Anti-allergic Effect of Bee Venom in an Allergic Rhinitis Mouse Model

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Bee venom (BV) has been used as an anti-inflammatory and immune modulating agent in Oriental medicine. This study used a mouse model to investigate the anti-allergic effect of BV, which is used in the treatment of various inflammatory diseases in traditional medicine. BV was obtained from the National Institute of Agricultural Science and Technology of Korea. Female BALB/C mice were sensitized by intraperitoneal injection of ovalbumin (OVA). BV was administered nasally prior to the intranasal instillation of OVA. Allergic behavior, serum OVA-specific immunoglobulin E (IgE), interleukin (IL)-4, IL-10, and interferon-gamma (INF- γ) levels in nasal lavage fluid were measured. Hematoxylin–eosin and periodic acid-Schiff staining were performed to evaluate histological change. BV attenuated nasal symptoms and inhibited the production of OVA-specific IgE and IL-4 in sensitized mice. The degree of inflammatory cell infiltration and goblet cell hyperplasia was attenuated by BV. Thus, BV effectively reduced allergic inflammation in a mouse model of allergic rhinitis, suggesting its potential as a useful therapeutic agent to treat allergic rhinitis.

Key words bee venom; allergic rhinitis; ovalbumin; mouse model

Allergic rhinitis (AR) is characterized by nasal mucosal inflammation resulting from immunoglobulin E (IgE) mediated hypersensitivity reaction. Allergen exposures stimulate infiltration of inflammatory cells within the nasal mucosa, including basophils, eosinophils, mast cells, and mononuclear cells. These inflammatory cells release several allergic mediators, such as histamine, cysteinyl leukotrienes, and prostaglandins, which sustain the inflammatory reaction and produce characteristic nasal symptoms of, sneezing, itching, rhinorrhea and nasal congestion. Animal models of the allergic response to inhaled allergens have been studied to elucidate the mechanisms leading to the development of inflammation and the therapeutic effect of newly developed anti-inflammatory agents. Repeated exposure of mice to ovalbumin (OVA) has been used to develop an allergic model with inflammatory cell infiltration and increased thickness of the epithelial layer.¹⁾

Bee venom (BV) consists of a various biologically active amines, peptides and nonpeptide components, and has radio-protective, antimutagenic, anti-inflammatory, antinociceptive, and anticancer activities.^{2,3)} Two main components of BV, melittin and adolapin, have anti-inflammatory activity that involve inhibition of cyclooxygenase-2 and, phospholipase A₂ expression, and decrease levels of tumor necrosis factor- α , interleukin (IL)-1, IL-6, and nitric oxide.⁴⁾ The anti-allergic activity is associated with marked inhibition of OVA-induced tracheal contraction and histamine release from lung tissue. The mast-cell degranulating peptide binds to the mast cell receptors and inhibits the binding of IgE and production of histamine.⁵⁾ BV also inhibits the release of inflammatory mediators similar to nonsteroidal anti-inflammatory drugs.

The anti-inflammatory effect of BV includes airborne allergen-induced cytokine production from nasal epithelial cells.^{6,7)} Depending on the type of treated cells, BV can exhibit time and dose dependent immunosuppressive and/or immunostimu-

lant activity. Given the anti-inflammatory effect of BV, it can also paradoxically cause a severe allergic or inflammatory reaction, depending on the administered concentration. Thus, the optimal concentration and dosage should be determined before clinical application of BV as a therapeutic agent. In this study, we used a mouse model of allergy rhinitis to evaluate the effect of BV instillation on nasal mucosal inflammation and allergic symptoms.

MATERIALS AND METHODS

Preparation of BV Pure honeybee (*Apis mellifera*) venom was obtained from the National Institute of Agricultural Science and Technology, Suwon, Korea. BV was collected using a specialized collector without damaging the honeybee by an established electric shock method. BV was dissolved in distilled water and centrifuged at 12000 $\times g$ for 10 min to remove insoluble materials. The BV was lyophilized by freeze drying and stored.⁸⁾

Animals and Experimental Protocol Female BALB/c mice, which were six-weeks old and free of murine specific pathogens, were obtained from Hyosung Science (Daegu, South Korea). They were maintained under standard laboratory conditions in a pathogen-free cage. Food and water were freely available and all animal experiments in this study were conducted in accordance with the guidelines of the Institutional Review Board of Daegu Catholic University Medical Center.

Mice were sensitized by administration of an intraperitoneal injection of OVA (75 μ g) in 200 μ L of phosphate buffer solution (PBS) containing 2 mg of aluminum hydroxide (Sigma-Aldrich, St. Louis, MO, U.S.A.) in a total volume of 200 μ L on days 0, 7, 14, and 21. On days 22–30 after initial sensitization, mice were challenged with nasal instillation of OVA 500 μ g in 20 μ L of PBS into bilateral nasal cavities. The control group was sensitized and challenged with PBS instead of OVA (Fig. 1). BV was dissolved in PBS and nasally administered by micropipette (20 μ L at 0.05, 0.5, 5, and 10 ng/mL), beginning 1 h before each challenge on days 22–30. The control group was

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Fig. 1. Schematic Diagram of Ovalbumin (OVA) Induced Allergic Rhinitis Model

BALB/c mice were sensitized with OVA and aluminum hydroxide on days 0, 7, 14, and 21. All groups except the negative control were sensitized with intranasal instillation with OVA on days 22 to 30. BV was nasally administered 1 h before each challenge at 0.05, 0.5, 5, and 10 ng/mL on days 22–30. Positive control group was treated with phosphate buffered saline.

treated with PBS.

Evaluation of Nasal Symptoms The number of sneezing and nose rubbing motions for 15 min after the final allergen challenge was recorded and compared with that of the control group.

Nasal Lavage Fluid Collection Nasal lavage fluid was collected 24 h after the last intranasal provocation with OVA. Nasal lavage by an 18-gauge catheter was performed after partial tracheal resection. The catheter was inserted into the tracheal opening in the direction of the upper airway and into the nasopharynx. Nasal passages were gently perfused with 1 mL cold PBS and collected in a tube. The collected fluid was centrifuged at 2000 rpm for 7 min at 4°C, and the supernatant was stored at -70°C. Amounts of IL-4, IL-10 and interferon-gamma (INF- γ) in nasal lavage fluid were measured using an ELISA quantitation kit (R&D Systems, Minneapolis, MN, U.S.A.).

Measurement of OVA-Specific IgE in Serum Blood specimens were collected from the inferior vena cava 24 h after the last intranasal provocation. Serum was obtained by centrifugation and stored at -70°C. OVA-specific IgE level in serum was measured using ELISA (Pharmingen, San Diego, CA, U.S.A.).

Histological Evaluation of Nasal Mucosa Mice were painlessly sacrificed with a lethal dose (120 mg/kg) of intra-peritoneally administered pentobarbital sodium 24 h after the last intranasal provocation. Decapitated heads were immersed in 10% neutral formalin overnight. The heads were then stripped of the eyes, skin, muscle, and the mandibles were excised. Specimens were decalcified until they were soft in 0.25 mol/L ethylenediaminetetraacetic acid for 24 h. The heads were trimmed with a fresh razor blade, with excision of the anterior portion of the nose and brain, leaving a portion of the nasal sinus, which measured approximately 8 mm in length from anterior to posterior. The resulting blocks were embedded in paraffin and sectioned anterior to posterior at 5- μ m thickness.

Three anatomically similar sections were chosen from each mouse for analysis. The first section, the most anterior, was at the level of the maxillary sinuses. The second section, more posterior, was at the end of the maxillary sinuses and the beginning of the complex ethmoid turbinates. The third section, more posterior, contained the brain superiorly.

Appearance of inflammatory cell infiltration and epithelial thickness was quantified in hematoxylin and eosin stained sections at $\times 200$ and $\times 400$ magnification. Goblet cell numbers

were quantified in periodic acid Schiff (PAS) stain at $\times 200$ magnification. All tissue sections were examined blindly with respect to the source of the tissue and counts were determined at three different mucosal areas for each of the three sections per mouse.

The presence or absence of submucosal inflammatory cell infiltration was quantified into four categories -0: no, 1: mild, occasional scattered inflammatory cells, 2: moderate, 3: severe, diffuse infiltration of inflammatory cells. Epithelial thickness was directly measured on a scale of $\times 400$ magnification and average number of goblet cells was counted at four different areas per mm² of nasal mucosa by an eyepiece reticule.

To determine the anti-inflammatory effect of BV, immunohistochemical staining was performed by using the avidin-biotin complex method. Deparaffinized sections were incubated with primary antibodies for 1 h at room temperature (nuclear factor (NF)- κ B p65, activator protein (AP)-1 c-Jun, Santa Cruz, CA, U.S.A.). Bounded antibody was visualized with avidin-biotin-peroxidase complex and color was developed by 3,3'-diaminobenzidine tetrahydrochloride. The morphometrical analysis was done with an image analyzer. A minimum of three sections were analyzed per mouse. Images were captured with a Nikon ECLIPSE 80i microscope (Nikon, Melville, NY, U.S.A.) and i-Solution (IMT i-Solution; ver. 11.0, Canada) was used to measure NF- κ B p65-positive, c-Jun-positive area in epithelial area.

Statistical Analysis All measured parameters are expressed as mean $\pm$ S.D. and are representative of five independent experiments. The Mann-Whitney *U* test (SPSS Inc., Chicago, IL, U.S.A.) was performed for statistical analysis of data. A probability value less than 0.05 was considered statistically significant.

RESULTS

Allergic Behavior The mean behavior score (total number of sneezing and nasal rubbing motions for 15 min after the final challenge) was 6.2 ± 3.5 in the control group, 36.4 ± 3.2 in the AR group, 22.6 ± 4.3 in the 0.05 ng/mL of BV group, 4.3 ± 1.4 in the 0.5 ng/mL group, 15.2 ± 2.6 in the 5 ng/mL group and 24.7 ± 9.4 in the 10 ng/mL group. Behavior scores were lower in the 0.5 and 5 ng/mL BV groups, compared with the AR group ($p < 0.05$) (Fig. 2).

Nasal Lavage Fluid Cytokine and Serum OVA-Specific IgE Repeated intranasal administration of BV resulted in a

significant decrease in serum OVA specific IgE antibody level at 0.5 and 5 ng/mL of BV ($p < 0.05$). At 10 ng/mL of BV, OVA specific IgE antibody level was similar with the non-treated AR group (Fig. 2). IL-4 and IL-10 levels in nasal lavage fluid displayed a significant increase in the AR group (14.9 ± 5.2 , 12.6 ± 4.7 pg/mL, respectively), compared with the control group (4.7 ± 1.2 , 2.9 ± 0.7 pg/mL, respectively). IL-4 production was significantly inhibited by BV at 0.05, 0.5, and 5 ng/mL ($p < 0.05$). However, IL-10 and INF- γ in nasal lavage fluid did not differ significantly among the AR, control, and BV treated group (Fig. 3).

Histological Changes Whereas no or minimal inflammation was noted in the control group, all experimental groups showed an increase in inflammatory cell infiltration of the

submucosal area. The degree of inflammatory cell infiltrations showed an increase in the AR group (1.7 ± 0.4). BV induced significant inhibition of inflammatory cell infiltration at 5 ng/mL (0.9 ± 0.3). Although at other concentrations of BV, the inflammatory cell infiltration was not statistically significantly inhibited, BV tended to decrease the infiltration of inflammatory cells in nasal mucosa. Mean inflammatory cell infiltration scores were 1.2 ± 0.4 with 0.05 ng/mL, 1.3 ± 0.3 with 0.5 ng/mL, and 1.1 ± 0.5 with 10 ng/mL of BV (Fig. 4).

Thickness of epithelial cells in nasal mucosa showed a significant increase in the AR group ($27.2 \pm 6.7 \mu\text{m}$) compared with the control group ($16.3 \pm 4.7 \mu\text{m}$). However, BV did not have a significant influence on the thickness of epithelial cells (Fig. 5).

To determine the change of mucin producing cells in nasal mucosa, PAS-positive cells were counted. Mucin producing goblet cells were significant increase in the AR group ($38.2 \pm 13.3 \text{ per mm}^2$), compared with the control group ($4.3 \pm 0.8 \text{ per mm}^2$). Mucin producing cells were significantly decreased in OVA-sensitized mice treated with BV at concentrations of 0.5 ng/mL ($15.5 \pm 3.2 \text{ per mm}^2$), 5 ng/mL ($15.2 \pm 5.3 \text{ per mm}^2$), and 10 ng/mL ($12.1 \pm 3.7 \text{ per mm}^2$) ($p < 0.05$). However, 0.05 ng/mL BV did not have a significant influence on PAS-positive cells ($24.8 \pm 13.2 \text{ per mm}^2$) (Fig. 5).

To determine the anti-inflammatory mechanism of BV, NF- κB and AP-1 expressions were determined with immunohistochemical stain. NF- κB expression was significantly suppressed with BV at concentrations of 0.5 ng/mL (48%), 5 ng/mL (41%), and 10 ng/mL (49%) ($p < 0.05$). However, AP-1 expression was not significantly suppressed with BV (Fig. 6).

Fig. 2. Effects of Bee Venom (BV) on Allergy Symptoms in Ovalbumin (OVA) Induced Allergic Rhinitis Mouse Model

Total number of sneezing and nasal rubbing motions were significantly inhibited with BV at concentration 0.5 and 5 ng/mL. NC; negative control, AR; non-treated allergic rhinitis, * $p < 0.05$ vs. AR group.

Fig. 3. Effects of Bee Venom (BV) on Production of Inflammatory Mediators in Ovalbumin (OVA) Induced Allergic Rhinitis Mouse Model

(A) OVA-specific IgE levels in serum and (B–D) interleukin (IL)-4, IL-10, and interferon (INF)- γ in nasal lavage fluid after intranasal instillation of BV. OVA-specific IgE and IL-4 production was significantly inhibited by BV. The values are mean $\pm$ S.D. of five independent experiments. NC; negative control, AR; non-treated allergic rhinitis, * $p < 0.05$ vs. AR group.

Fig. 4. Effects of Bee Venom (BV) on Inflammatory Cell Infiltration in Histologic Sections of Sinonasal Tissue of Ovalbumin (OVA) Induced Allergic Rhinitis Mouse Model (H&E Stain, $\times 200$)

(A) Negative control tissue, (B) non-treated allergic rhinitis, (C) treated with 0.05 ng/mL of BV, and (D) treated with 5 ng/mL of BV. (B) shows inflammatory cells with eosinophils were infiltrated in subepithelial layer of sinus mucosa. (D) shows significantly decreased inflammatory cells infiltration.

Fig. 5. Effects of Bee Venom (BV) on the Histological Change in Ovalbumin (OVA) Induced Allergic Rhinitis Mouse Model

Epithelial thickness and the number of periodic acid-Schiff (PAS) positive cells of nasal mucosa of each study group. (A) Epithelial thickness was not influenced by intranasal instillation of BV. (B) The number of PAS positive cells was decreased decrease at 0.5, 5, and 10 ng/mL of BV. The values are mean $\pm$ S.D. of five independent experiments. NC; negative control, AR; non-treated allergic rhinitis, * $p < 0.05$ vs. AR group.

DISCUSSION

AR is the most common chronic inflammatory condition within the nasal mucosa, affecting 10% to 30% of adults and 20% to 40% of children.⁹ Chronic inflammation of the nasal mucosa is associated with pathophysiologic change in the epithelial cells, goblet cell hyperplasia, and inflammatory cell infiltration. The presentation of an allergen to lymphocytes leads to the release of Th2 cytokines, which promote IgE production and subsequent release of allergic inflammatory mediators, including histamine and leukotrienes from basophils and eosinophils. Pharmacological and immunological treatment modalities for allergy rhinitis include, antihistamines, corticosteroids, decongestants, mast cell stabilizers, leukotriene modifiers, anti-IgE antibodies, phosphodiesterase inhibitors, intranasal heparin, and immunotherapy. Although these therapies are effective and safe, the search for more effective and safe way to conclusively alter the pattern of treatment of AR is desirable.

In this study, we used BV collected by electric shock method without scarifying the honey bees. BV concentrations ranging from 0.05 to 10 ng/mL did not influence the morphology of nasal mucosa and survival of mice. Although up to 3 μ g/mL of BV is not reportedly cytotoxic to nasal epithelial cells, more than 500 ng/mL of BV is lethal to mouse.⁶ In this experiment, we used less than 10 ng/mL of BV. BV contains at least 18 active components, including enzymes, peptides, and biogenic amines. Peptides of BV have anti-inflammatory effects due to their ability to inhibit prostaglandin synthesis, stabilize mast cells, and inhibit the activity of cyclooxygenase.¹⁰ However, at higher concentrations of BV, enzymatic components that enhance the inflammatory response might influence immune response. BV induces neurogenic inflammation and tissue edema due to the toxins containing in BV.¹¹ For use in humans, the optimal concentration of BV that exerts maximal anti-inflammatory effects without harmful effects must be determined. Although BV is heat-stable and strongly affects

Fig. 6. Effects of Bee Venom (BV) on the Expression of Nuclear Factor (NF)- κ B and Activator Protein (AP)-1 in Immunohistochemical Section of Sinonasal Tissue of Ovalbumin (OVA) Induced Allergic Rhinitis Mouse Model ($\times 200$)

(A) and (B) show NF- κ B expression of non-treated (A) and treated with 5 ng/mL of BV (B). (C) and (D) show AP-1 expression of non-treated (C) and treated with 5 ng/mL of BV (D). NF- κ B expression was significantly suppressed with BV at concentrations of 0.5, 5, and 10 ng/mL ($p < 0.05$). However, AP-1 expression was not significantly suppressed with BV. NC; negative control, AR; non-treated allergic rhinitis, * $p < 0.05$ vs. AR group.

the mucous membrane, BV components are easily weakened by digestive enzymes, such as pepsin, pancreatin, and lentin. Hence, oral administration is ineffective. Intranasal application was used in this study.

Although BV has anti-inflammatory characteristics, its anti-allergic characteristic has not been commonly studied. Our previous study reported that BV inhibits airborne allergen-induced cytokine production from nasal epithelial cells by inhibiting the NF- κ B and AP-1 pathways. *In vitro*, the anti-allergic effect of BV has been demonstrated. Presently, we tried to confirm the anti-allergic properties of BV using a mouse model of AR. The model displayed characteristic allergic behaviors, tissue eosinophilia, and increased thickness of lamina propria and epithelium.¹² BV decreased allergic symptom score, inflammatory cell infiltration of nasal mucosa, and PAS positive cell counts. Although the exact mechanism of the anti-allergic effect of BV was not determined, we can suggest several possible mechanisms. First, BV can inhibit the production of chemical mediators from nasal epithelial cells. These chemical mediators influence the accumulation of inflammatory cells and Th2 immune responses in nasal mucosa. BV suppresses leukocyte migration and reduces cytokine production upon uptake of the antigen by dendritic cells.¹³ Second, BV inhibits the production of Th2 cytokines from lymphocytes. Phytohaemagglutinin and lipopolysaccharide induced TNF- α and INF- γ production from peripheral blood mononuclear cells are significantly inhibited by BV.¹⁴ Although *in-vitro* study with nasal epithelial cells, anti-inflammatory effect of

BV was associated with the inhibition of NF- κ B and AP-1 pathways, the anti-allergic effect of BV in mouse model was associated with the inhibition of NF- κ B pathways. So, BV might induce an anti-inflammatory effect by the direct inhibition of transcription factors, such as NF- κ B or AP-1. Melittin, the major constituent of BV, inhibits the DNA-binding activity of NF- κ B by inhibiting I κ B phosphorylation. Apamin inhibits both OVA-induced tracheal contraction and histamine release from lung tissue with a mast cell stabilizing effect. The mast-cell-degranulating peptide binds to the mast cell receptors and partially inhibits the binding of IgE to its receptor.¹⁵ Adolapin also has anti-inflammatory activity through its ability to inhibit prostaglandin synthesis. The eukaryotic transcription factor NF- κ B is involved in the regulation of the arachidonic acid pathway.

In the present study, nasal instillation of BV inhibited OVA specific IgE antibody production in sensitized mice and the levels of Th2 cytokine and IL-4 in nasal lavage fluid. IL-10 is an anti-inflammatory cytokine produced by monocytes and, to a lesser extent, by lymphocytes. Although IL-10 concentration was not significantly decreased by BV treatment, production tended to decrease. IL-10 suppression may lead to up-regulation of Th1 cytokines like INF- γ , IL-3, and TNF. At 5 and 10 ng/mL BV, INF- γ concentration in nasal lavage fluid was higher than in the non-treated group, suggesting that BV inhibits nasal allergic inflammation by inhibiting cytokine production from inflammatory or structural cells. However, intranasal application of BV did not influence the thickness

of epithelial layer. In allergen induced inflammation, many mediators have been implicated in the tissue remodeling and structural change with thickness of the airway wall.¹⁾ Th2 delivered cytokines are capable of inducing subepithelial fibrosis and TGF- β also plays an important role in the progression of fibrotic events. The prolonged allergen challenge of sensitized mice results in persistent remodeling of the airway. In this study, BV inhibited the expression of IL-4 and inflammatory cell infiltration in allergen sensitized mice, it perhaps not sufficiently to suppress the fibrotic change or tissue remodeling process in nasal mucosa. The airway structural change is hard to reverse with anti-allergic medication.¹⁶⁾

In summary, BV has significant anti-allergic effect in an animal model of human AR. The anti-allergy effect of BV is associated with the inhibition of Th2 cytokine production, inflammatory cell infiltration in nasal tissue and mucin production. The pharmacokinetics of the anti-allergic effect of BV has not been studied and is not fully understood. About 5 ng/mL of BV is enough to control AR in this model. Although further studies are necessary prior to clinical use, BV may be a useful therapeutic strategy to treat AR due to the anti-inflammatory effect. The combination of natural products, like BV, with modern anti-allergic medications, might enhance the therapeutic potency and minimize adverse effects.

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