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Action of Enzyme food, green life enzyme on systemic and local anaphylaxis

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SUMMARY

We studied the inhibitory effect of Green Life Enzyme (GLE) on compound 48/80-induced anaphylactic response in a murine model. GLE inhibited compound 48/80-induced systemic anaphylactic shock at the dose of 10 g/kg by 87.5%. When GLE was given as pre-treatment at concentrations ranging from 0.01 to 1.0 g/kg, it inhibited passive cutaneous anaphylaxis activated by anti-dinitrophenyl (DNP) IgE. In addition, GLE (0.1 mg/ml) inhibited anti-DNP IgE-induced tumor necrosis factor-α production from mast cells by 69% compared to saline value. These results indicate that GLE may possess anti-anaphylactic and anti-inflammatory activity.

Key Words: Green Life Enzyme; Systemic anaphylactic shock; Passive cutaneous anaphylaxis; Tumor necrosis factor-α

INTRODUCTION

As part of our continuing search for biologically active anti-allergic agents from the medicinal resources, the Green Life Enzyme (GLE) was investigated. GLE was an enzyme food that fermented, cultured and matured raw grains consisted of unpolished rice, wheat, coicis semen, etc. So, it has been recently used a substitute for rice. Moreover, GLE had not only improvement of health but also therapeutic effect of allergic disorders in Korea. However, it is still unclear by which mechanism this enzyme food inhibits allergic disorders and how much effect it has on allergic reactions in experimental models.

Allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, and food allergy afflict up to 20% of the human population in most countries and are believed to be increasing in prevalence (Wuthrich, 1989). Recently, the number of allergy patients sensitized to environmental and food allergens have been increased rapidly. The mast cells have been thought to play a major role in the development of many physiologic changes during allergic responses (Kim *et al.*, 1999). Mast cells activation both by IgE-dependent and IgE-independent stimuli, bring about the process of degranulation that results in the fusion of the cytoplasmic granule membranes with the plasma membrane. This is accompanied by the fast external release of granule-associated stored mediators (histamine, neutral proteases, acid hydrolyses, proteoglycans, chemotactic factors, cytokines, etc.) as well as by the generation and release of newly generated mediators, such as products of arachidonic acid metabolism (Metcalfe *et al.*, 1981) and, at later times, by the production and release of an array of cytokines (Church *et al.*, 1997). 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 (Petersen *et al.*, 1996).

Mast cells degranulation can be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells (Lagunoff *et al.*, 1983). The most potent secretagogues include the synthetic compound 48/80 (Ennis *et al.*, 1980). The compound 48/80 is a mixture of polymers synthesized by condensing N-methyl-p-methoxyphenyl

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ethylamine with formaldehyde (Baltzly *et al.,* 1949) and its hypotensive effect was shown by Paton (Paton, 1951) to be the result of histamine release. An appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylaxis (Kim *et al.,* 1998). The secretary response of mast cells can also be induced by aggregation of their cell surface-specific receptors (FcεRI) for IgE by the corresponding antigen (Chen *et al.,* 2000). It has been established that the anti-IgE antibody (Ab) induces passive cutaneous anaphylaxis (PCA) as a typical model for mast cell-dependent local immediate type anaphylaxis. Although mast cells also store small amounts of cytokines in their granules (Gordon *et al.,* 1990), these cells dramatically increase the production of tumor necrosis factor-α (TNF-α), interleukin 6 and other cytokines after their surface FcεRI are cross-linked with specific antigen (Kim *et al.,* 1999, 2000).

In the present study, we examined the effects of GLE on mast cell-mediated anaphylaxis. We found that GLE inhibited both compound 48/80-induced systemic anaphylactic shock and anti-dinitrophenyl (DNP) IgE-induced PCA. We also found that GLE inhibited anti-DNP IgE-induced TNF-α production from mast cells.

MATERIALS AND METHODS

Reagents

Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), metrizamide, *o*-phthaldialdehyde (OPA), and Evans blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The a-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, anti-mouse TNF-α were purchased from R&D systems Inc, USA.RPMI medium 1640 was from Life Technologies (Gaitherburg, MD).

The original stock of male ICR mice and male Sprague Dawley rats (7 week old) 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±1°C and relative humidity of 55±10% throughout the study.

Preparation of GLE

The GLE was obtained from Green Life Co. Ltd. GLE powder was dissolved in sterile saline (100 mg/ml).

Compound 48/80-induced systemic anaphylaxis

Mice were given an i.p. injection of 8 mg/kg compound 48/80. The control group (n=5) was orally administered with saline, and the experimental group (n=5) was orally administered with various doses of GLE 1 h before compound 48/80 injection. Mortality was monitored for 24 min after the induction of anaphylactic shock.

PCA reaction

PCA reaction was generated as previously described (Kim *et al.,* 1999). GLE or saline (control) was orally administered 1 h before the challenge. After 40 min of the challenge, the mice were killed and the dorsal skins were removed for measurement of the pigment amount of area. The number of mice used in each group was four, and all data were measured from two independent experiments.

Preparation of rat peritoneal mast cells (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 (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 sec. 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 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.* (Yurt *et al.,* 1977). 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×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. RPMCs 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.

Compound 48/80-induced histamine release

Mast cells suspensions (2×10^5 cells/ml) were pre-incubated for 10 min at 37°C before the addition with compound 48/80 (6 mg/l) for stabilization. The cells were pre-incubated (30 min) with the GLE or saline (control) and then incubated (20 min) with compound 48/80. The reaction was stopped by cooling the tubes in 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.

Assay of histamine release

The histamine content was measured by the OPA spectrofluorometric procedure of Shore et al (Shore *et al.*, 1959). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in a spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation:

%Inhibition=(a-b)×100/a

where a is histamine release without GLE and b is histamine release with GLE.

Cell cultures

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

Assay of TNF-α production

TNF-α assay was performed as previously described (Kim *et al.*, 1998). The RBL-2H3 cells were sensitized with anti-DNP IgE (1 mg/l) and incubated for 16 h in the absence or presence of GLE before the challenge with DNP-HSA (0.1 mg/l) for 4 h.

%Inhibition=(a-b)×100/a

where a is cytokine release without GLE and b is cytokine release with GLE.

Statistical analysis

Each data were presented as the mean and standard error of the mean (SEM) of the different experiments under the same conditions. The Independent Samples Test was used to make a statistical comparison between the groups. Results with $P<0.05$ were considered statistically significant.

RESULTS

Effect of GLE on compound 48/80-induced systemic anaphylaxis

To assess the contribution of GLE in anaphylaxis, we first used the in vivo model of systemic anaphylaxis. We used compound 48/80 (8 mg/kg) as a systemic fatal anaphylactic inducer. After the injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. As shown in Table 1, an oral injection of 200 μl saline as a control induced 100% mortality. When the GLE was orally administered at a concentration of 0.1 g/kg, the mortality was reduced by 12.5% without any histological differences.

Effect of GLE on PCA

Another way to test anaphylaxis is to induce PCA (Wershil *et al.*, 1987). Local extravasation was induced by skin injection of anti-DNP IgE followed

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

Dose[a] (g/kg)	Compound 48/80[b] (8 mg/kg)	Mortality (%)[c]
None (saline)	+	100.0
0.01	+	40.0
0.1	+	12.5
1	+	40.0
1	-	0.0

[a]The groups of mice were orally pretreated with 200 μl of saline or GLE was given at various doses 1 h before 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×100/Total no. of experimental mice. Each datum represents the mean±SEM of three independent experiments.

Table 2. Effect of GLE on 48 h passive cutaneous anaphylaxis in mice

Dose (g/kg)[a]	Amount of dye (ig/site)[b]	Inhibition (%)
None (saline)	1.5961±0.12008	-
0.0001	1.0237±0.14007*	35.8*
0.001	1.4460±0.16997	9.4
0.01	1.1281±0.20307	29.3

[a]GLE dissolved with saline was orally administered to mice 1 h before the challenge; [b]Each value indicates mean±SEM; *P<0.05: significantly different from the saline value.

by an intravenous antigenic challenge. Anti-DNP IgE was injected in the right dorsal skin sites. As a control, the left dorsal skin sites of the mouse were injected with saline alone. After 48 h, the mouse was injected intravenously with DNP-HSA plus Evans blue. The cutaneous anaphylaxis was best visualized by the extravasation of the dye. Oral administration of GLE (0.0001 g/kg) showed a significant inhibition rate in PCA reactions (Table 2).

Effect of GLE on histamine release from RPMCs

We next examined the effect of GLE on compound 48/80-induced histamine release from RPMCs. Unfortunately, the inhibitory effects of GLE on compound 48/80-induced histamine release from RPMCs were not significant (data not shown).

Effect of GLE on TNF-α production from RBL-2H3 cells

To assess the effect of GLE on IgE-induced TNF-α production, RBL-2H3 cells were pre-treated with GLE for 30 min prior to antigenic stimulation. Culture supernatants were assayed for TNF-α protein levels by the described ELISA method. Our results showed that pre-treatment of cells with GLE resulted in inhibition of TNF-α production (Table 3).

DISCUSSION

We have demonstrated that GLE pre-treatment profoundly affected compound 48/80-induced systemic anaphylaxis and anti-DNP IgE-induced PCA reaction. GLE inhibited compound 48/80-induced fatal reaction up to 87.5%. And 35.8% inhibition of anti-DNP IgE-induced local allergic reaction was obtained by GLE. These results indicate that mast cell-mediated immediate-type allergic reactions are inhibited by GLE. However, GLE did not significantly inhibit the release of histamine from RPMCs. To our knowledge, this was caused by peculiarity of enzyme food. A case similar to GLE was observed on studies of Tongkyutang (Na *et al.,* 2002). Therefore, further studies on this point are needed.

Stimulation of mast cells with compound 48/80 initiates the activation of signal-transduction pathway which leads to histamine release. Some previous studies have shown that compound 48/80 and other polybasic compounds are able to activate G-proteins (Mousli *et al.,* 1990a; Mousli *et al.,* 1990b). The evidence indicates that the protein is Gi-like and that the activation is inhibited by benzalkonium chloride (Bueb *et al.,* 1990). Compound 48/80 also increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane (Tasaka *et al.,* 1986). The membrane permeability increase may be an essential trigger for the release of the mediators from mast cells. GLE might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80.

GLE also inhibited IgE-mediated cutaneous anaphylaxis and TNF-α production. Antigen stimulation of mast cells via FcεRI elicits release of numerous mediators containing histamine in minutes and cytokines in hours. Consequently, these mediators induce immediate or late allergic

Table 3. Effect of GLE on IgE-induced TNF-a secretion from RBL-2H3 Cells

GLE Treatment (mg/ml)	Anti-DNP IgE+DNP-HSA	TNF-α secretion (ng/ml)	Inhibition (%)
None (saline)	+	0.1808±0.00913	0.0
0.1	+	0.0555±0.01087	69.3

Effect of GLE on IgE-induced TNF-α secretion from RBL-2H3 Cells. TNF-α levels in supernatant were measured using ELISA method. Each datum represents the mean±SEM of independent experiments.

reaction. It is likely that GLE regulates the degranulation of the mast cells in mouse skin by stabilizing membrane fluidity. In addition, GLE may inhibit late allergic reaction, such as inflammation, by blocking TNF-α production. The effect of GLE 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.

Our results showed that GLE might contain compounds with actions that inhibit mast cell-mediated anaphylaxis *in vivo* and *in vitro*. Therefore, we can suggest that GLE has an anti-allergic effect. We believe that administration of GLE may has clinical applicability to the allergic reactions.

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