Longitudinal study of effects of oral dosage of Bifidobacterium bifidum G9-1 on Japanese cedar pollen-induced allergic nasal symptoms in guinea pigs

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ABSTRACT

Previous studies using experimental animal models have reported the beneficial effects of probiotics on allergic responses; however, their long-term effects on allergic nasal symptoms in clinical settings have not yet been elucidated in detail. In the present study, a guinea pig allergic rhinitis model involving repeated inhalation challenges with a natural allergen, Japanese cedar pollen, was used to examine the longitudinal effects of Bifidobacterium bifidum G9-1 (BBG9-1) on allergic nasal symptoms. BBG9-1 was administered orally once a day. Amelioration of nasal blockage was consistently observed throughout the experimental period in the BBG9-1-treated group. Although challenge-induced sneezing was not significantly inhibited in the BBG9-1-treated group, prolonged treatment with BBG9-1 slightly reduced the frequency of sneezing. Antigen-specific IgE antibody production was also not inhibited in the BBG9-1-treated group. Increases in the numbers of eosinophils and neutrophils in nasal cavity lavage fluid collected after pollen challenge were almost completely suppressed by BBG9-1 treatment, whereas those in mast cell mediators, histamine and cysteinyl leukotrienes were not. In contrast, increases in the levels of nitric oxide metabolites were potently suppressed. Furthermore, prolonged BBG9-1 treatment markedly suppressed exogenous leukotriene D₄-induced nasal blockage. Thus, prolonged oral administration of BBG9-1 suppresses Japanese cedar pollen-induced allergic nasal symptoms. The inhibitory mechanisms responsible may involve reductions in the responsiveness of target organs, such as endothelial cells in nasal mucosal blood vessels, to chemical mediators.

Key words allergic rhinitis, *Bifidobacterium bifidum*, cedar pollen, probiotics.

Evidence that the intestinal microbiota is closely related to the induction of allergic diseases has recently accumulated. Ouwehand *et al.* have reported differences in microbiota composition between children with allergies and healthy infants: they showed that infants with atopic dermatitis have large populations of adult type *Bifidobacterium* microbiota with a high prevalence of *B. adolescentis*, whereas the microbiota in healthy infants mainly comprises *B. bifidum*, which is typically detected in breast-fed infants (1). Odamaki *et al.* identified differences in intestinal microbiota, especially in *Bacteroides fragilis*, between individuals with allergies and non-allergic individuals during the pollination period (2).

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List of Abbreviations: AUC, area under the response curve; BBG9-1, *Bifidobacterium bifidum* G9-1; cys, cysteinyl; i.p., intraperitoneal; LT, leukotriene; NCL, nasal cavity lavage; NCLF, nasal cavity lavage fluid; NO, nitric oxide; OVA, ovalbumin; sRaw, specific airway resistance.

Previous studies have demonstrated that supplementation with viable bacteria as probiotics has beneficial effects on allergic diseases. Many kinds of organisms have been used as probiotics, the most common being the lactic acid bacteria, lactobacilli and bifidobacteria (3). The beneficial effects of probiotics against allergic responses have been shown in experimental animal models (4). Sunada et al. (5) and Kawase et al. (6) recently reported that some Lactobacillus strains suppress antigen-induced nasal symptoms in mouse and guinea pig models of allergic rhinitis. We have also previously found that oral treatment with BBG9-1 suppresses serum total and antigen-specific IgE production in mice (7). Unlike mice, sensitized guinea pigs clearly exhibit both sneezing and nasal blockage induced by intranasal antigen challenge (8-10). Thus, we evaluated the efficacy of BBG9-1 in ameliorating sneezing and nasal obstruction in a guinea pig model of experimental allergic rhinitis (11). Allergic rhinitis is a common disease, affecting more than 500 million people worldwide (12). Its main symptoms, namely sneezing, rhinorrhea and nasal blockage, have a negative impact on affected individual's quality of life (12). The pathogenesis of allergic rhinitis is characterized by Th2 cell-type allergic airway inflammation manifesting as IgE-mediated mast cell activation and airway eosinophilia associated with activation of Th2 cells (13). These mechanisms have been elucidated using multiple sensitizations and long-term challenges with air-borne natural antigens such as cedar pollen and house dust mite. However, the guinea pig model (11) evaluating effects of BBG9-1 was developed by sensitizing animals with a non-air-borne antigen, OVA, and a subsequent single topical challenge with OVA solution. To determine longitudinal effects of BBG9-1 on allergic rhinitis in humans, it is desirable to use an animal model that reproduces the morbidity of human allergic rhinitis.

We previously established an experimental allergic rhinitis model in guinea pigs that uses repetitive inhalation challenges over several months with the air-borne natural antigen, Japanese cedar pollen (10, 14–16). The sensitized, repeatedly challenged guinea pigs produced large serum

concentrations of IgE antibodies that were specific to *Cry j* 1 and *Cry j* 2, antigenic proteins of Japanese cedar pollen (10, 14). The guinea pigs' nasal symptoms were similar to those reported for allergic rhinitis patients and were measured longitudinally after challenges with pollen inhalation: 1) Frequent sneezing was induced immediately after antigen provocation (10), 2) biphasic-like nasal blockage was induced after antigen challenge (10), 3) nasal responsiveness to histamine (15) and LTD4 (16) was markedly increased after multiple challenges. In the present study, in order to establish the clinical and scientific bases of the effects of BBG9-1 on allergic rhinitis,

the longitudinal effects of BBG9-1 were evaluated in a guinea pig model of cedar pollen-induced allergic rhinitis.

MATERIALS AND METHODS

Preparation of bacterial cells

Bifidobacterium bifidum G9-1 was prepared in the laboratory of Biofermin Pharmaceutical Co., Ltd. (Kobe, Japan) by isolating it from infant feces. BBG9-1 was culturedat37°C for 20 hrin *viandeet foie* broth (beef-liver extract) supplemented with 1% glucose and 0.04% cystine, then dried with sodium glutamate and dextrin. These preparations of BBG9-1 contained 2.2×10^{11} CFU/g.

Animals

Male Hartley guinea pigs (3 weeks old) were purchased from Japan SLC (Hamamatsu, Japan) and housed in an air-conditioned room at 23Tl °C and 60T 10% humidity, with lights on from 08:00 to 20:00. Access to a standard laboratory diet (Labo G standard, Nosan, Yokohama, Japan) and water was provided *ad libitum*. Sensitization of the animals began 2 weeks after arrival. All study protocols were approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University (Kyoto, Japan).

Sensitization and challenge

The study protocol is shown in Figure 1. Animals (5 weeks old) were randomly divided into two groups $(n\frac{1}{4}10)$, and sensitized with Japanese cedar pollen according to the method described by Nabe et al. (10). Briefly, the animals were sensitized by intranasal instillation of Japanese cedar pollen extract adsorbed onto an Al(OH)3 gel at a concentration of 0.3 mg protein/0.3 mg Al(OH) $_3$ / 3 mL/nostril twice a day for 7 days. Prior to each sensitization, the upper airway mucosal surface was anesthetized by 5 min inhalation of a 4% lidocaine hydrochloride solution mist generated with an ultrasonic nebulizer to prevent rapid elimination of the antigen by ciliary movement. The sensitized animals were then intranasally challenged once a week for 48 weeks by inhalation of cedar pollen using an inhalation apparatus (14). The apparatus was loaded with 3 mg of pollen and positioned in one nostril of a conscious guinea pig for 1 min so that approximately 1.8 mg of the pollen was inhaled with spontaneous breathing. During the inhalation, the other nostril was plugged with a finger. The procedure was repeated a second time for the other nostril.

As a negative control, a sensitized-non-challenged group $(n \quad 10)$ was prepared for NCL and assessment of nasal responsiveness to LTD₄.



Fig. 1. Schedules for sensitization and challenge with Japanese cedar pollen, longitudinal treatment with BBG9-1, and assessment of nasal allergic responses. Guinea pigs were sensitized by intranasal instillation of pollen extracts adsorbed onto an $Al(OH)_3$ gel twice a day for 7 days, then intranasally challenged once a week by inhalation of pollen. BBG9-1 was orally administered six times a week from 1 week before the first sensitization at a dose of 0.05 (10^7 CFU) mg/mL/day/animal. Nasal blockage (NB) was assessed by measuring sRaw and sneezing frequency was counted at the indicated times. Nasal responsiveness to intranasally applied LTD₄ was assessed 2 days after the indicated challenges. NCL was performed before and after the indicated challenges. Nasal mucosa was isolated for histological examination before or after the 48th challenge. NB, nasal blockage; SNZ, sneezing.

Oral administration of BBG9-1

As shown in Figure 1, BBG9-1 was suspended in saline and orally administered 6 times a week from 1 week before the first sensitization at a dose of 0.05 (10⁷ CFU) mg/mL/day/animal using a polyvinyl chloride tube (ATOM indwelling feeding tube for infants, 4 Fr; Atom Medical, Tokyo, Japan). A control group received oral saline.

In our previous study, the effects of BBG9-1 on OVAinduced allergic rhinitis of guinea pigs were evaluated at various doses (0.05, 0.5 and 5 mg/animal) and it was found that 0.05 mg/animal of BBG9-1 was sufficient to suppress nasal obstruction (11). Thus, we selected a dose of 0.05 mg/animal in the present study.

Measurement of nasal blockage

To evaluate the degree of nasal blockage, sRaw was measured in conscious guinea pigs immediately before and at 1 and 4 hr after the fourth, eighth, 12th and 16th antigen challenges by using a two-chambered doubleflow plethysmograph system according to the method of Pennock *et al.* (17) with a data analyzer, Pulmos-I II III (M.I.P.S., Osaka, Japan). Changes in sRaw are expressed as sRaw value minus baseline value for sRaw (obtained immediately before each challenge). At the fourth and16th antigen challenges, detailed timecourse changes in sRaw were also measured. The degrees of early and late phase nasal blockage are expressed as the AUC for changes in sRaw between before and 2 hr (early phase) and 3–10 hr (late phase) after the challenge.

Determination of sneezing frequency

Sneezing frequency was measured by observing general symptoms and listening to expiratory sounds in the interval of 0–1 hr after the fifth, seventh, ninth, 11th, 13th and 15th pollen inhalation challenges.

Measurement of *Cry j* 1-specific lgE and lgG₁ antibodies in sera

Serum samples were obtained 1 day before the fifth and 17th inhalation challenges.

Cry *j* 1-specific IgE antibodies were quantitated using an ELISA kit (Guinea pig IgE ELISA MARUPI; Dainippon Pharmaceutical, Osaka, Japan). Because this kit is for measuring guinea pig total IgE, we modified the method provided by the manufacturer to measure Crv *j* 1-specific IgE, as previously reported (18). Diluted sera were added to the wells of a microtiter plate pre-coated with an anti-guinea-pig IgE antibody and the plate was incubated for 1 hr at room temperature. After washing, 100 ng/mL of biotinylated Cry j 1 (Asahi Food and Healthcare, Tokyo, Japan) was added at a volume of 100 mL/well, followed by incubation for 1 hr at room temperature. The plate was then washed and avidin-HRP conjugate (BD Pharmingen, San Diego, CA, USA) was added. Following incubation for 30 min and subsequent washing, the substrate was added and the enzyme reaction was developed for 30 min at room temperature. The reaction was stopped with a stop solution and absorbance values were measured at 450 nm. Values for Cry j 1-specific IgE levels in tested sera are expressed in arbitrary units relative to the value of pooled standard serum from sensitized-challenged guinea pigs.

Cry j 1-specific IgG₁ antibodies were determined by an ELISA kit (Guinea pig IgG1-measuring kit; Morinaga Institute of Biological Science, Kanagawa, Japan). Because this kit is for measuring of guinea-pig total IgG_1 , we modified the method provided by the manufacturer to measure Cry j 1-specific IgG₁. Diluted sera were added to the wells of a microtiter plate precoated with an anti-guinea-pig IgG₁ antibody, after which the plate was incubated for 1 hr at room temperature. After washing, 100 ng/mL of biotinylated Cry j 1 (Asahi Food and Healthcare) was added at a volume of 100 mL/well, followed by incubation for 1 hr at room temperature. The plate was then washedand avidin-HRP conjugate (BD Pharmingen, San Diego, CA, USA) was added. Following incubation for 30 min and subsequent washing, the substrate was added, and the enzyme reaction was developed for 30 min at room temperature. The reaction was stopped with a stop solution and absorbance values measured at 450 nm. Values for Cry j 1-specific IgG₁ levels in tested sera are expressed in arbitrary units relative to the value of a pooled standard serum from sensitized-challenged guinea pigs.

Standard serum was prepared by i.p. injections of pollen extract adsorbed onto Al(OH)₃ once every week for a total of nine times in na€iveguinea pigs. Sera were

collected 2 weeks after the last sensitization, and all sera obtained pooled. *Cry j* 1-specific IgE and IgG₁ titers of the pooled serum were regarded as 1000 AU/mL.

Nasal cavity lavage and counting leukocytes

Nasal cavity lavage, which was performed as previously described (14, 19, 20), was only performed once on each guinea pig. In brief, each guinea pig was anesthetized i.p.

with pentobarbital sodium (30 mg/kg) at a predetermined time point, namely either before or 30 min or 5 hr after the 19th or 29th pollen inhalation challenge. Silicon tubing connected to an air pump was accurately

positioned in the left nostril and then maintained under slightly reduced pressure. Washing with 1 mL of physiological saline prewarmed to 37 °C was performed from the right to the left nostril. This NCL technique achieved recovery of approximately 75% of NCLF (14). The recovered NCLF was centrifuged at 120 g for 5 min at 4 °C. The resultant supernatant was stored at 80 °C until assays for histamine, CysLTs, and NO₂and NO₃⁻ concentrations. The resultant cell pellet was suspended in a defined volume (200 mL/sample) of physiological saline. The number of total leukocytes in the NCLF was determined by staining with Turk's solution. To obtain differential leukocyte counts, a 50 mL aliquot of the cell suspension was centrifuged on a Settling chamber (Neuro Probe, Cabin John, MD, USA) at 50 g for 30 s at 4 °C, after which the settled leukocytes were stained with Diff-Quik solution (International Reagents, Kobe, Japan). A minimum of 300 cells were counted under the microscope and classified as mononuclear cells, eosinophils, and neutrophils based on morphological criteria. All microscope slides were coded before the analysis and read blind to

Measurement of histamine, CysLTs, and NO_2^- and NO_3^- in NCLF

avoid observer bias.

Histamine in NCLF was assayed fluorometrically by HPLC according to a previously described method (21, 22). The amount of CysLTs was measured after extraction of the sample. As reported previously (19), a 1.6 mL volume of ice-chilled ethanol was added to 0.4 mL of the sample and the mixture allowed to stand at 0 °C for 30 min. After centrifugation at 15,000 g for 15 min at 4 °C, 1.8 mL of the supernatant, to which 40 mL of a 0.2% gelatin solution had been added, was evaporated to dryness under reduced pressure. The residue was then dissolved in 0.36 mL of 0.1 M phosphate buffer (pH 7.5) containing 0.1% BSA. The amount of CysLTs in the solution was measured using a leukotriene C4/D4/E4/ enzyme-immunoassay system (Amersham International plc, Buckinghamshire, UK). The amounts of NO_2^- and NO_3^- were measured with an NO_3^- assay kit-F (fluorometric) (Dojindo Lab, 2 3 Kumamoto, Japan).

Histological examination

For histological examination of the nasal mucosa, guinea pigs were anesthetized i.p. with pentobarbital sodium (30 mg/kg), and then exsanguinated from the abdominal aorta 5 hr after the 48th antigen challenge. Immediately after death, the head of each guinea pig was taken from the carcass, and the skin, lower jaw, muscle and other soft tissues removed. The nasal cavities were flushed via a cannula through the nasopharyngeal orifice with 20 mL of 10% neutral buffered formalin and the skulls subsequently fixed in 10% neutral buffered formalin and then decalcified in 5% formic acid solution. After decalcification, a transverse tissue block containing the nasal airway, which was trimmed at the incisive papilla, was embedded in paraffin wax and cut into 4 mm-thick sections that were stained with Luna and examined by light microscopy to assess eosinophil infiltration.

Measurement of nasal responsiveness to LTD₄

Two days after the eighth, 10th, 14th and 16th pollen inhalation challenges, nasal responsiveness to intranasally instilled LTD₄ was measured as previously described (16). At 20 min intervals, increasing doses (10 mL/each nostril) of the LTD₄ solution (10^{-8} and 10^{-6} M) were applied bilaterally. sRaw was measured 10 min after each of the agonist dose. A sensitized–non-challenged group was prepared as a negative control.

Statistical analyses

Data are presented as the meanTSEM. Results for sneezing were statistically evaluated using the Mann– Whitney U test. Results for nasal blockage were statistically evaluated using the Student's *t*-test or Welch's *t*-test after evaluation with the F-test. Probability values of P < 0.05 were considered significant. All statistical calculations were performed with SPSS software (SPSS, Chicago, IL, USA) in our laboratory.

RESULTS

Effects on nasal blockage

We have previously reported that allergen-induced nasal blockage, detected by increases in sRaw in this Japanese cedar pollen-induced allergic rhinitis model, consisted of early and late phases that peaked 1 and 4 hr, respectively, after a pollen challenge (10). Thus, we investigated the effects of BBG9-1 on nasal blockage by measuring sRaw 1 and 4 hr after antigen challenges. Prolongedoral

administration of BBG9-1 almost consistently inhibited increases in sRaw 1 and 4 hr after the fourth, eighth, 12th, and 16th challenges; however, the degree of this inhibition was sometimes significant and sometimes not (Fig. 2a).

We also measured detailed time-course changes in sRaw at the fourth and 16th pollen inhalation challenges. In the control group, pollen inhalation challenges induced increases in sRaw that lasted for 6-10 hr (Fig. 2b). We have reported that long-lasting nasal blockage may consist of early and late phases peaking at around 1 and 4–6 hr, respectively (10). Nasal blockage was ameliorated in the BBG9-1-treated group during all measurement periods (Fig. 2b). Early and late phase nasal blockage are expressed as the AUC for changes in sRaw between before and 2 hr (early phase) and 3-10hr (late phase) after each challenge. BBG9-1 inhibited early and late phase nasal blockage by 59% and 34%, respectively, at the fourth challenge, and by 64% and 55%, respectively, at the 16th challenge. We observed significant differences in early phase nasal blockage at both challenges (Fig. 2b).

Effects on sneezing

As previously reported (10), most sneezing is induced within 1 hr of pollen inhalation challenge. In contrast to the effects observed on nasal blockage, oral administration of BBG9-1 did not markedly affect the occurrence of sneezing induced by the fifth–11th inhalation challenges (Fig. 3a). At the 13th and 15th pollen inhalation challenges, the BBG9-1 treatment inhibited the frequency of sneezing by 39% and 44%, respectively, whereas this degree of inhibition was not significant (Fig. 3a).

Effects on *Cry j* 1-specific lgE and lgG₁ production

Cry j 1-specific IgE and IgG₁ antibodies were detected in the serum of sensitized-challenged animals on day 6 after the fourth challenge and were markedly increased on day 6 after the 16th challenge, which is consistent with our previous findings (10). Oral administration of BBG9-1 did not inhibit production of IgE or IgG₁ (Fig. 3b).

Effects on leukocyte infiltration into the nasal mucosa

The numbers of leukocytes, including eosinophils, neutrophils and mononuclear cells (mainly consisting



Fig. 2. Effects of prolonged oral BBG9-1 on nasal blockage. (a) Changes in sRaw, an indicator of nasal blockage, 1 and 4 hr after the indicated challenges with pollen are shown. (b) At the 4th and 16th challenges, time-course changes in sRaw were measured until 10 hr after each challenge. The inserted panels in Figure 2b represent the AUC for time-course changes in sRaw 0–2 hr and 3–10 hr after the fourth and 16th challenges. BBG9-1 was administered orally six times a week from one week before the first sensitization. Each point and column represents the mean T.S.E. for 10 animals. m, P < 0.05; mm, P < 0.01.

of lymphocytes, macrophages and dendritic cells) were significantly increased in NCLF 5 hr after the 19th pollen inhalation challenge, which is consistent with our previous findings (20). The antigen-induced increases in the numbers of all these leukocytes were completely suppressed by oral administration of BBG9-1 (Fig. 4a).

We conducted pollen inhalation challenges and the oral administration of BBG9-1 until the 48th challenge; the nasal mucosa was isolated 5 hr after this challenge. Figure 4b shows representative light microscopic photographs of the nasal mucosa after Luna staining. As previously reported (20), histological examination revealed that antigen inhalation challenge caused marked accumulation of inflammatory cells, mainly eosinophils, in the nasal mucosa (Fig. 4b), whereas this did not occur in sensitized-non-challenged animals. Consistent with the results of NCL, BBG9-1 treatment potently suppressed antigen-induced eosinophil accumulation (Fig. 4b).

Effects on increases in amounts of mast cell mediators, histamine, and cysteinyl leukotrienes, and NO₂⁻/NO₃⁻ in NCLF

As reported previously (19), there were large amounts of mast cell mediators, histamine and cysteinyl leukotrienes in NCLF 30 min after the 29th pollen challenge (Fig. 5a). These increases were not inhibited by prolonged treatment with BBG9-1 (Fig. 5a).



Fig. 3. Effects of prolonged oral BBG9-1 on frequency of sneezing and amounts of serum Cry j 1-specific IgE and IgG1 antibody. (a) Sneezing was counted at indicated challenges with pollen. (b) Sera for measuring antibody were collected 6 days after the 4th and 16th challenges. BBG9-1 was administered orally six times a week from one week before the first sensitization. Each column represents the mean T.S.E. for 10 animals.

As shown in Figure 5b, the NO metabolites NO -/NO -

were significantly increased after the 29th challenge. Treatment with BBG9-1 significantly suppressed these increases in amounts of NO $_{2}$ /NO $_{3}$ (Fig. 5b).

Effects on exogenous LTD₄-induced nasal blockage

We have previously reported that non-challenged guinea pigs show only slight or no increase in sRaw 10 min after intranasal instillation of a high concentration (10^{-6} M) of LTD₄, whereas repeatedly challenged animals showed marked increases in sRaw with intranasal administration of LTD₄, even at 100–1000-fold lower concentrations, on day 2 after a pollen challenge (16). In the present study, nasal responsiveness to LTD₄ was longitudinally assessed



Fig. 4. Effects of the longitudinal oral dosing of BBG9-1 on (a) increases in numbers of leukocytes, mononuclear cells, eosinophils and neutrophils in NCLF and (b) nasal mucosal inflammation manifested as eosinophilia. (a) NCL was performed 5hr after the 19th challenge BBG9-1 was administered orally six times a week from one week before the first sensitization. Each column represents the mean T.S.E. for 10 animals. ^m, P < 0.05; ^{mm}, P < 0.01 for sensitized-non-challenge versus control group. (b) The nasal mucosa was isolated 5 hr after the 48th challenge for Luna staining.

2 days after the eighth, 10th, 14th, and 16th pollen challenges. As reported previously (16), the degree of nasal hyperresponsiveness was gradually augmented by the multiple pollen challenges (Fig. 6). Development of nasal hyperresponsiveness at the 14th and 16th inhalation challenges was almost completely suppressed by prolonged oral administration of BBG9-1 (Fig. 6).

DISCUSSION

The longitudinal effects of BBG9-1 on induction of allergic rhinitis symptoms induced by inhalation of Japanese cedar pollen were assessed using a guinea pig model. Prolonged treatment with BBG9-1 consistently suppressed pollen-induced nasal blockage, which persists for 6–10 hr after a pollen challenge, whereas induction of sneezing was not significantly suppressed by the treatment. On the other hand, the increase in nasal responsiveness to exogenous LTD₄ during multiple pollen challenges was markedly suppressed by the longitudinal treatment. Thus, we herein experimentally



Fig. 5. Effects of prolonged oral BBG9-1 on changes in amounts of (a) mast cell mediators, histamine, and cysteinyl leukotrienes, and (b) NO $^{-3}$ NO $^{-3}$ in NCLF. NCL was collected 30 min after the 29th challenge to measure mast cell mediators and NQ $^{-7}$ NQ $^{-2}$. Each

column represents the mean T S.E. of 10 animals. Significantly different from the sensitized-non-challenge group ($^{+}P < 0.01$) and control group ($^{m}P < 0.05$).

demonstrated that BBG9-1 alleviates the nasal blockage and relieves the development of nasal hyperresponsiveness induced by a natural allergen.

We previously reported that BBG9-1 alleviates not only nasal blockage, but also sneezing induced by the antigen OVA in guinea pigs (11). We are currently unclear why the present results differ from previous findings on the influence of BBG9-1 on allergic sneezing. However, one prominent difference is the antigen used. In contrast to the purified protein, OVA, the natural antigen, cedar pollen cracks after landing on the mucosal surface, leading to elution of various antigenic proteins, including Cryj 1 and Cryj 2. Therefore, the mechanisms by which nasal allergic sneezing is induced by a pollen allergy may not be identical with those involved in an OVA-induced allergy.

To investigate the inhibitory mechanisms of polleninduced nasal blockage, the effects of BBG9-1 on serum concentrations of antigen-specific antibodies were assessed. Increase in serum concentrations of antigenspecific IgE and IgG1 antibodies, which are both anaphylactic antibodies in guinea pigs, were not inhibited by BBG9-1. This result is not consistent with the general consensus that probiotics have the capacity to create conditions that facilitate re-direction of allergeninduced Th2-skewed responses toward a healthier, regulated Th1/Th2 balance (4). Previous studies have reported that Lactobacillus strains suppress antigenspecific IgE production in murine models (5, 23–25). However, another Lactobacillus strain did not significantly inhibit antigen-specific IgE production in sensitized guinea pigs (6). Thus, there may be species differences between mice and guinea pigs in the effectiveness of probiotics on antigen-specific IgE production. We and others have demonstrated that allergen-induced sneezing is markedly suppressed by histamine H1 receptor antagonists (19, 26), indicating that sneezing is mainly mediated by histamine, which is released from airway mast cells immediately after binding of the antigen to antigen-specific IgE. In the present study, BBG9-1 did not inhibit sneezing or histamine release in the nasal mucosa, which is consistent with the lack of effect of BBG9-1 on IgE production. Therefore, inhibition of nasal blockage by BBG9-1 cannot be attributable to IgE production or mast cell activation, but must involve other mechanisms, as speculated below.

Despite its lack of effect on release of mast cell mediators, BBG9-1 treatment reduced production of NO

by nasal tissue. We have previously demonstrated that

NO contributes to pollen antigen-induced nasal blockage responses through vasodilation and plasma extravasation by the nasal mucosa (27). We have also reported that locally produced NO may be derived from activation of constitutive NO synthase in nasal vascular endothelial cells (27). Furthermore, we have shown that exogenously applied LTD₄ causes both nasal blockage and nasal NO production in sensitized, repeatedly challenged, guinea pigs (16), indicating that endogenously released CysLTs may also cause NO production in endothelial cells. Taken together, these findings suggest that prolonged treatment with BBG9-1 may reduce the responsiveness of blood vessels to chemical mediators, resulting in



reduction in NO, which is consistent with the inhibition of development of nasal hyperresponsiveness to LTD₄ by BBG9-1. The suppressive effects of BBG9-1 on the nasal responsiveness were strengthened as the duration of the treatment was extended.

In the present study, increases in leukocyte counts in nasal lavage fluid were potently suppressed by BBG9-1 treatment. Eosinophils are known to play a role in exacerbating allergic rhinitis (13). However, BBG9-1 treatment not only reduced the numbers of eosinophils, but also those of neutrophils and morphologically identified mononuclear cells, including lymphocytes, macrophages and dendritic cells. Thus, we consider that BBG9-1 treatment alters the nasal tissue such as to reduce production of not only Th2 cytokines, IL-4, IL-5, and IL-13, but also of other molecules that recruit neutrophils and mononuclear cells. Because appropriate anti-guinea pig cytokine antibodies are not yet commercially available, it is not yet possible to quantitate guinea pig cytokines. Based on the effectiveness of BBG9-1 on both leukocyte infiltration and NO production, we speculate that longitudinal treatment with BBG-1 alters nasal mucosal vascular endothelial cells to reduce their production of NO, possibly through inhibiting leukocyte infiltration into the nasal tissues. The reduced production of NO may not only inhibit nasal blockage responses induced by the allergen, but also by exogenously applied LTD₄.

The mechanisms of action by which BBG9-1 alters nasal blood vessel responses currently remain unclear. Previous studies have reported that intestinal bacteria influence the development of the gut immune system and play important roles in both health and disease (28, 29). Furthermore, during the pollination period differences have been detected in intestinal microbiota, especially in *Bacteroides fragilis*, between individuals with and without allergies (2). Furthermore, oral administration of *Bifidobacterium longum* inhibits this fluctuation, preserving the proper microflora (2). Additionally, probiotics reportedly directly stimulate the gut immune system and modulate innate and adaptive responses (30, 31). Collectively, previous findings and the results of the present study suggest that some of the mechanisms by which BBG9-1 suppresses antigen-induced nasal symptoms are associated with improvements in the intestinal microbiota and/or the gut immune system. Further studies are required to clarify this relationship.

In conclusion, longitudinal oral dosing with BBG9-1 significantly inhibits Japanese cedar pollen-induced nasal blockage and development of nasal hyperresponsiveness in an experimental allergic rhinitis model, the pathogenesis of which is similar to that which occurs in clinical settings. The inhibitory mechanism is likely associated with reduction in leukocyte infiltration and NO production in the nasal tissue. The results of the present study suggest that BBG9-1 has potential as a treatment for the nasal symptoms of patients with allergic rhinitis.

DISCLOSURE

Satoru Tsunemine, Yasuhiro Isa, Hiroshi Ohno and Hideki Yamamura are employees of Biofermin Pharmaceutical Co., Ltd., Kobe, Japan. Takeshi Nabe, Satoko Hagino, and Nobuaki Mizutani have no conflicts of interest.

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