

Inhibitory Effect of Honeybee-Collected Pollen on Mast Cell Degranulation *In Vivo* and *In Vitro*

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ABSTRACT Bee-collected pollen (bee pollen [BP]) has been used as a folk medicine for centuries against various diseases, including allergy. There is no study elucidating how BP exerts such an anti-allergic effect. Since mast cells play a central role in the pathogenesis of various allergic diseases, we investigated the effect of BP on mast cell activation elicited by the Fc immunoglobulin E (IgE) receptor (FcεRI)-mediated pathways. The *in vivo* effect of orally administered BP on cutaneous mast cell activation was examined by passive cutaneous anaphylaxis reaction. *In vitro* mast cell degranulation and IgE binding to mast cells and the status of protein tyrosine phosphorylation were examined using bone marrow-derived mast cells. Daily oral administration of BP to mice significantly reduced the cutaneous mast cell activation elicited by IgE and specific antigens. BP also reduced *in vitro* mast cell degranulation and tumor necrosis factor-α production by inhibiting IgE binding to FcεRI on mast cells. The inhibitory effect of BP on mast cell degranulation by preventing IgE binding was confirmed by the reduced levels of protein tyrosine phosphorylation, which occurred as downstream events in activated mast cells via FcεRI. These results first revealed that the anti-allergic action of BP was exerted by inhibiting the FcεRI-mediated activation of mast cells, which plays important roles, not only in the early phase, but also in the late phase of allergic reactions.

KEY WORDS: • allergy • bee pollen • Fc immunoglobulin E receptor • immunoglobulin E • mast cell

INTRODUCTION

BEE POLLEN (BP) is flower pollen collected from selected flower species by the honeybee, *Apis mellifera*. It provides a source of protein, carbohydrate, vitamins, and minerals for the developing larvae. In addition to these general elements, it contains a variety of secondary plant product such as flavonoids, carotenoids, and terpenes.^{1,2} Because of its nutrient-rich components, it has been used as a folk medicine for centuries, to alleviate or cure conditions such as colds, flu, ulcers, premature aging, anemia, colitis, enteritis, and allergic reactions.^{2,3} Key components providing such activity are likely to be flavonoids, since the anti-inflammatory actions of flavonoids in various foods or plants have been well documented for improving many disease-associated symptoms.⁴ It has been shown that BP ingestion by rats decreased the levels of the lipid oxidation in erythrocytes.⁵ These authors also reported immunomodulatory activity of BP in rabbits,⁶ but direct evidence showing that flavonoids

in BP are responsible for exerting these activities is completely lacking. The German Federal Board of Health has recently officially recognized pollen as a medicine, and it is currently marketed widely in Europe, America, and Asia as a tonic primarily appealing to the elderly to ameliorate the effects of aging.^{1,2} Cernilton, an extract from eight different kinds of pollen, has been used in treatment of chronic prostatitis, for its presumed anti-inflammatory and anti-androgenic effects.^{7,8} Although the precise mode of action is still obscure, evidence gained from experiments suggests that the extract's lipid-soluble fraction contains the material responsible for inhibiting the biosynthesis of prostaglandins and leukotrienes.⁹ Thus, BP is one alternative therapy utilizing plant-derived products (phytotherapy) as the treatment of choice for many chronic conditions and is gaining popularity.

Allergic diseases have increased recently in developed countries. The mast cells activated by cross-linkage of Fc immunoglobulin E (IgE) receptors (FcεRI) by multivalent antigens play critical roles in allergic reactions by secreting granule-stored mediators, as well as *de novo* synthesis of cytokines. There is an increasing amount of data both supporting and refuting the use of dietary supplements in the treatment of inflammatory diseases that involve mast cells, such as allergies, arthritis, and chronic pelvic pain.¹⁰ The

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most well-documented evidence published is on the inhibitory action of flavonoids on mast cell and allergic symptoms.⁴ Although it has been reported that BP was administered orally to patients suffering from pollen allergy aiming to induce oral desensitization,¹¹ the direct effect of BP on mast cell function along with its effect on intracellular signaling event has not been investigated. Thus, the aim of this study is to analyze the anti-allergic action of BP *in vivo* and *in vitro*, especially targeting mast cells, to explore the possible usage of BP as a dietary supplement to suppress the symptoms of chronic inflammatory or allergic diseases.

MATERIALS AND METHODS

BP

BP from alfalfa and red clover was provided by BeeBest Co. Ltd. (Tokyo, Japan). BP was harvested by beekeepers from Michigan during 2 months in 2005 and processed for human consumption by partial drying. Table 1 gives a broad outline of the components of the mixed pollen used in this study. BP was stored at -20°C until analysis.

Mice

BALB/c (5–7-weekold) mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed under the approval of the Institutional Review Board of Juntendo University.

Generation of bone marrow-derived mast cells (BMMCs)

BMMCs were generated from the femoral bone marrow cells of mice and maintained in RPMI 1640 (Sigma-Aldrich Japan KK, Tokyo) supplemented with 10% heat-inactivated fetal calf serum, 100 μM 2-mercaptoethanol, 10 μM Minimum Essential Medium-nonessential amino acids, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 10% pokeweed mitogen-stimulated spleen-conditioned medium as a source of mast cell growth factors as previously described.¹² After 4 weeks of culture, more than 98% of cells were identifiable as mast cells as determined by toluidine blue stain-

ing and fluorescence-activated cell sorting (FACS) analysis of cell surface expressions of both *c-kit* and Fc ϵ RI.

β -Hexosaminidase release assay

In vitro degranulation of mast cells was determined by β -hexosaminidase release assay as previously described,¹³ since it has been reported that the release of β -hexosaminidase was well correlated with that of histamine, a major component of mast cell granules.¹⁴ Briefly, BMMCs were sensitized with 1 $\mu\text{g}/\text{mL}$ trinitrophenol (TNP)-specific mouse IgE (BD PharMingen, San Diego, CA) in the presence or absence of various concentrations of BP at 4°C for 1 hour. After the cells were washed with Tyrode's buffer (10 mM HEPES, 130 mM NaCl, 5 mM KCl, and 5.6 mM glucose), BMMCs (1×10^6 cells/mL) in Tyrode's buffer containing 0.1% bovine serum albumin (BSA), 1 mM CaCl_2 , and 0.6 mM MgCl_2 were stimulated with 0.5 $\mu\text{g}/\text{mL}$ anti-mouse IgE (BD PharMingen) for 40 minutes at 37°C . Cell supernatants and total cell lysate solubilized with 1% Triton[®] X-100 (Sigma-Aldrich Japan KK) were collected, and β -hexosaminidase in the supernatants and cell lysates was quantified by spectrophotometric measurement of hydrolysis of *p*-nitrophenyl-*N*-acetyl- β -D-glucopyranoside (Sigma-Aldrich Japan KK) in 0.1 M sodium citrate buffer (pH 4.5). The reaction was terminated by addition of 0.2 M glycine (pH 10.7). The percentage of β -hexosaminidase release was calculated using the following formula: percentage release = (optical density of the stimulated supernatant – optical density of the unstimulated supernatant) $\times$ 100/(optical density of the total cell lysate – optical density of the unstimulated supernatant).

Measurement of cytokine concentrations

BMMCs (5×10^6 cells/mL) were sensitized with TNP-specific mouse IgE as described above in the presence or absence of various concentrations of BP at 4°C for 1 hour. After the cells were washed, BMMCs (2×10^6 cells/mL) in complete culture medium were stimulated with 0.5 $\mu\text{g}/\text{mL}$ anti-mouse IgE (BD Biosciences, San Jose, CA) for 6 hours at 37°C . The level of tumor necrosis factor (TNF)- α in the supernatant was measured using an enzyme-linked immunosorbent assay kit (R & D, Minneapolis, MN).

FACS analysis

The Fc receptor γ (Fc γ R) of BMMCs was first blocked by incubation with anti-Fc γ RII/III (2.4G2; BD PharMingen). Various concentrations of BP and then 1 $\mu\text{g}/\text{mL}$ mouse IgE antibody (BD PharMingen) were incubated with BMMCs (1×10^6 mL) at 4°C for 1 hour. After 1 hour of incubation, the cells were washed twice with phosphate-buffered saline, incubated with fluorescein isothiocyanate (FITC)-anti-mouse IgE (BD PharMingen) at 4°C for 30 minutes, and then analyzed by a FACSCalibur[™] flow cytometer (BD, Franklin Lakes, NJ). Cells incubated without IgE in the presence of BP at the indicated concentrations and

TABLE 1. VARIATIONS IN POLLEN COMPONENTS COLLECTED IN DIFFERENT YEARS

Element	% dry weight in year ^a		
	2004	2005	2006
Sugars	48	44.9	45.4
Proteins	21.6	24.1	22.6
Fiber	10.6	9.7	10.2
Fat	10.2	12.1	12.2
Water	6.5	6	6.4
Ash	3.1	3.2	3.2

^aAs analyzed by Japan Food Research Laboratories, Tokyo.

then stained with FITC-anti-mouse IgE were used as a negative control. To analyze the expression of Fc ϵ RI on BMMC, an FITC-labeled monoclonal antibody specific to the α chain of mouse Fc ϵ RI (MAR-1, eBioscience, San Diego) was used.

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 orally administered saline or 250 μ g per head of BP for 14 days. Fourteen days after oral administration, 25 μ L (10 μ g/mL) of anti-TNP mouse IgE (BD PharMingen) or saline was injected intradermally into the left or right ear of the mice, respectively. Twenty-four hours after sensitization with IgE, the mice were challenged intravenously with 0.5 mL (1 mg/mL) of TNP-BSA (Cosmo Bio Co. Ltd., Tokyo) containing 0.5% Evans blue dye (Wako Pure Chemical Industries Ltd., Osaka, Japan). In some experiments, BP (250 μ g per head) was administered orally just 3 hours before antigen challenge. Thirty minutes after antigen challenge, mice were sacrificed, and the ears were removed and weighed. To measure the amount of Evans blue dye in the exudate, ears were dissolved in 200 μ L of KOH and incubated at 37°C for overnight. Dissolved tissue solution was then added to a mixture of acetone and phosphoric acid (5:13 vol/vol), and optical density 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 (in μ g) per tissue weight (in mg).

Western blot analysis

BMMCs were sensitized with mouse IgE in the presence or absence of various concentrations of BP at 4°C for 1 hour as described for the β -hexosaminidase assay. After the cells were washed with phosphate-buffered saline, BMMCs were stimulated with 1 μ g/mL anti-IgE at 37°C for 5 minutes. The reaction was terminated by addition of cold phosphate-buffered saline and removal of the supernatant, and then cells were lysed by direct addition of 20 μ L of sample buffer (2% sodium dodecyl sulfate, 20% glycerol, and 0.01% bro-

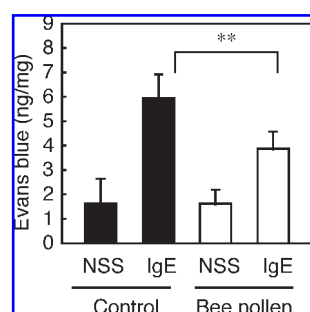


FIG. 1. *In vivo* effect of BP on cutaneous IgE-dependent anaphylaxis reaction. Extravasations of Evans blue into ears after stimulation with anti-TNP-IgE and specific antigen (TNP-BSA) was examined in mice orally administered normal saline solution (NSS) or BP (250 μ g per head per day) for 14 days as described in Materials and Methods. Data are mean $\pm$ SD values from five mice. ** P < .01, when compared with the value for saline-administered mice.

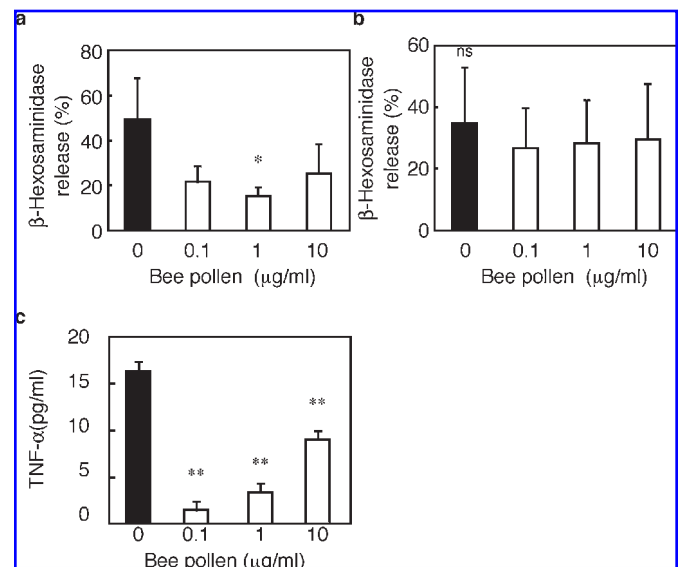


FIG. 2. *In vitro* effect of BP on mast cell degranulation and cytokine production. Various concentrations of BP were added to BMMCs when cells were sensitized with IgE at 4°C for 1 hour (a) or after IgE sensitization (b). β -Hexosaminidase release following stimulation with anti-IgE (0.5 μ g/mL) was measured and expressed as a percentage of release as described in Materials and Methods. Data are mean $\pm$ SD values of three experiments using different BMMCs. (c) BMMCs were sensitized with IgE with various concentrations of BP as described above. After two washes of the cells, BMMCs (2×10^6 cells/mL) in complete culture medium were stimulated with anti-IgE (0.5 μ g/mL) for 6 hours at 37°C. The levels of TNF- α in the supernatant were measured by an enzyme-linked immunosorbent assay kit (R & D). Data shown are one representative result of three experiments. ** P < .01, * P < .05, when compared to control BMMCs sensitized with IgE in the absence of BP.

mophenol blue in 0.125 M Tris-HCl, pH 6.8). A sample of 1×10^6 BMMCs was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the resolved protein was then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). For the detection of protein phosphotyrosine in whole cell lysate, mouse monoclonal antibody against phosphotyrosine (4G10, Upstate Biotechnology, Charlottesville, VA) was used as the primary antibody, and then Alexa Fluor 680-conjugated goat anti-mouse IgG (Invitrogen, Tokyo) was used as the secondary antibody. To confirm an equal load of protein for each sample, anti-actin antibody (C-2, Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Fluor 680-conjugated goat anti-rabbit IgG (Invitrogen) were used. Infrared fluorescence on membranes was detected by a LI-COR Odyssey[®] infrared imaging system (model ODY-9201-SC, LI-COR Bioscience, Lincoln, NE).

Statistical analysis

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

RESULTS

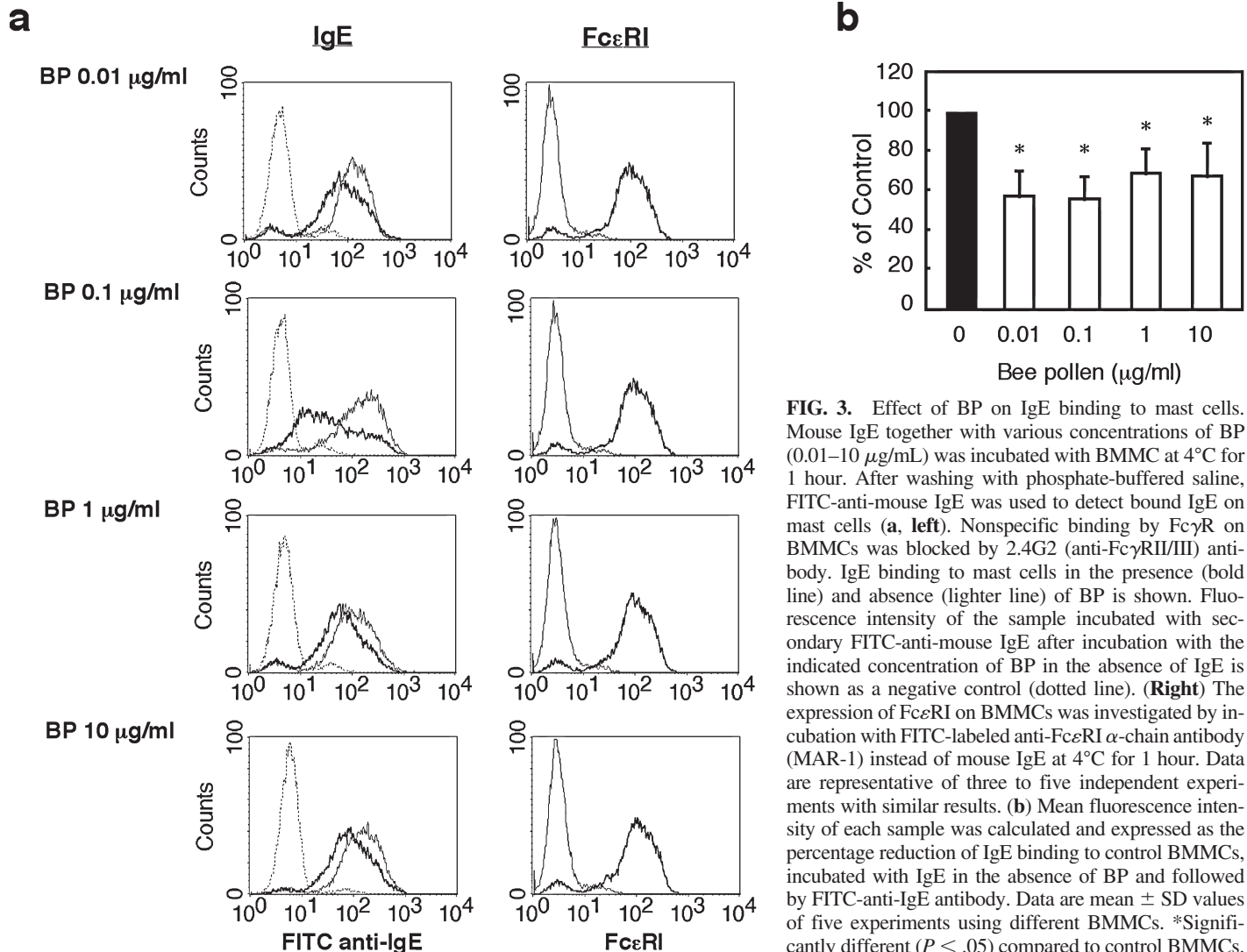
In vivo effect of BP on cutaneous IgE-dependent anaphylaxis reaction

To analyze the anti-allergic effect of BP, we first examined the effect of orally administered BP on the cutaneous IgE-dependent anaphylaxis reaction. As shown in Figure 1, oral administration of BP (for 14 days) before IgE sensitization and antigen challenge significantly inhibited dye extravasations into the tissues, because of the activation of cutaneous mast cells via the cross-linkage of bound IgE with specific antigens.¹⁵ Administration of BP after IgE sensitization and 3 hours before antigen challenge did not inhibit the PCA reaction significantly (data not shown).

In vitro effect of BP on mast cell degranulation and cytokine production

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

we examined the direct effect of BP on mast cells cultured *in vitro*. BMMCs, which can be used to evaluate mast cell function *in vitro*, resulted in degranulation after the cross-linkage of IgE bound on mast cells by specific antigens or anti-mouse IgE. Addition of various concentrations (0.1–10 $\mu\text{g/mL}$) of BP to IgE-sensitized mast cells resulted in significant inhibition of mast cell degranulation following stimulation with anti-IgE (Fig. 2a). Although all concentrations of BP tended to inhibit degranulation (49.1–68.9%), only the effect of 1 $\mu\text{g/mL}$ BP was significant. Addition of BP, after sensitization of mast cells with IgE and before stimulation with anti-IgE, did not significantly affect degranulation (Fig. 2b). In addition, preincubation with various concentrations of BP with mast cells in the culture medium for up to 3 days followed by washout before IgE sensitization did not result in significant inhibition of mast cell degranulation (data not shown). Furthermore, the production of the cytokine TNF- α after stimulation with IgE and anti-IgE was reduced by the incubation of BMMCs with BP, especially at lower concentration (Fig. 2c).



Effect of BP on IgE binding to mast cells

To clarify the mechanisms of the inhibition of mast cell degranulation by BP addition at the time of IgE sensitization, we examined the effect of BP on IgE binding to mast cells. Consistent with the results of mast cell degranulation, BP addition to mast cells at the time of IgE sensitization significantly inhibited IgE binding to mast cells. The inhibitory effect of BP was prominent when 0.1 or 0.01 $\mu\text{g/mL}$ BP was used and less prominent when higher concentrations of BP (1 or 10 $\mu\text{g/mL}$) was used (Fig. 3a, left column). The mean fluorescence intensity (as a percentage of control) of five experiments is shown in Figure 3b. Although the effect of higher concentrations (1 or 10 $\mu\text{g/mL}$) of BP on IgE binding to mast cells was not obvious from the histogram, the statistical evaluation of mean fluorescence intensity showed significant inhibition of IgE binding to mast cells at all concentrations (0.01–10 $\mu\text{g/mL}$) of BP. The same BP concentrations, added before or after IgE sensitization, did not significantly affect the binding of IgE to mast cells (data not shown). Also, BP addition did not change the expression of Fc ϵ RI itself when evaluated by a monoclonal antibody specific for the α -chain of Fc ϵ RI (Fig. 3a, right column).

Effect of BP on Fc ϵ RI-mediated protein tyrosine phosphorylation of mast cells

To elucidate whether reduced IgE binding and degranulation of mast cells in the presence of BP at the time of IgE sensitization were correlated with signaling events induced by cross-linkage of Fc ϵ RI on mast cells, we next examined the status of protein tyrosine phosphorylation essential for Fc ϵ RI-mediated mast cell activation. Cross-linkage of Fc ϵ RI on mast cells induced tyrosine phosphorylation of a number of proteins within 5 minutes in cells sensitized with IgE in the absence of BP. Addition of various concentrations of BP at the time of IgE sensitization significantly reduced the levels of tyrosine phosphorylation of several proteins (Fig. 4). The most prominent protein, with a molecular size of around 70 kDa, is Syk, an Src family protein tyrosine kinase, which plays a critical role in the initiation of downstream signal events following Fc ϵ RI-mediated mast cell activation.¹⁶ These results indicate that the inhibition of IgE binding and degranulation of mast cells by BP were followed by the reduction of several tyrosine kinase activities of mast cells.

DISCUSSION

BP clearly inhibited the *in vitro* degranulation of mast cells when it was added to cells at the time of IgE sensitization. This was clearly demonstrated by FACS analysis, showing that various concentrations of BP inhibited IgE binding to mast cells without affecting the expression of Fc ϵ RI itself. The detailed mechanism of this inhibition was not clear from this experiment, but it is unlikely that pollen particles disturbed IgE binding nonspecifically since inhibi-

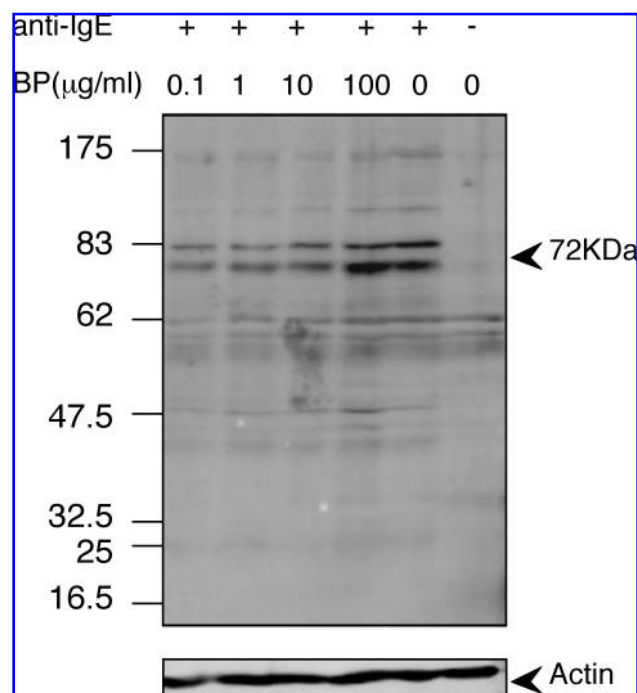


FIG. 4. Effect of BP on Fc ϵ RI-mediated protein tyrosine phosphorylation of mast cells. BMMCs were incubated with various concentrations of BP with mouse IgE at 4°C for 1 hour. Five minutes after anti-mouse IgE stimulation, tyrosine phosphorylation of resolved protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was analyzed by anti-phosphotyrosine specific antibody (4G10) and secondary Alexa Fluor 680-conjugated goat anti-mouse IgG using a LI-COR Odyssey infrared imaging system. Anti-actin (C-2) antibody and Alexa Fluor 680-conjugated goat anti-rabbit IgG were used to confirm equal protein loading in each lane.

tion was more obvious at lower concentrations, and binding of the specific antibody to the Fc ϵ RI α -chain was not affected by addition of BP (Fig. 3a). Consistent with this, BP also inhibited the production of TNF- α from mast cells at lower concentrations (0.1–1 $\mu\text{g/mL}$). Preincubation of BP with mast cells, washed out before IgE sensitization, did not result in the inhibition of mast cell degranulation, suggesting that the action of BP was more targeted toward IgE than Fc ϵ RI on mast cells or mast cells themselves. Similar inhibition of IgE binding to mast cells has been reported in BMMCs treated with polyphenol-enriched apple extracts, where highly polymeric but not low-molecular-weight procyanidins could inhibit the interaction of IgE and Fc ϵ RI on mast cells.^{17,18} Thus, a similar polymeric component may be responsible for the inhibition of IgE binding to mast cells by BP. Further detailed analysis will be required to elucidate this inhibitory mechanism by BP.

BP is a mix of bee-collected floral pollen, which varies widely in composition depending on the species of pollen and the collection area. We used BP selectively collected from alfalfa and red clover in North America. In addition to the general components shown in Table 1, BP contains a va-

riety of secondary plant products such as flavonoids, carotenoids, and terpenes.¹ Flavonoid profiles in pollen are proportional to the ratio of individual pollen in BP, since individual pollen has species-specific flavonoid profiles. We also measured the flavonoid/phenolic profiles of BP used in this experiment. Major flavonoids are isoflavones, consisting of more than 0.5% (wt/wt) of BP (data not shown), but the amount of flavonols, such as quercetin, which has been reported to have a strong inhibitory activity against mast cell degranulation, was less than 0.01% (wt/wt). Among the flavonoids, flavones (e.g., luteolin) and flavonols (e.g., quercetin, myricetin) have been shown to have inhibitory activity against human mast cells via inhibition of the intracellular activation of Syk tyrosine kinase or other mechanisms,^{19,20} whereas flavanones (e.g., naringenin), and isoflavones (e.g., genistein, daidzein) have either weak or no effect on Syk tyrosine kinase activity.²⁰ Since our BP contained mostly isoflavone-classified flavonoids and no significant inhibition of mast cell degranulation was observed when it was preincubated with mast cells and washed or added just before antigen stimulation, inhibition by BP may not be mediated by these flavonoids.

We also observed significant inhibition of IgE-mediated cutaneous mast cell activation by oral administration of BP. Since in the PCA reaction the extravasation of the dye to the tissue is caused by allergic mediators, such as histamine, serotonin, and leukotrienes, which are released as a result of mast cell activation by passively transferred IgE,¹⁵ anti-allergic action of BP in this system is caused either through the inhibition of IgE binding to tissue mast cells and subsequent inhibition of signal transduction pathways as observed *in vitro* BMMC experiments, or by other unknown mechanisms. We also observed significant reduction of serum IgE levels in ovoalbumin-immunized mice by the daily oral administration of BP (authors' manuscript in preparation), suggesting that the anti-allergic actions of BP are exerted in both the acquired immune phase and the effector phase. The mechanisms of *in vivo* inhibition by BP are more complex than *in vitro*. Since one oral administration of BP 3 hours before antigen challenge did not affect the PCA reaction, digestive and metabolic products or systemic changes after daily uptake of BP may be involved in this inhibition. 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 and total SH groups as well as the activities of glutathione peroxidase and glutathione reductase.⁵ In addition, 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.²¹ We also have examined the plasma concentration of malondialdehyde as an indicator of lipid peroxidation in mice after daily oral administration of BP. The plasma levels of malondialdehyde were significantly reduced in mice treated with BP (17 ± 16 nM) for 14 days compared with control mice (109 ± 83 nM). Since the redox status has been reported to be very critical for the consequence of immune cell or FcεRI-mediated

ated mast cell activation,^{22,23} changes in the antioxidative system in mast cells or systemically in the tissue environment where mast cells are located may contribute to this phenomenon. Again, since orally administered flavonoids have also been reported to inhibit mast cell activation *in vivo*,^{24,25} further examination will be required to establish whether flavonoids in our BP were responsible for this *in vivo* inhibition. Also, it would be interesting to know whether the oxidative status of mast cells in cutaneous or other tissues was changed after ingestion of BP.

In summary, we demonstrated that oral intake of BP had an anti-allergic effect by preventing FcεRI-mediated activation of mast cells. This may be caused by direct inhibition of IgE binding to mast cells or by systemic antioxidative action of BP on mast cells and other tissues. This is the first report showing a specific anti-allergic 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 other chronic inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis.

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