

Lipid-soluble Components of Honeybee-collected Pollen exert Antiallergic Effect by inhibiting IgE-mediated Mast Cell Activation *In Vivo*

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It was shown previously that bee-collected pollen (bee pollen, BP), inhibited *in vitro* murine mast cell activation. This study further analysed the antiallergic effect of BP *in vivo* by measuring cutaneous mast cell activation using a passive cutaneous anaphylaxis reaction. Daily oral administration of BP to mice, dose-dependently reduced the cutaneous mast cell activation elicited by IgE and specific antigens. Administration of BP also reduced the plasma concentration of malondialdehyde (MDA), an indicator of lipid peroxidation. The inhibitory effect of BP was mostly in a lipid- but not in water-soluble fraction. The HPLC analysis of isoflavones in BP revealed that genistein was a major isoflavone. However, administration of genistein alone at the concentration found in BP, did not show an inhibitory effect as observed in whole BP, suggesting that component(s) other than genistein would be responsible for the inhibitory effect of BP. These results first reveal that lipid-soluble components of BP exert an antiallergic action by inhibiting the Fc ϵ RI-mediated cutaneous mast cell activation. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: mast cell; bee-pollen; Fc ϵ RI; allergy.

INTRODUCTION

Bee pollen (BP) is a flower pollen collected from selected flower species by the honeybee, *Apis mellifera*. It is rich in vitamins, minerals, trace elements, enzymes and amino acids, and provides a good nutritional source for drone bees. In addition to these general elements, it contains a variety of secondary plant products such as flavonoids, carotenoids and terpenes (Campos *et al.*, 1997; Linskens and Jorde, 1997). Because of its nutrient-rich components, BP has been used as a folk medicine in certain cultures for thousands of years. It has become increasingly popular over recent years by recognition of its potential health benefits, including the relief of constipation and prostatic conditions, and the amelioration of several immune related diseases (Middleton, 1998; Traidl-Hoffmann *et al.*, 2003). However, limited information is available to reveal the components responsible for exerting those activities and the precise mode of action. Also limited clinical information is available to support the use of BP to treat any condition. Cernilton, an extract from eight different kinds of pollen, has been used in the treatment of chronic prostatitis, for its presumed antiinflammatory and anti-androgenic effects (Buck *et al.*, 1989; Shoskes, 2002). Although the precise mechanism is unknown, the extract's lipo-soluble fraction reportedly contains the material responsible for inhibiting the biosynthesis of prostaglandin and leukotrienes (Loschen and Ebeling, 1991).

Allergic diseases have increased recently in developed countries. There are increasing amounts of data both to support or dispel the use of dietary supplements in the treatment of inflammatory diseases, such as allergies, arthritis and chronic pelvic pain (Theoharides and Bielory, 2004). There was a report that the use of BP relieves the symptoms of patients suffering from pollen allergy aiming to induce oral desensitization (Stickl and Kerscher, 1980), however, the mechanisms of the anti-allergic action of BP have not been fully evaluated. It was noticed that BP exerts anti-allergic effects comparatively early after oral uptake. Since the mast cells play critical roles especially in immediate hypersensitivity by secreting granule-stored mediators, as well as *de novo* synthesis of cytokines after cross-linkage of Fc immunoglobulin E receptors (Fc ϵ RI) by multivalent antigens, we previously focused on mast cells and found that BP inhibited mast cell degranulation and cytokine production *in vitro* by inhibiting IgE-binding to mast cells (Ishikawa *et al.*, 2008). Thus, this study examined the *in vivo* effect of BP mainly on cutaneous mast cell activation. Also examined was the antioxidative effect of BP by measuring the levels of plasma malondialdehyde (MDA), as a marker of lipid-oxidation, since oxidative status has been suggested to affect various immune cells, including mast cells (Suzuki *et al.*, 2005; Swindle and Metcalfe, 2007; Terada, 2006). It has been reported that BP ingestion by rats decreased the levels of lipid oxidation in erythrocytes (Dudov and Starodub, 1994). The authors also reported the immunomodulatory activity of BP in rabbits (Dudov *et al.*, 1994), however, the relationship between antioxidative effects and the immunomodulatory effect of BP, and again the component of BP exerting such effects are not clear.

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Thus, this study investigated the effect of orally administered BP on the activation of cutaneous mast cells *in vivo* and lipid oxidation as a characteristic of antioxidants, and the possible component of BP responsible for such activities, to explore the possible use of BP as a dietary supplement to ameliorate the symptoms of chronic inflammatory or allergic diseases.

MATERIALS AND METHODS

Bee pollen. Bee pollen from a mixture of alfalfa and red clover was provided by BeeBest Co. Ltd (Tokyo, Japan). BP was harvested by beekeepers from Michigan, North America during 2 months in 2005 and processed for human consumption by partial drying. A broad outline of the components of the mixed pollen used in this study was described in a previous study (Ishikawa *et al.*, 2008). BP was stored at -20°C until analysis. BP was generally suspended in normal saline solution (NSS) and sonicated with Bioruptor[®] (Cosmo Bio Co. Ltd., Tokyo, Japan) at 200 W for 15 min (1 min sonication at 30 s intervals). Following centrifugation at 3000 rpm for 30 min, the supernatant was used as the water-soluble fraction. The resultant pellets were dissolved in olive oil and used as a lipid-soluble fraction.

Mice. BALB/c (6 weeks) mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed under the approval of the Institutional Review Board of Juntendo University.

Passive cutaneous anaphylaxis (PCA) reaction. The effect of BP on an IgE-dependent cutaneous anaphylaxis reaction was measured as follows. Six-week-old male BALB/c mice were administered orally with saline or various concentrations (10, 50, 250 $\mu\text{g}/\text{head}$) of BP every day for 14 days. Fourteen days after oral administration, 25 μL (10 $\mu\text{g}/\text{mL}$) of anti-TNP mouse IgE (BD PharMingen) or saline was injected intradermally into the left or right ear of mice, respectively. Twenty-four hours after sensitization with IgE, the mice were challenged intravenously with 0.5 mL (100 $\mu\text{g}/\text{mL}$) of TNP-BSA (Cosmo Bio Co. Ltd, Tokyo, Japan) containing 0.5% Evans blue dye (Wako Pure Chemical Industries Ltd, Osaka, Japan). In some experiments, BP (250 $\mu\text{g}/\text{head}$), which was separated into water- or lipid-soluble fraction as described above, or genistein (1.25 ng/head) (Calbiochem, EMD Chemical Inc., Darmstadt, Germany) dissolved in olive oil was administered for 14 days. Thirty minutes after antigen challenge, the mice were killed and the ears were removed and weighed. To measure the amount of exudate Evans blue, the ears were dissolved in 200 μL of KOH and incubated at 37°C overnight. The dissolved tissue solution was then added to a mixture of acetone and phosphoric acid (13:5) and the OD was measured at 620 nm. The amount of exudate dye was calculated from the standard curve of a known concentration of Evans blue and expressed as the amount of dye ($\mu\text{g}/\text{tissue weight (mg)}$). For compound 48/80-induced skin inflammation, 20 μL of 100 $\mu\text{g}/\text{mL}$ of compound 48/80 (Sigma Aldrich Japan KK, Tokyo, Japan) or PBS was injected intradermally into the ears of mice, and 0.5% Evans blue dye (Wako Pure Chemical Industries Ltd, Osaka, Japan) was challenged intravenously. The ears

were removed 30 min after compound 48/80 injection, and proceeded as described above.

Measurement of plasma malondialdehyde (MDA). The concentration of plasma MDA was measured using a spectrophotometric assay kit, Bioxytech[®]MDA-586[™] (Oxis Research[™], CA, USA) according to the manufacturer's instruction.

Measurement of isoflavones in BP. The quantification of the isoflavones in BP was conducted by Japan Food Research Laboratories (Tokyo, Japan), using high-performance liquid chromatography (HPLC). The water- or lipid-soluble fractions of BP were prepared as described above.

Statistical analysis. Statistical analysis was performed using Student's *t*-test.

RESULTS

Orally administered BP inhibits cutaneous IgE-dependent anaphylaxis reaction *in vivo*

To analyse the antiallergic effect of BP, the study first examined the effect of orally administered BP on the cutaneous IgE-dependent anaphylaxis reaction. As shown in Fig. 1, oral administration of various concentrations of BP (14 days) before IgE sensitization and antigen challenge, dose-dependently inhibited dye extravasations to the tissues, due to the activation of cutaneous mast cells via the cross-linkage of bound IgE with specific antigens (Koda *et al.*, 1990). The administration of BP (250 $\mu\text{g}/\text{head}$) significantly inhibited the PCA reaction. The same concentration of BP administration once, 3 h just before antigen challenge after IgE sensitization did not inhibit the PCA reaction (data not shown).

Lipid-soluble components of BP have inhibitory effects on mast cell activation

To further elucidate the mechanisms of the inhibitory effect of BP on cutaneous IgE-dependent mast cell

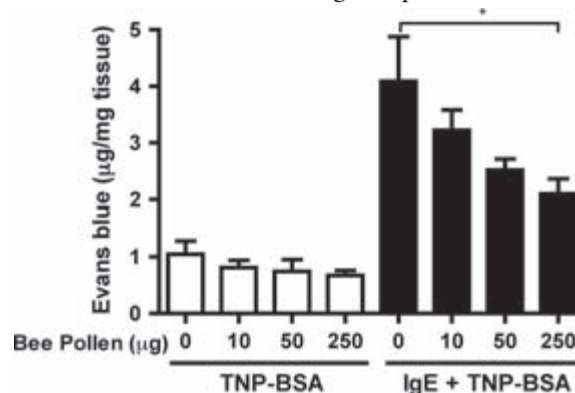


Figure 1. Orally administered BP inhibits cutaneous IgE-dependent anaphylaxis reaction *in vivo*. Extravasation of Evans blue into ears after stimulation with anti-TNP-IgE and specific antigen (TNP-BSA) was examined in mice orally administered with saline or BP (10–250 $\mu\text{g}/\text{head}/\text{day}$) for 14 days as described in Materials and Methods. Data are the mean $\pm$ SD of five mice. * $p < 0.05$, when compared with the value of saline-administered mice.

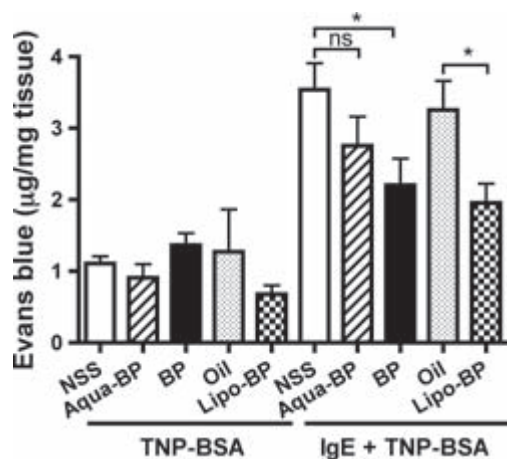


Figure 2. Lipid-soluble components of BP have inhibitory effects on mast cell activation. BP (250 µg/head) in water- (Aqua-BP)- or lipid-(Lipo-BP) soluble fraction was prepared as described in Materials and Method. Fourteen days after daily administration of each fraction of BP, the PCA reaction was elicited as described in the legend of Fig. 1. Normal saline solution (NSS) or olive oil (Oil) was used as the vehicle control for the water- or lipid-soluble fraction of BP, respectively. Data are the mean $\pm$ SD of five mice. * $p < 0.05$, when compared with the value of saline- or olive oil-treated mice.

activation, BP was separated into water- or lipid-soluble components and the same experiments were performed. As shown in Fig. 2, only lipid-soluble components, but not water-soluble components, of BP exerted an inhibitory effect against cutaneous IgE-dependent mast cell activation. Olive oil alone did not have any effect on PCA reaction.

Genistein is a main component in lipid-soluble component of BP

The most probable components in the lipid-soluble fraction of BP would be isoflavones. Since the anti-inflammatory actions of flavonoids in various foods or plants have been well documented for improving many disease-associated symptoms, and isoflavones are reported to have inhibitory actions on mast cells and allergic symptoms (Middleton, 1998), the concentration of each isoflavone in BP was analysed. As shown in Table 1, among the isoflavones, BP contained genistein,

Table 1. The concentration of isoflavones in BP^a

Isoflavone	Whole BP	Water-soluble	Lipo-soluble
Daidzin	ND	ND	ND
Glycitin	ND	ND	ND
Genistin	ND	ND	ND
6''o-malonyl daidzin	ND	ND	ND
6''o-malonyl glycitin	ND	ND	ND
6''o-malonyl genistin	ND	ND	ND
6''o-acetyl daidzin	ND	ND	ND
6''o-acetyl glycitin	ND	ND	ND
6''o-acetyl genistin	ND	ND	ND
Daidzein	ND	ND	ND
Glycitein	ND	ND	ND
Genistein	0.5 mg/100 g	ND	0.7 mg/100 g

ND, not detected.

^a Japan Food Research Laboratories, Tokyo.

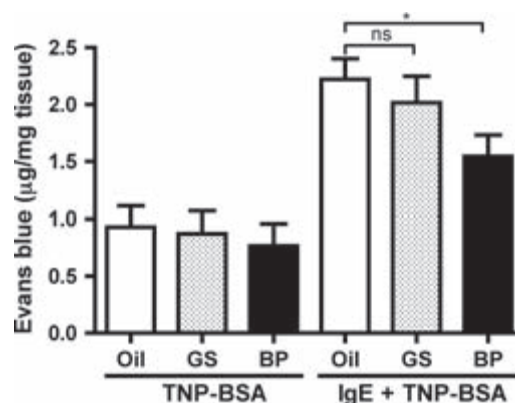


Figure 3. Administration of genistein does not lead to inhibition of cutaneous mast cell activation. Genistein (GS: 1.25 ng), which was dissolved in olive oil, was administered orally to mice for 14 days. PCA reaction was elicited as described in the legend of Fig. 1. Olive oil was used as the vehicle control. Whole BP in olive oil was used as a positive control. Data are the mean $\pm$ SD of five mice. * $p < 0.05$, when compared with the value of olive oil-treated mice.

but not other isoflavones. Since genistein is a well-known tyrosine kinase inhibitor (Akiyama *et al.*, 1987) and is reported to inhibit IgE-mediated mast cell activation (Lavens *et al.*, 1992), the study next examined whether genistein alone was sufficient to exert an antiallergic action of BP against cutaneous mast cell activation.

Administration of genistein does not lead to inhibition of cutaneous mast cell activation

The same dosage (1.25 ng) of genistein dissolved in olive oil, which was included in 250 µg of BP was administered for 14 days. As shown in Fig. 3, this dosage of genistein did not, but whole BP (250 µg/head), which was also dissolved in olive oil, exerted inhibitory activity against cutaneous IgE-dependent mast cell activation.

Oral administration of BP does not inhibit cutaneous mast cell activation elicited by compound 48/80

To further elucidate the mechanisms of the antiallergic action of BP on cutaneous mast cells, the effects of BP were investigated on cutaneous mast cell activation elicited by compound 48/80, a direct G-protein activator. The administration of the same dose (250 µg/head) of BP for 14 days showed a tendency to inhibit skin inflammation elicited by compound 48/80, but not significantly (Fig. 4). These results suggested that BP was more likely to inhibit mast cell activation by acting in early signaling events associated with FcεRI-signaling, such as phosphorylation of Syk, MAPKs and AKT etc.

Orally administered BP inhibits lipid peroxidation

Another major role of isoflavones in immune system would be an antioxidative effect. In fact, it has been reported that the function of mast cells is inhibited by treatment of mast cells with antioxidants (Suzuki *et al.*, 2005). Thus, the antioxidative effect of BP was evaluated by measuring the lipid peroxidation of plasma

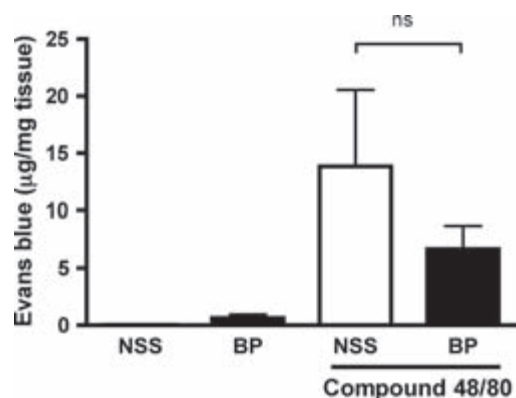


Figure 4. Oral administration of BP does not inhibit cutaneous mast cell activation elicited by compound 48/80. BP (250 µg) or normal saline solution (NSS) was administered orally to mice for 14 days. Twenty µL of compound 48/80 (100 µg/mL) was injected into the ears intradermally, and 0.5% of Evans blue was intravenously administered. The determination of the amount of extravasated dye was performed 30 min after injection of compound 48/80 as described in Materials and Methods. Data are the mean ± SD of 6–7 mice.

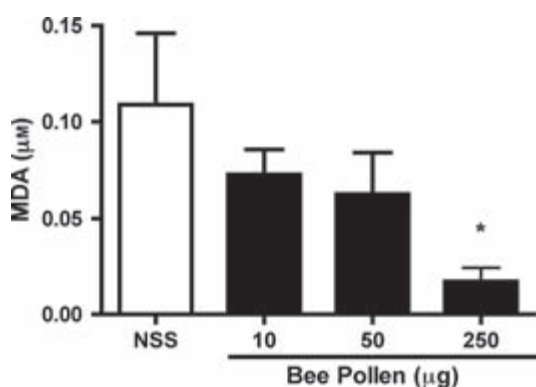


Figure 5. Orally administered BP inhibits lipid peroxidation. Normal saline solution (NSS) or BP (10–250 µg/head/day) was administered orally to mice for 14 days as described in Materials and Methods. Plasma was collected from these mice at 2 weeks after administration of BP. Plasma MDA was measured by the ELISA kit. Data are the mean ± SD of eight mice. * $p < 0.05$, when compared with the value of saline-treated mice.

malondialdehyde. Consistent with the results of the PCA reaction, the concentration of MDA was decreased by administration of BP dose-dependently (Fig. 5). The dosage of 250 µg/head of BP significantly decreased plasma MDA concentration at 14 days, but not at 7 days after administration (Fig. 5 and data not shown). When BP was separated into water- or lipid-soluble fractions, both fractions of BP significantly reduced the plasma MDA concentrations at 14 days after BP administration, which was in contrast to the effect of BP against PCA reaction (Fig. 6). Again the administration of genistein alone at the concentration found in 250 µg of BP did not affect the plasma MDA concentration (Fig. 7).

DISCUSSION

Daily administration of BP clearly inhibited the degranulation of mast cells via FcεRI-cross-linking *in vivo*. This result was consistent with our previous finding

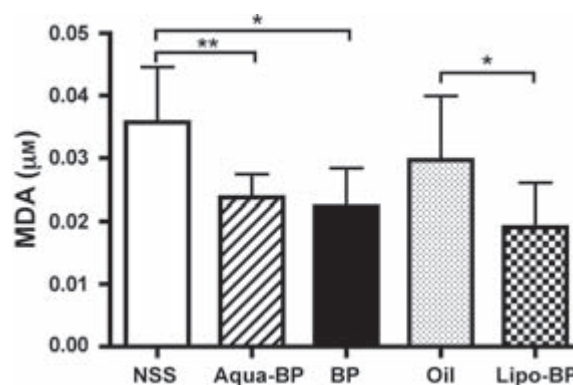


Figure 6. Both lipid- and water-soluble fraction of BP significantly reduce the plasma MDA concentrations. BP (250 µg/head) in water-(Aqua-BP) or lipid-soluble fraction (Lipo-BP) was prepared as described in Materials and Method. Fourteen days after daily administration of each fraction of BP, the plasma concentration of MDA was measured using the ELISA kit. Data are the mean ± SD of 5–8 mice. * $p < 0.05$, ** $p < 0.01$, when compared with the value of saline (NSS)- or olive oil (Oil)-treated mice.

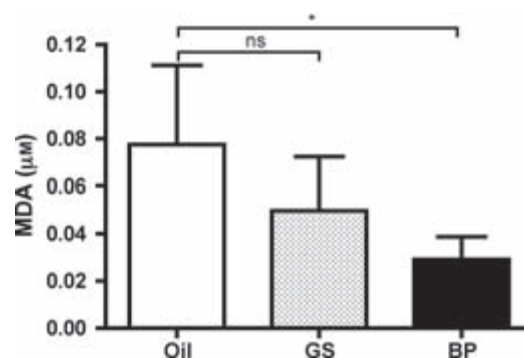


Figure 7. Genistein does not affect the levels of plasma MDA. Genistein (GS; 1.25 ng), which were dissolved in olive oil, was orally administered to mice for 14 days. Fourteen days after daily administration, plasma concentration of MDA was measured by the ELISA kit. Olive oil was used as the vehicle control. Whole BP in olive oil was used as the positive control. Data are the mean ± SD of 5–8 mice. * $p < 0.05$, ** $p < 0.01$, when compared with the value of olive oil (Oil)-treated mice.

(Ishikawa *et al.*, 2008). The detailed mechanism of this inhibition was not clear from this experiment, but it is likely that the lipid-soluble fraction of BP was responsible for this inhibition. To further elucidate the mechanisms of BP in the molecular events leading to inhibition of mast cell degranulation, the study investigated whether mast cell degranulation elicited by compound 48/80, a direct G protein activator, which bypassed the early receptor-stimulated signaling events, was inhibited by BP administration. The administration of BP showed a tendency to inhibit compound 48/80-induced cutaneous mast cell activation, but not significantly. Thus, BP mainly appeared to exert an antiallergic action by acting in early signaling events associated with FcεRI-mediated pathways, such as phosphorylation of Syk, MAPKs and AKT or IgE-binding to tissue mast cells. However, the inhibitory activity of BP on cutaneous mast cell activation elicited by compound 48/80 was not null, it is possible that BP also weakly affect mast cell activation pathways shared by, or independent of, FcεRI-mediated pathways.

The redox status has been reported to be critical for the consequence of immune cell or FcεRI-mediated

mast cell activation (Gushchin *et al.*, 1990; Suzuki *et al.*, 2005). It has been reported that feeding BP to rats for 1 month resulted in a change in the antioxidant system of erythrocytes by increasing the content of glutathione, total SH groups as well as the activities of glutathione peroxidase and glutathione reductase (Dudov and Starodub, 1994). Furthermore, the introduction of BP extract has been reported to prevent small x-irradiation-induced activation of lipid peroxidation and the antioxidant enzyme system in mouse liver (Bevzo and Grygor'eva, 1997). When the plasma concentration of malondialdehyde (MDA) was examined as an indicator of lipid peroxidation in mice after daily oral administration of BP, the plasma levels of MDA were significantly reduced in mice treated with BP for 14 days. Thus, changes in the antioxidative system in mast cells or in the tissue environment where mast cells are located, may contribute to the antiallergic action of BP. However, the results of fractionation of BP suggest that the inhibitory effect against mast cells was not solely related to the oxidative status of cells or the tissue environment. Since both the lipid- and water-soluble fraction of BP inhibited plasma lipid peroxidation, in contrast, only the lipid-soluble fraction of BP inhibited cutaneous mast cell activation (Fig. 6 vs Fig. 2). BP is a mixture of floral pollen collected by bees. Its composition varies depending on the species of pollen and the collection area. However, the major components such as sugars, proteins, fiber, fat, water and ash in BP were shown to be quite stable when those of BP collected from different years at the same area were compared (Ishikawa *et al.*, 2008). This study used BP selectively collected from a mixture of alfalfa and red clover in North America. To explore the component of BP exerting antiallergic or antioxidative activities, the profile of flavonoids in our BP was examined, since the profiles of flavonoids in BP are proportional to the ratio of individual pollen contained in BP and individual pollen has species-specific flavonoids profiles. Flavonoids are low molecular weight compounds composed of a three-ring structure with various substitutions, and are nearly ubiquitous in plants. Flavonoids can be subdivided according to their substituents into flavonols, anthocyanidins, isoflavones and flavones, flavanones and chalcones (Middleton, 1998; Middleton *et al.*, 2000). Among flavonoids, flavonols (e.g. quercetin, myricetin) and flavones (e.g. luteolin) have been reported to have a strong inhibitory activity against mast cell degranulation via prevention of the intracellular activation of Syk tyrosine

kinase or other mechanisms (Kempuraj *et al.*, 2005; Shichijo *et al.*, 2003), however, flavanones (ex; naringenin), and isoflavones (ex; genistein, daidzein) have either weak or no effect on Syk tyrosine kinase activity (Shichijo *et al.*, 2003). Major flavonoids in our BP were isoflavones, which compose more than 0.5% (w/w) of BP. In contrast, the amount of flavonols was less than 0.01%. First genistein was shown to be the major isoflavone contained in this BP. Although orally administered flavonoids have been reported to inhibit mast cell activation *in vivo* (Foreman, 1984; Kimata *et al.*, 2000), genistein, major flavonoids contained in this BP, alone did not exert an antiallergic effect on cutaneous mast cell activation or antioxidative effect. Thus, it is possible that additional lipid component(s), in addition to flavonoids, such as vitamin E and carotene, both of which are contained in BP and reported to prevent type-I allergic reaction *in vivo* (Bando *et al.*, 2003; Sato *et al.*, 2004), would be responsible for antiallergic and antioxidative effects. In addition, the water-soluble fraction of BP may also be responsible for the antioxidative activity of BP.

The BP was administered for 14 days. The duration required to exert the inhibitory effect of BP on cutaneous mast cell activation is not known. When the plasma MDA was measured, BP significantly reduced plasma MDA at 14 days but not at 7 days. Administration of BP 3 h before antigen challenge did not inhibit the following PCA reaction, suggesting that at least some duration is required to exert the antiallergic effects of BP.

In summary, it was demonstrated that oral administration of BP had an antiallergic effect by inhibiting the FcεRI-mediated activation of mast cells. It was also demonstrated that lipid-soluble components of BP other than genistein were responsible for this inhibition. This is the first report showing a specific antiallergic effect of BP focusing on mast cell function. Further investigation will reveal various benefits of BP not only for mast cell-associated allergic disease, but also for other chronic inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis.

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