory substances released on the degranulation of mast cell, histamine remains the best characterized and most potent vasoactive mediator implicated in the acute phase of anaphylaxis.^[10] In IgE-dependent allergic disorders, including anaphylaxis, allergic asthma, rhinitis, and atopic dermatitis,^[11,12] mast cell activation induces many of acute changes observed. The secretory response of mast cells can be induced by aggregation of their cell surface-specific receptors (FcεRI) for IgE by corresponding antigen.^[13–15] One of the important cytokines is tumor necrosisfactor-α (TNF-α). TNF-α has an exceptionally broad spectrum of biological activities, with roles in many immunologic and inflammatory processes. Activated mast cells also produce the multipotent cytokine tumor necrosisfactor-α (TNF-α).^[16,17] Inhibition of this cytokine secretion from mast cells can provide us with a useful therapeutic strategy for allergic inflammatory disease. In the skin, mast cells appear in the greatest number near blood vessels, hair follicles, and sebaceous glands. Human skin contains approximately 10,000 mast cells per cubic millimeter.^[18]

Yunbutang (YB) is an Oriental herbal prescription, which has been applied for the treatment of various allergic disorders in Korea, mainly atopic dermatitis in clinical medicine. However, its effect in experimental models remains unknown on mast cells. Studies of the effect of YB on mast cell dependent-allergic reactions and TNF-α secretion were carried out.

MATERIALS AND METHODS

Reagents

Compound 48/80, anti-DNP IgE, metrizamide, o-phthaldialdehyde (OPA), and α-minimal essential medium was purchased from Flow Laboratories (Irvine, UK). Fetal bovine serum (FBS) was purchased from Life Sciences (Grand Island, NY, USA). Recombinant TNF-α, biotinylated TNF-α and anti-mouse TNF-α were purchased from R&D systems Inc, USA.



Animals

The original stock of ICR mice and Wistar rats were purchased from the Damul Experimental Animal Center (Daejeon, 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.

Preparation of YB

The Extract of YB was prepared by decocting the dried prescription of herbs with boiling distilled water. The extract decocted for approximately 3 h has been filtered, lyophilized, and kept at 4°C . The ingredients of YB include 12.0 g of dried shelled chestnut (fruit of *Castanea crenata*), 12.0 g of Semen Coicis, 4.0 g of Fructus Schizandrae, 4.0 g of Radix Platycodi, 4.0 g of Semen Raphani, 4.0 g of Tuber Liriodopsis, 4.0 g of Rhizoma Acori Graminei. These plants materials were purchased from the Oriental drug store (College Oriental Pharmacy, Iksan, South Korea).

Compound 48/80-Induced Systemic Anaphylactic Reaction

Mice were given an intraperitoneal injection of the mast cell degranulator compound 48/80 (8 mg/kg). YB was dissolved in saline and administered orally 1 h before the injection of compound 48/80. Mortality was monitored for 20 min after induction of anaphylactic reaction.

Ear Swelling Response

Compound 48/80 was freshly dissolved in saline and injected intradermally into the dorsal aspect of a mouse ear using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a digimatic micrometer (Mitutoyo, Japan) under mild anesthesia. Ear swelling response represented an increment in thickness above baseline control values, and was determined 40 min after compound 48/80 or vehicle injection (100 $\mu\text{g}/\text{site}$). YB was administered orally 1 h before the compound 48/80-injection. The values obtained would appear to represent the effect of compound 48/80 rather than the effect of the vehicle injection (physical swelling), since the ear-swelling response evoked by physiologic saline returned to almost baseline thickness within 40 min.

Preparation of Rat Peritoneal Mast Cells (RPMCs)

RPMCs were isolated as previously described.^[5] 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, St. Louis, MO, USA) 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. RPMCs 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.^[19] 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, St. Louis, MO, USA) 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. RPMC 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 YB for 20 min, and then incubated for 15 min with compound 48/80 (6 $\mu\text{g/ml}$). The reaction was stopped by cooling. 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 OPA spectrofluorometric procedure of Shore et al.^[20] 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 YB and B is histamine release with YB.

Cell Cultures

RBL-2H3 cells, a rat basophilic leukemia cell line, were obtained from the Korean cell line bank (Daejeon, Korea). The cells were grown in RPMI 1640 (Life Technologies) with 10% FBS at 37°C in 5% CO_2 .

TNF- α Secretion Assay

RBL-2H3 cells were pretreated with various concentration of YB for 30 min prior to anti-DNP IgE antibody stimulation. Culture supernatants were assayed for TNF- α protein levels by a modified enzyme-linked immunosorbent assay (ELISA), as described.^[1] Sandwich ELISA for TNF- α was carried out in duplicate in 96-well ELISA plates (Nunc, Denmark) coated with each of 100:1 aliquots of mouse anti-human TNF- α monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) at 1.0 $\mu\text{g/ml}$ in PBS at pH 7.4 and was incubated overnight at 4°C . The plates were washed in PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO, USA) and blocked with PBS



containing 1% BSA, 5% sucrose and 0.05% NaN₃ for 1 h. After additional washes, sample or recombinant TNF- α standards were added and incubated at 37°C for 2 h. After 2 h of incubation at 37°C, the wells were washed and then each of 0.2 μ g/ml of biotinylated anti-human TNF- α was added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C. Wells were again washed and ABTS substrate (Sigma, St. Louis, MO, USA) was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant mouse TNF- α (R&D Systems) in serial dilutions.

Statistical Analysis

The results were expressed as the mean $\pm$ standard error of the mean (SEM) for the number of experiments. Statistical significance was compared between each treated group and control by the independent t-test. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of YB on Compound 48/80-Induced Systemic Anaphylaxis

To assess the contribution of YB in anaphylactic reactions, we first used the in vivo model of systemic anaphylaxis. As a nonimmunologic stimulator, compound 48/80 (8 mg/kg) was used. After the injection of compound 48/80, the mice were monitored for 20 min, after which the mortality was determined. As shown in Table 1, an oral administration of saline as a control induced a fatal reaction in 100% of each group. YB was orally administered at concentrations of 0.01–1.0 g/kg 1 h before compound 48/80 injection. The results showed that the mortality was reduced at concentration of 1.0 g/kg significantly, when compared with the control.

Table 1. Effect of YB on compound 48/80-induced systemic anaphylactic reaction in mice.

YB ^a (g/kg)	Compound 48/80 ^b (0.008 g/kg)	Mortality (%) ^c
None (saline)	+	100
0.01	+	33.3 $\pm$ 7.0
0.1	+	50.0 $\pm$ 9.6
1.0	+	33.3 $\pm$ 6.5*
1.0	—	0

^aThe groups of mice were orally pretreated with 200 μ l of saline or YB was given at various doses 1 h before the compound 48/80 injection.

^bThe compound 48/80 solution was intraperitoneally given to the groups of mice.

^cMortality (%) is presented as the number of dead mice $\times$ 100/Total number of experimental mice. Each datum represents the mean $\pm$ SEM of three independent experiments. * $P < 0.05$: significantly different from the saline value.



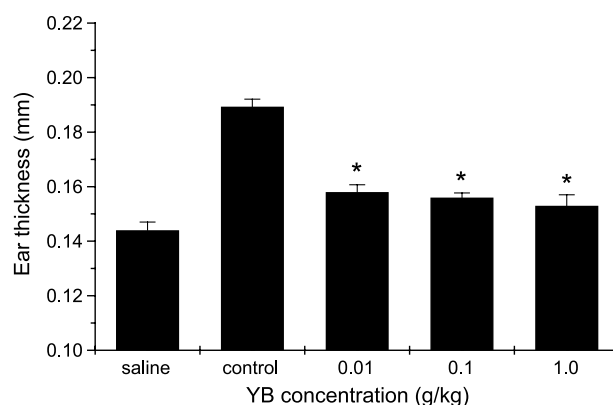


Figure 1. Effect of YB on compound 48/80-induced ear swelling response in mice. Twenty microliters of compound 48/80 (100 $\mu\text{g}/\text{site}$) were applied intradermally. The mice were orally administered with the indicated concentration of YB for 1 h prior to the compound 48/80 application. Each datum represents the mean $\pm$ SEM of three independent experiments. * $P < 0.05$: significantly different from the saline value.

Effect of YB on Ear Swelling Response

The fact that intradermal application of compound 48/80 at the dose of 100 $\mu\text{g}/\text{site}$ can induce an ear-swelling response in normal mice has been confirmed in previous studies.^[21] We chose a concentration of 100 $\mu\text{g}/\text{site}$ for compound 48/80-induced optimal ear-swelling response in this experiment. As shown in Fig. 1, when mice were pretreated with YB for 1 h, the ear-swelling responses derived from compound 48/80 were reduced in dose-dependent manner.

Effect of YB on Histamine Release from RPMCs

The inhibitory effects of YB on compound 48/80-induced histamine release from RPMCs are shown in Table 2. RPMCs (2×10^5 cells/ml) were preincubated with the YB at 37°C for 15 min prior to the challenge with compound 48/80. YB

Table 2. Effect of YB on compound 48/80-induced histamine release from RPMC.

YB (g/kg)	Compound 48/80	Inhibition rate (%)
None (saline)	+	0
0.01	+	31.95 $\pm$ 2.1*
0.1	+	33.23 $\pm$ 2.7*
1.0	+	30.73 $\pm$ 1.6*
1.0	—	100

RPMCs (2×10^5 cells/ml) were preincubated with various concentrations of YB at 37°C for 10 min prior to incubation with compound 48/80. Each datum represents the mean $\pm$ SEM of six independent experiments. * $P < 0.05$: significantly different from the control value.



Table 3. Effect of YB on IgE-induced TNF- α secretion from RBL-2H3.^a

YB (mg/ml)	TNF- α secretion (ng/ml)	Inhibition rate (%)
None (distilled water)	0.257 $\pm$ 0.016	–
1.0	0.197 $\pm$ 0.018*	32.08 $\pm$ 2.6

^aEffect of YB on IgE-induced TNF- α secretion from RBL-2H3. TNF- α levels in supernatant were measured using ELISA method. Each datum represents the mean $\pm$ SEM of three independent experiments. * P < 0.05: significantly different from the control value.

dose-dependently inhibited compound 48/80-induced histamine release from RPMCs. At the concentration of 0.01–1.0 mg/ml, the inhibition rate reached up to 30.73% significantly (P < 0.05), but the experiment had to be stopped at 1 mg/ml of concentration because of the difficulty to get the data. Cell viability has not been affected at these concentrations (data not shown).

Effect of YB on TNF- α Secretion from RBL-2H3 Cells

RBL-2H3 cells were stimulated with anti-DNP IgE antibody. To assess the effect of YB in anti-DNP IgE antibody induced TNF- α secretion, the cells were pretreated with 0.01, 1.0 mg/ml of YB for 30 min prior to stimulators. Our results showed that the pretreatment of the cells with YB resulted in inhibition of TNF- α secretion. Especially YB (1.0 mg/ml) showed inhibition rate of 32.08% when TNF- α was secreted by anti-DNP IgE stimulation. There was no significant effect of YB on TNF- α secretion in the lower concentrations (0.01, 0.1 mg/ml) (Table 3).

DISCUSSION

We have demonstrated that YB pretreatment profoundly affected compound 48/80-induced systemic allergic reaction and anti-DNP IgE induced local allergic reaction. These results indicate that non-specific and specific allergic reactions are inhibited by YB. YB inhibited compound 48/80 induced histamine release from RPMCs. Compound 48/80 induced ear swelling has also been inhibited by YB.

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.^[22–24] Recently Chadi et al.^[25] 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. The report that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane^[26] indicates that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells. So, it is possible to suppose that YB might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80.



YB-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 YB inhibits the initial phase of immediate type allergic reactions, probably through interference with the degranulation system.

RBL-2H3 cells were useful cells for studying cytokine activation pathways.^[27] Our data demonstrated that YB inhibited anti-DNP IgE-induced TNF- α secretion from RBL-2H3 cells. The effect of YB 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.

In conclusion, the results obtained in the present study provide evidence that YB inhibited the mast cell-dependent allergic reactions and inflammatory cytokine secretion. To our knowledge, this is the first report of such selective inhibition of mast cell-dependent immediate type allergic reaction by YB. Further work should address the possibility that such compounds may also be active in the inhibition of human mast cell degranulation and, therefore, in the treatment of various allergic disorders. The amounts of YB which have been used in this study are large, raising the possibility that the active agents in the extract represent a small component of the total mass. Studies on the purified components of YB are in progress.

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