

Suppression of Allergic Diarrhea in Murine Ovalbumin-Induced Allergic Diarrhea Model by PG102, a Water-Soluble Extract Prepared from *Actinidia arguta*

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Key Words

Actinidia arguta · Antiallergy · Diarrhea · Food allergy · IgE · IL-6 · IL-10 · MCP-1 · PG102

Abstract

Background: Allergic reactions to food can involve diarrhea, vomiting, nausea and abdominal pain. PG102 has previously been shown to control various factors involved in allergy pathogenesis, including IgE and various Th1 and Th2 cytokines, in vivo as well as in vitro [Park EJ, et al.: J Allergy Clin Immunol 2005;116:1151–1157; Park EJ, et al.: J Invest Dermatol 2007;127:1154–1160]. These data indicate that PG102 might have antiallergic effects on allergic diarrhea. Here, we investigated whether PG102 could prevent allergic diarrhea in the murine ovalbumin (OVA)-induced allergic diarrhea model. **Methods:** BALB/c mice were orally treated with PG102, dexamethasone or water for 9 days on a daily basis, followed by subcutaneous injection with OVA on day 0. Animals were orally administered with OVA from day 7, 3 times a week, over a period of approximately 20 days. Incidence of diarrhea, serum, OVA-restimulated splenocytes and lamina propria lymphocytes were analyzed. **Results:** Oral adminis-

tration of PG102 could suppress the incidence of diarrhea in a murine allergic diarrhea model. The amelioration of allergic diarrhea by PG102 was accompanied with the inhibition of mast cell infiltration into the large intestine. The serum level of IgE, IL-6 and MCP-1 was decreased in PG102-treated mice. When splenocytes were isolated from respective groups and cultured in the presence of OVA, cells from PG102-administrated animals produced lesser amounts of IL-6 and MCP-1. **Conclusions:** PG102 has the potential to be used as a preventive for food allergic diseases.

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Introduction

Diarrhea is a representative intestinal allergic symptom resulting from the abnormal absorption of nutrients and water and/or the intestinal secretion of fluid by inflammatory responses [1]. Most common food allergies are caused by IgE-mediated reactions to food, known as type I hypersensitivity reactions [2–4]. In the susceptible individual, allergens processed by antigen-presenting cells promote the differentiation of naïve T helper (Th0)

cells to Th2 phenotype and lead to the induction of an isotype switching to IgE production in plasma cells [3, 4]. IgE can tightly bind to high-affinity receptors, FcεRI, present on mast cells and basophils in the tissue and the blood. Binding of the antigen to IgE cross-links these receptors, which provokes activation of the mast cells and the basophils, resulting in the releases of preformed and newly synthesized inflammatory molecules, such as histamine, leukotrienes, cytokines and chemokines [1, 3]. Thereafter, the released mediators recruit more leukocytes including eosinophils, mast cells, basophils and Th2 lymphocytes to the site of the allergic inflammation. These cells are major contributors to a chronic allergic inflammation, triggering tissue damage [3, 4].

We previously found that PG102, a water-soluble extract from *Actinidia arguta*, commonly called hardy kiwifruit, modulates the level of IgE and Th1/Th2 cytokines in in vitro cell culture systems as well as in 3 different allergy models [5, 6, submitted]. It was subsequently found that oral administration of PG102 could improve asthma conditions in a murine model, probably by regulating the level of IgE and IL-5 [7]. In these experiments, PG102 was orally administrated after [5] or simultaneously with [6, 7] induction of the allergy. All these in vitro and in vivo data strongly suggested that PG102 might be an effective antiallergic agent for various allergic diseases. In this study, a murine OVA-induced diarrhea model was used for testing the preventive effects of PG102 on food allergies. Our data indicated that oral administration reproducibly and effectively suppressed the incidence of diarrhea. PG102 regulated various molecules related to allergic reactions, IgE, IL-6 and MCP-1 in the serum, and IL-6, IL-10 and MCP-1 in ovalbumin (OVA)-restimulated splenocytes. Furthermore, the recruitment of mast cells into the large intestine was found to be inhibited in PG102-treated animals. These results suggested that oral administration of PG102 might be a useful preventive and/or therapeutic for food allergies as well as other allergic diseases such as dermatitis and asthma.

Material and Methods

Preparation of PG102

The hardy kiwifruits used in this study were purchased from a company specializing in this fruit (Vital Berry Marketing SA, Santiago, Chile). Total water-soluble extract of the dried fruits, PG102T in our previous reports [5], has been named PG102 in this study. PG102 was prepared from these hardy kiwifruits as described previously [5]. Briefly, the dried fruits were extracted by boiling in distilled water for 3 h. The extract was filtered with Whatman filter paper (No. 2, 110 nm), and concentrated using a

rotary evaporator, followed by a freeze-drying process. Powdered PG102 was dissolved in distilled water at a concentration of 200 mg/ml and stored at -80°C until use. The dry form of PG102 contains 2.4% protein (as determined by semimicro-Kjeldahl method), 91.6% carbohydrate (by phenol-sulfuric acid method), 182 IU/100 g vitamin A (by SbCl_3 method) and 680 mg/100 g vitamin C (by DNP method). Lipid was not detected, and acidity of PG102 was pH 7.4 as lactic acid. All PG102 preparations contained undetectable levels of Gram-negative bacterial endotoxin, as determined by the limulus amoebocytes lysate assay (Cambrex, Walkersville, Md., USA). PG102 was also tested to be negative for heavy metals, residual pesticides and microorganisms.

Bioassay for PG102

To determinate the biological activity of PG102, RBL-2H3 cells, a rat mast cell line, were plated at 2×10^5 cells/well in a 24-well culture plate and grown in 0.5 ml MEM (Sigma, St. Louis, Mo., USA) supplemented with 15% FBS (Gibco, Grand Island, N.Y., USA) at 37°C under 5% CO_2 for 6 h. Cells were treated with various concentrations of PG102 for 30 min before stimulation with $1 \mu\text{M}$ of A23187 (Sigma). Twelve hours later, the supernatants were taken to measure the level of IL-4 by ELISA (R&D Systems, Minneapolis, Minn., USA).

Murine Allergic Diarrhea Model

Female BALB/c mice (6 weeks old) were purchased from Orientbio Inc. (Seongnam, Korea), bred under aseptic conditions in facilities at Seoul National University, and acclimated for at least 1 week before use. All experimental procedures were performed in compliance with the guidelines set by the University Animal Care and Use Committee at Seoul National University. Two groups of mice were gastrically administered with PG102 (200 mg/kg/day) or dexamethasone (Dex; 2.5 mg/kg/day; Sigma) in the volume of 200 μl , from day -9 to day 26 (or to day 33, depending on the status of diarrhea induction), while another group of mice was fed with 200 μl of water alone. As another control, normal mice were fed with 200 μl of water without immunization. The first 3 groups of mice were sensitized by subcutaneous injection with 1 mg OVA (Fraction V; Sigma) emulsified in 100 μl complete Freund's adjuvant (Sigma) on day 0. One week after sensitization, mice were orally administered with 100 mg of OVA dissolved in 300 μl of PBS, 3 times per week for 3 or 4 weeks, depending on the experiments. Two hours following the last administration, the mice were sacrificed and sera, spleens as well as intestines were obtained [8, 9].

Isolation and Culturing of Splenocytes

Splenocytes were prepared by the mechanical dissociation method [9] and resuspended in RPMI-1640 (Sigma) containing 10% FBS (Gibco). Splenocytes were seeded at 5×10^6 cells/ml/well, and incubated with 100 $\mu\text{g}/\text{ml}$ of OVA for 3 days [5, 6, 9]. Collected culture supernatants were used for the analysis of inflammatory mediators.

Measurement of Immunoglobulins, Cytokines and Chemokines

The total level of IgE was determined using a mouse IgE detection kit (Shibayagi, Gunma, Japan). The level of OVA-specific IgE was measured by the sandwich ELISA method (BD Biosciences, San Jose, Calif., USA; Pierce, Rockford, Ill., USA) as described by

Hirano et al. [5, 6, 10]. The levels of IL-6, IL-10 and MCP-1 in the serum and the restimulated splenocytes were measured by commercially available ELISA kits (Pierce; R&D Systems).

Measurement of Mast Cell Infiltration into Large Intestine

Mononuclear cells were dissociated from the large intestines using 0.5 mg/ml of collagenase (type IV; Sigma) and purified using a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) as described by Kweon et al. [8, 9]. The separated cells were pooled and labeled by the PE-conjugated anti-CD117 antibody and the FITC-conjugated mouse IgE (BD Biosciences). The labeled cells were analyzed by flow cytometry analysis using a FACS calibur (BD Biosciences) [5, 8, 11].

Statistics

Data are presented as the means $\pm$ SEM and evaluated by the Student's *t* test for unpaired samples. $p < 0.05$ was considered to be statistically significant.

Results

Preparation of PG102 from *A. arguta*

A water-soluble extract, code-named PG102, was prepared from *A. arguta*, as described previously [5]. PG102 has previously been shown to regulate the expression of Th1 and Th2 cytokines as well as that of IgE [5, 6]. The active compounds contributing to these properties have not yet been precisely identified. To quantitatively perform experiments and also to obtain an experimental reagent in a consistent manner, reliable bioassay systems have been developed. One such assay is the use of a cell line together with IL-4 as a biomarker for the quality control of PG102. IL-4 plays key roles in the pathogenesis of almost all allergies [4]. The rat mast cell line, RBL-2H3, was treated with various concentrations of PG102, in the presence of calcium ionophore, A23187. When cells were treated with A23187, the level of IL-4 was highly increased from the undetectable basal level. The production of IL-4 was inhibited by PG102 in a dose-dependent manner (fig. 1). In all concentrations used in this assay, no cytotoxic effects were found. The IC_{50} value of PG102 used in this study was 1.50 mg/ml. This method was used for the quality control of experimental samples as well as the study of the molecular mechanism underlying the effects of PG102.

Oral Administration of PG102 Suppressed Antigen-Specific Allergic Diarrhea

It was first examined whether oral administration of PG102 could prevent food allergy conditions using murine OVA-induced allergic diarrhea model [8]. BALB/c mice were sensitized once with OVA by subcutaneous in-

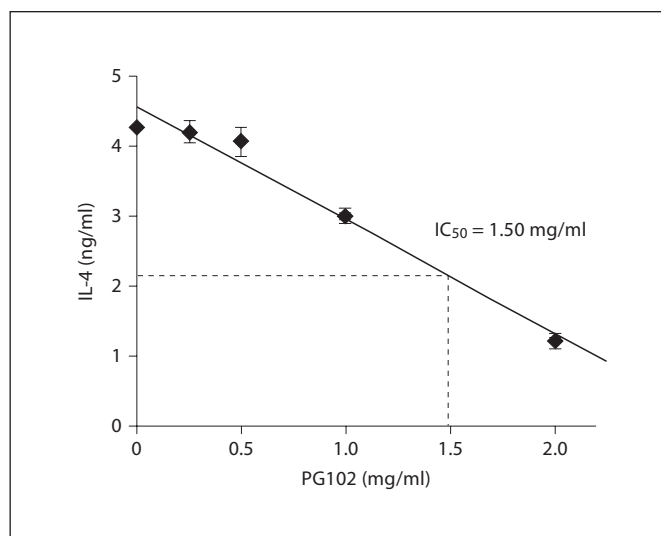


Fig. 1. Effect of PG102 in the bioassay system using RBL-2H3 cells. Rat RBL-2H3 mast cells were stimulated with PG102 (0, 0.5, 1 and 2 mg/ml) and A23187 (1 μ M). Twelve hours later, the level of IL-4 was measured in the supernatant by ELISA kit. The results are shown as means $\pm$ SD measured in the duplicate microplate wells. More than 3 independent sets of experiments were performed.

jection on day 0, and intragastrically administrated with excess OVA 3 times a week from day 7 to the sacrifice day. For the purpose of comparison, animals were divided into 3 groups, each orally treated with PG102, Dex or water alone, once a day from day -9 to the sacrifice day, as shown in figure 2. As another control, 1 group of mice was grown without OVA immunization and with water administration only.

When animals were orally administrated with OVA from day 7, water-treated and OVA-immunized mice (OVA/water group) began to experience loosening of the bowels from the second treatment. After the eighth oral challenge on day 24, all mice from the OVA/water group suffered from severe diarrhea (fig. 3). In the PG102-treated and immunized group (OVA/PG102 group), the first lax stools were observed at the sixth oral administration with OVA, and at the end of the experiment, the symptom of diarrhea was observed in only 20–40% of mice in 4 independent experiments (fig. 3). No diarrhea-related symptom was observed in the nonsensitized animals (normal group) and the Dex-treated and immunized mice (OVA/Dex group; fig. 3). Although Dex completely blocked the induction of allergic diarrhea, mice treated with this glucocorticoid showed significant side effects, including reduction of body weight by more than 10%

Fig. 2. Protocols for OVA sensitization, challenge and PG102 administration. Mice were sensitized by subcutaneous (s.c.) injection with 1 mg of OVA plus CFA on day 0, and then orally challenged with 100 mg of OVA 3 times a week. Animals were divided into 3 groups, and administered with PG102, Dex or water, on a daily basis from day -9 to day 26. As a control, 1 group of mice was fed with water only without OVA sensitization. Between 30 min and 2 h after each oral challenge with OVA, mice were observed to determine whether they had loose stools or not. On day 26, mice were sacrificed and samples were analyzed.

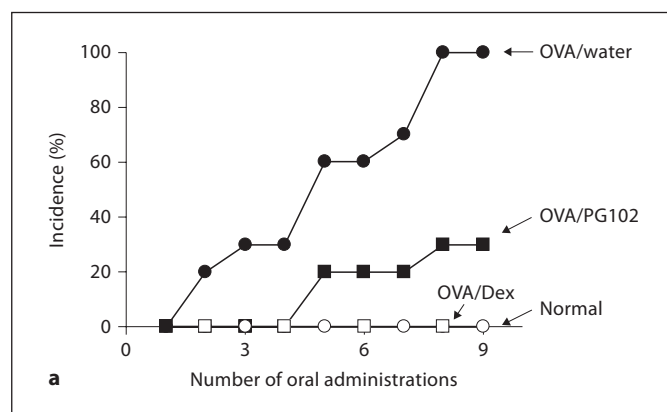
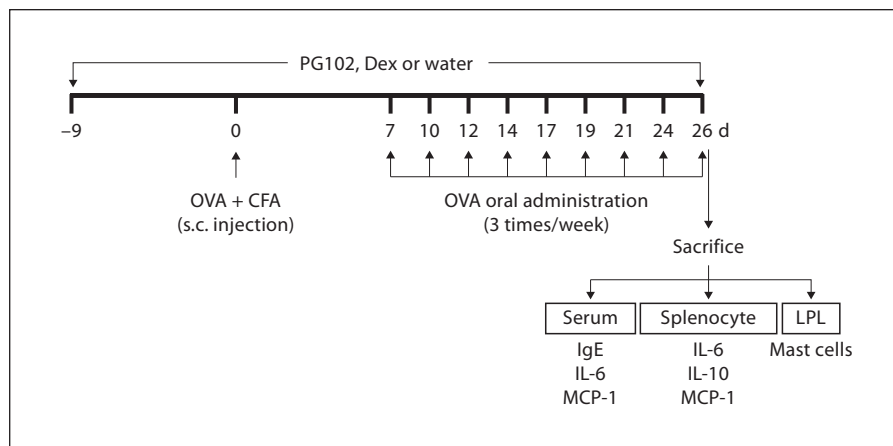
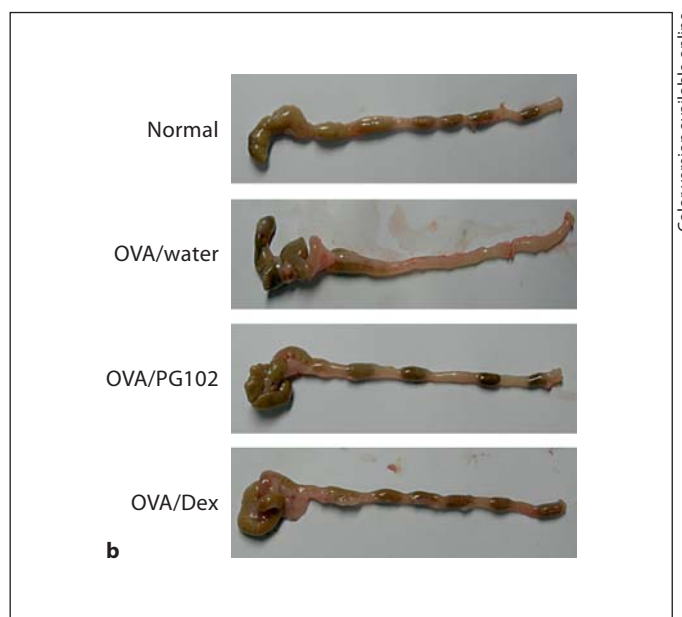


Fig. 3. Effects of PG102 in allergic diarrhea. Diarrhea was induced by OVA sensitization and repeated challenges (10 mice/group). **a** The effect on diarrhea incidence is shown. **b** The effect on macroscopic status of large intestine is shown. This figure provides 1 representative result of 4 separate sets of experiments.



and serious loss of thymus (data not shown). On the contrary, mice administered with PG102 maintained regular body weights, as compared with that of OVA/water as well as normal groups, and did not show any noticeable side effects (data not shown). These data indicated that oral administration of PG102 could ameliorate allergic diarrhea without side effects.

Effect on the Serum Level of IgE, IL-6 and MCP-1

Allergen-specific IgE mediates a type I hypersensitivity, triggering inflammatory responses in the local tissue and eventually generating various allergic symptoms, including food allergy [2–4, 12]. Next, we examined the se-

rum level of total and OVA-specific IgE, IL-6 and MCP-1, which are all known to play key roles in the establishment of allergic diseases and related inflammatory reactions [2, 4, 13–15]. The immunization with OVA increased the level of total and OVA-specific IgE in a statistically significant manner, compared with that from normal mice (fig. 4a and b). More than 30% reduction in the level of total IgE was observed in OVA/PG102 animals. In particular, it is worth noting that the magnitude of OVA-specific IgE suppression mediated by PG102 appeared to be similar to that in the OVA/Dex group (fig. 4b). The serum level of IL-6 and MCP-1 was also dramatically increased by 3- to 4-fold when animals were immunized with OVA.

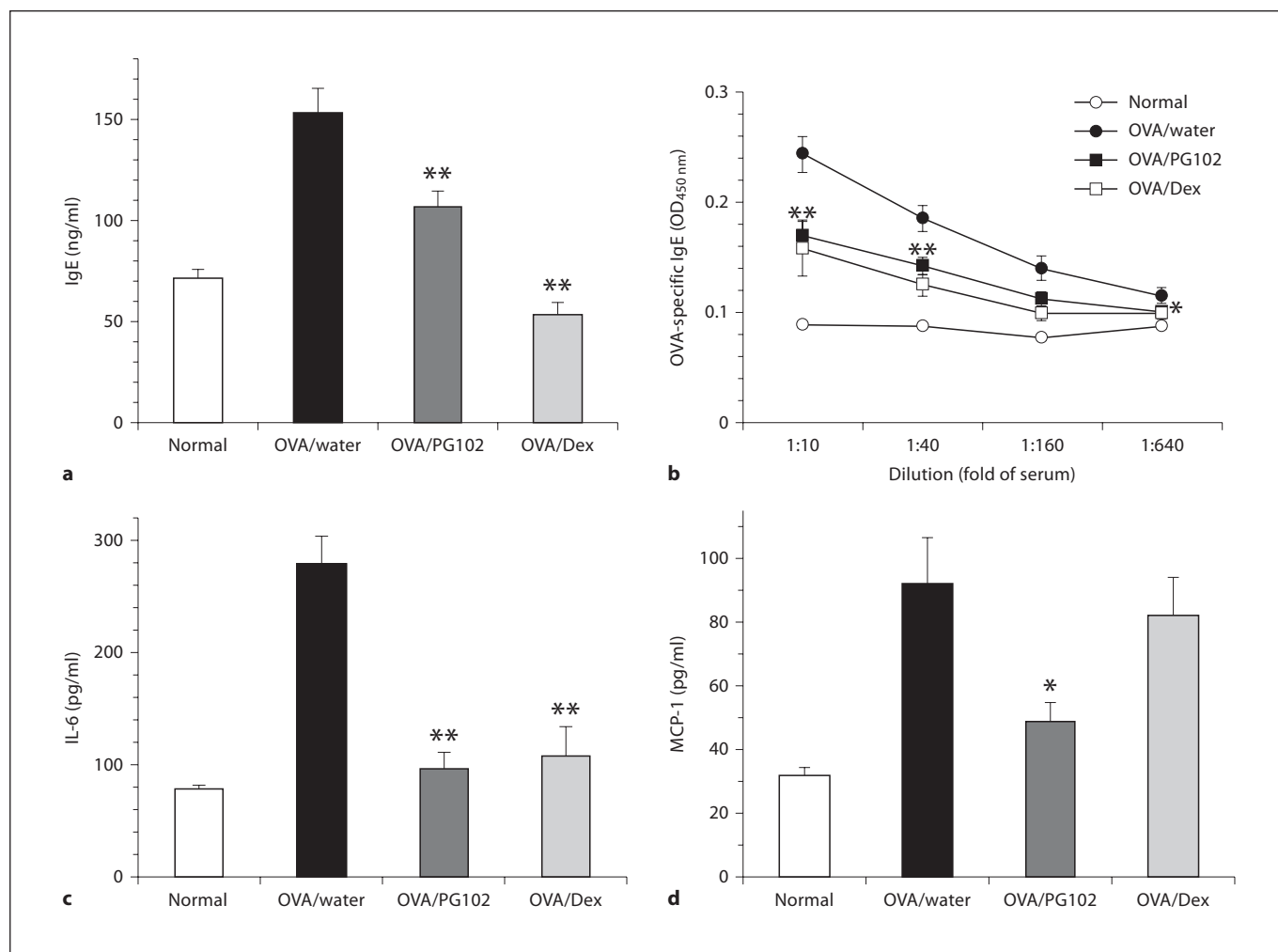


Fig. 4. Effects of PG102 on the serum level of total and OVA-specific IgE, IL-6 and MCP-1. **a, c, d** The level of total IgE, IL-6 and MCP-1 in the serum was determined using respective ELISA kits. **b** Because OVA-specific IgE antibody is unavailable, the relative level of OVA-specific IgE was represented as absorbance at OD_{450 nm} using a manual ELISA method. Values are shown as means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. OVA/water mice (Student's *t* test).

The production of IL-6 was lowered by approximately 3-fold in both OVA/PG102 and OVA/Dex groups, returning to an almost normal level (fig. 4c). In the case of MCP-1, only PG102 but not Dex could reduce its level (fig. 4d). These data suggested that PG102 could downregulate the serum level of 3 key factors involved in the early and late stages of allergic disease.

Effects of PG102 on Cytokine and Chemokine Production in Splenocytes from OVA-Sensitized Mice

To be certain that the above observation could be reproduced in an *in vitro* system, splenocytes were isolated from the mice of each group and cultured with OVA for

3 days. The culture supernatants were taken to measure the level of IL-6, IL-10 and MCP-1. Splenocytes isolated from the OVA/water group produced a large amount of IL-6, IL-10 and MCP-1 (fig. 5). However, cells from PG102-treated mice showed a significantly lower level of IL-6 (fig. 5a). The level of MCP-1 was also decreased, though the difference was not statistically significant (fig. 5b). The suppressive effect of PG102 on these inflammatory mediators was comparable to that of Dex (fig. 5a and b). On the contrary, the level of IL-10 produced from the splenocytes of PG102-fed mice was higher than that of the OVA/water group, while it was lower in the OVA/Dex group (fig. 5c). These data suggested that PG102

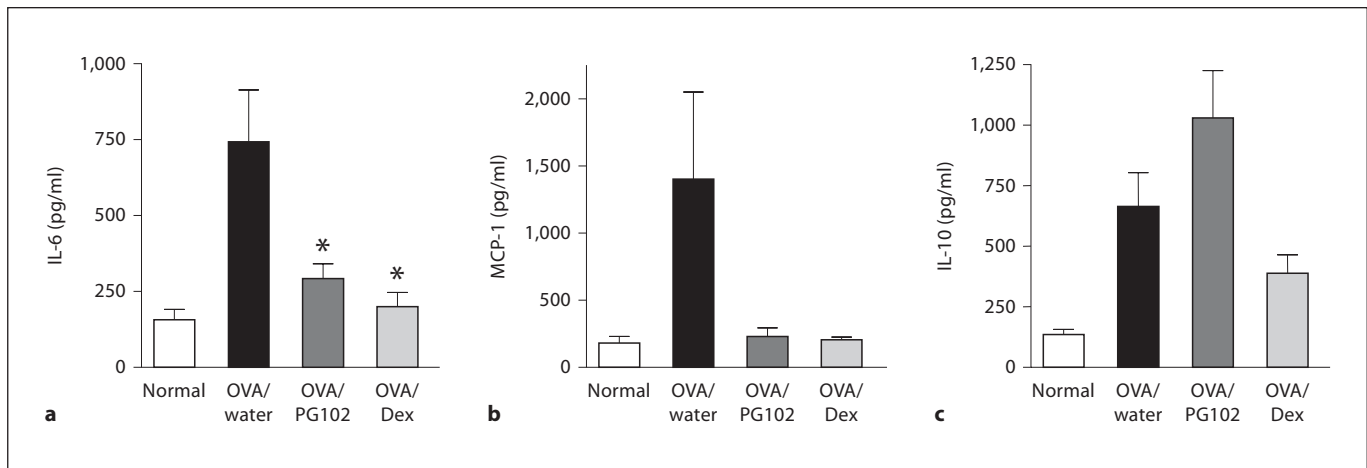


Fig. 5. Effects of PG102 on the production of IL-6, IL-10 and MCP-1 in OVA-restimulated splenocytes. Isolated splenocytes were cultured in the presence of 100 µg/ml of OVA for 3 days. The culture supernatants were taken to measure the level of IL-6 (a), MCP-1 (b) and IL-10 (c) using respective ELISA kits. The results are presented as means ± SEM. * $p < 0.05$ vs. OVA/water mice (Student's t test).

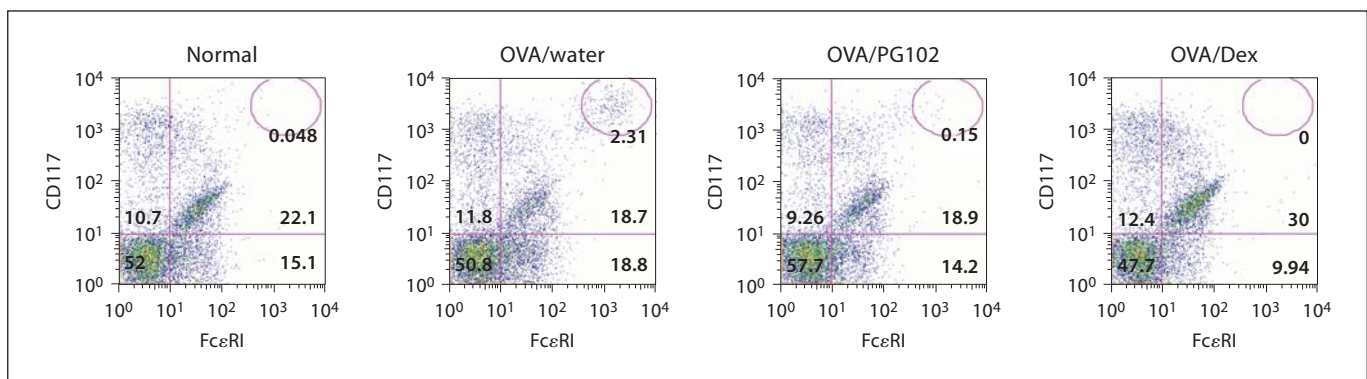


Fig. 6. Analysis of mast cell (CD117^{hi}FcεRI^{hi}) infiltration into large intestine. LPLs were isolated from the large intestines, labeled with dye-conjugated antibodies specific to CD117 and FcεRI, and analyzed by flow cytometry. The upper right corner of CD117^{hi}FcεRI^{hi} represents mast cells that infiltrated into the large intestine. This result is representative of 2 independent experiments each containing 2 mice.

might control pro- and anti-inflammatory molecules in a selective manner, unlike Dex, which suppressed the production of cytokines in an indiscriminate manner.

Oral Administration of PG102 Inhibits the Infiltration of CD117^{hi}FcεRI^{hi} Mast Cells into the Large Intestine

The lamina propria is a constituent layer of mucosa. Many different kinds of leukocytes exist in this region, and they play important roles in inducing various immune reactions to food [12, 16]. To test the infiltration status of mast cells in the large intestine, lamina propria

lymphocytes (LPLs) were isolated and analyzed by flow cytometry [8, 9, 11]. Mast cells highly express CD117 and FcεRI on their surface [11]. As revealed in figure 6, the proportion of infiltrating mast cells (CD117^{hi}FcεRI^{hi}) in the large intestine was increased to 2.31% in OVA/water mice from 0.048% in normal mice. However, oral administration of PG102 and Dex decreased the fraction of mast cells to 0.15 and 0%, respectively (fig. 6). These results indicated that PG102 might suppress an allergic inflammation by inhibiting the infiltration of CD117^{hi}FcεRI^{hi} mast cells into the allergen-sensitized large intestine.

Discussion

Specific foods can provoke adverse reactions in a susceptible individual by hypersensitivity reaction, which is termed food allergy [1–3]. Food allergens are derived from a limited number of foods, such as cow's milk, eggs, soy, wheat, peanuts and seafood, so the management of food allergy generally focuses on the avoidance of such causal food(s) from the diet [2, 3, 12]. However, the elimination of ubiquitous allergens from food is very difficult and time-consuming, and even careful individuals may accidentally ingest causal food(s) through cross-contamination. A recent clinical trial showed that the use of humanized monoclonal anti-IgE antibody, called TNX-901, could significantly increase the threshold for individuals' sensitivity to peanuts [17]. Theoretically, this approach should be protective against multiple food allergens, however, it requires continued treatment and is too expensive to be used for the purpose of prevention. Therefore, there has been a strong need for the development of a convenient, cost-effective and efficacious preventive or therapeutic agent.

We have previously shown that PG102 could regulate the expression of IgE and Th1 and Th2 cytokines involved in the allergy pathogenesis, using the OVA-sensitized mouse, NC/Nga dermatitis mouse, magnesium-deficient hairless rat and OVA-induced asthmatic mouse models [5–7]. These *in vivo* findings were also strongly supported by the data from various *in vitro* cell culture experiments [5, 7]. All these results suggested that PG102 might have preventive or therapeutic effects on a broad range of allergic diseases. In this study, we tested whether PG102 could prevent allergic diarrhea in a murine OVA-induced allergic diarrhea model. Oral administration with PG102 could sharply decrease the incidence of diarrhea. Our data suggested that PG102 might have suppressed diarrhea incidence by lowering the level of IgE, subsequently inhibiting the infiltration of CD11^{hi}FcεRI^{hi} mast cell to the large intestine (as supported by the flow cytometry analysis of LPLs), and suppressing the inflammation as evidenced by the decreased level of IL-6 and MCP-1 *in vitro* as well as *in vivo*.

PG102 highly decreased the level of IL-6, both *in vivo* and *in vitro*, while greatly lowering the amount of MCP-1 in isolated splenocytes and, to a lesser extent, in the serum. The magnitude of suppressive effects was similar to that of Dex. IL-6 is deeply involved in fever induction and the acute-phase response, while MCP-1 induces the recruitment of T lymphocytes, monocytes, eosinophils and basophils, generating a series of inflammatory reactions

[14, 15, 18, 19]. The fact that PG102 could efficiently downregulate the production of IL-6 and MCP-1 suggested that PG102 might be effectively used to control inflammatory reactions.

The effect of PG102 on IL-6 and IL-10 is interesting in the context of tolerance. IL-6 secreted by dendritic cells, together with unidentified factors produced by myeloid dendritic cells, are thought to interrupt the anergy status of CD4+CD25+ regulatory T (Treg) cells in the spleen [13, 20, 21]. IL-10 plays a key role in the differentiation of naïve CD8 T cells into Tr1 cells, a subset of Treg cells, and the induced Tr1 cells secrete a large amount of IL-10 [22–25]. That is, the tolerance could be inhibited by IL-6 but induced by IL-10, through the regulation of Treg cells. For this reason, it is tantalizing to hypothesize that one mechanism of how PG102 controls food allergies might be via the induction of immunological tolerance.

PG102 is a water-soluble extract prepared from an edible fruit. An active compound has yet to be found, and the underlying mechanism remains to be unraveled. However, several bioassays based on *in vitro* cell culture systems have been developed. In this study, the effect of PG102 on IL-4 expression was used to prepare PG102 in a consistent manner, using RBL-2H3 cells. The use of such an assay system helps to identify the active compounds and elucidate the molecular mechanisms at molecular and cellular levels as well as to control experimental reagents. Our extensive safety experiments have shown that PG102 is very safe. Together with efficacy data obtained from animal disease models, PG102 demonstrates great potential as a safe and effective reagent for food allergies as well as other allergic diseases, including dermatitis and asthma.

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