

Oral Administration of *Bifidobacterium bifidum* G9-1 Suppresses Total and Antigen Specific Immunoglobulin E Production in Mice

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Recent studies have suggested that oral bacteriotherapy with probiotics might be useful in the management of allergic diseases. We investigated the effect of oral administration of *Bifidobacterium bifidum* G9-1 (BBG9-1) on immunoglobulin (Ig) E production in BALB/c mice. Live BBG9-1 was orally administered to mice for 2 weeks from 1 week before ovalbumin (OVA)-immunization. The treatment of BBG9-1 significantly reduced serum total IgE level. In addition, BBG9-1 significantly and largely reduced the serum level of OVA-specific IgE without lowering of the specific IgG1 and increasing of the specific IgG2a. We also examined T helper type (Th) 1 and Th2 cytokine production from OVA-immunized splenocytes by restimulation with OVA *in vitro*. Productions of interferon (IFN)- γ , interleukin (IL)-4 and IL-5 from the splenocytes of mice given BBG9-1 were weaker than those of control mice. We conclude that oral administration of BBG9-1 selectively and powerfully suppresses total and antigen specific IgE production in mice. It is suggested that BBG9-1 is useful for the prophylactic treatment in IgE-dependent allergic diseases.

Key words *Bifidobacterium bifidum*; immunoglobulin E; ovalbumin (OVA); oral administration; allergic disease

Probiotics are well known to be live bacteria or components of bacteria that have a beneficial effect on human health.¹⁾ Some probiotics, such as lactic acid bacteria, have been used to improve symptoms resulting from changes in the intestinal flora.^{2,3)} Furthermore, several reports have revealed that these probiotics can act in helping to protect from intestinal infections,^{4,5)} alter immunostimulation⁶⁾ and decrease elevated serum levels of cholesterol^{7,8)} and glucose^{8–10)} on several animal models.

There have been several recent reports on regulation of allergic antibody production. Intraperitoneal injection of probiotics, heat-killed *Lactobacillus casei* strain Shirota (LC)¹¹⁾ and heat-killed *Lactobacillus plantarum* L-137¹²⁾ suppressed antigen-specific immunoglobulin (Ig) E production in a food allergy model in mice. Moreover, the oral feeding of probiotics, heat-killed LC,¹³⁾ heat-killed *Lactobacillus paracasei* KW3110,¹⁴⁾ live *Lactobacillus acidophilus* L92 and *Lactobacillus fermentum* CP34¹⁵⁾ were effective in inhibiting total IgE production and/or ovalbumin (OVA)-specific IgE production in serum of immunized mice. These reports suggested that the mechanism of the probiotic action might be mediated via the effect of T helper type (Th) 1 cytokine interferon (IFN)- γ production through interleukin (IL)-12.^{11–14,16)}

Bifidobacterium is one of the major intestinal microflora components. During the period when the microflora of the gut becomes established, breast-feeding can promote the intestinal colonization of *Bifidobacterium*. Recently, He *et al.*¹⁷⁾ have reported that allergic infants were found to have lower levels of *Bifidobacterium bifidum* than that of healthy infants.

In the present paper, we examined the effect of *Bifidobacterium bifidum* G9-1 (BBG9-1) on IgE production in OVA-immunized mice and cytokine production of splenocytes from the mice orally administered BBG9-1.

MATERIALS AND METHODS

Bacteria BBG9-1 and *Enterococcus faecalis* JCM8726 (EFJCM8726) were obtained from our laboratory and the

Japan Collection of Microorganisms (Wako, Japan), respectively.

BBG9-1 and EFJCM8726 were cultured at 37 °C for 20 h in VF broth supplemented with 1% glucose and 0.04% cystine. For *in vitro* experiments, heat-killed bacteria were obtained by heating the suspension of the bacteria at 100 °C for 10 min.

For *in vivo* experiments, BBG9-1 was dried with sodium glutamate and dextrin. These preparations of BBG9-1 contained 2.5×10^{11} colony forming units (cfu)/g.

Mice Male, 4-week-old BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were housed in plastic cages in a room kept at 22 ± 3 °C and $55 \pm 5\%$ humidity, on a 12 h light/dark cycle under pathogen-free conditions. The mice were fed a standard diet (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and were allowed free access to water throughout the experimental period.

This animal study was approved by the Experimental Animal Care and Use Committee of Biofermin Pharmaceutical Co., Ltd.

Oral Administration of Live Bacteria and Immunization (Fig. 1) Live BBG9-1 were orally given to mice ($n=6$ per group) at 10^{10} cfu/0.2 ml/d/animal for 2 weeks from 1 week before the 1st immunization by intraperitoneal injection of 1 μ g OVA (Sigma Grade V; Sigma Aldrich Co., St. Louis, MO, U.S.A.) absorbed on 2 mg of Al(OH)₃ gel (alum). Once a week after the 2nd immunization with above mentioned OVA absorbed on alum, blood was collected from orbital cavity or the carotid artery during deep anesthesia. The

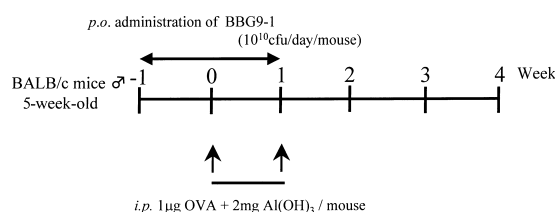


Fig. 1. Schedule for Immunization with OVA and Oral Administration of BBG9-1 to Mice

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sera were prepared by centrifugation at $10,000\times g$ at 4°C for 10 min and were frozen at -80°C until the determination of immunoglobulins.

Cytokine Production from Splenocytes *in Vitro*
Spleens were removed from non-immunized or OVA-immunized mice during anesthesia, and single-cell suspensions were prepared by squeezing them between two glass slides in Hanks' balanced salt solution (Nissui Pharm. Co., Ltd., Tokyo, Japan). After the removal of the red blood cells using Tris-buffered NH_4Cl solution, the cells were washed three times in cold Hanks' balanced salt solution and then resuspended in RPMI 1640 medium (Gibco, Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 units/ml penicillin, 55 μM 2-mercaptoethanol and 25 mM HEPES.

The non-immunized or OVA-immunized splenocytes (4×10^5 cells) were seeded into each well of 96 Micro Well Plates (Nunc, Roskilde, Denmark) and then stimulated with heat-killed bacteria from 0.001 to 1 $\mu\text{g}/\text{ml}$ or OVA at a final concentration of 50 $\mu\text{g}/\text{ml}$ at 37°C for 24 h (for IL-12 and IL-5) or 72 h (for IFN- γ and IL-4). Following filtration through a 0.22 μm pore-size filter, the resultant filtrate was frozen at -80°C until the determination of cytokine.

Determination of Cytokines and Immunoglobulins
IFN- γ , IL-4 and IL-5 were measured using a commercial ELISA kit purchased from Genzyme Techne (Minneapolis, MN, U.S.A.). IL-12 and total IgE were measured using a commercial ELISA kit purchased from Pierce Biotechnology, Inc. (Rockford, IL, U.S.A.) and OptEIA ELISA set purchased from BD Bioscience Pharmingen (San Diego, CA, U.S.A.), respectively.

To measure OVA-specific IgE, anti-mouse IgE antibody (Technopharm Biotechnology, Villejuif Cedex, France) was coated onto Maxisorp microtiter plates (Nunc, Roskilde, Denmark) in bicarbonate coating buffer overnight at 4°C . After blocking by phosphate buffered saline containing 10% fetal bovine serum (PBS-10% FBS) and washing, the test samples were added and incubated at 37°C for 1 h. After washing, bound samples were detected using biotin-conjugated OVA at 37°C for 1 h. Then, avidin-horseradish peroxidase (HRP) solution (BD Bioscience Pharmingen, San Diego, CA, U.S.A.) was added to each well and incubated at 37°C for 1 h. After washing, 3,3',5,5'-tetramethylbenzidine (TMB)/Peroxidase substrate solution was added, and the plates were allowed to develop at room temperature for 30 min. Color reaction was stopped by the addition of 1 mol/l H_2SO_4 . Absorbances were measured at 450 nm.

To measure OVA-specific IgG1 and IgG2a levels, OVA was coated onto Maxisorp microtiter plates (Nunc, Roskilde, Denmark) in bicarbonate coating buffer, overnight at 4°C . After blocking PBS-10% FBS and washing, the test samples were added and incubated at 37°C for 1 h. After washing, bound samples were detected using HRP-conjugated goat anti-mouse IgG1 or IgG2a (Bethyl Laboratories Inc., Montgomery, TX, U.S.A.) at 37°C for 1 h. The absorbances were measured after addition of TMB/peroxidase substrate solution as described above.

The standard serum for assays of OVA-specific IgE, IgG1 and IgG2a were obtained from serum of mice immunized three times with intraperitoneal administration of OVA ab-

sorbed on alum at the intervals of 1 week. The serum determined 1/300 titer by rat heterogenous passive cutaneous anaphylaxis, was arbitrarily expressed as 10000 units/ml.

Statistical Analysis Differences in results were analyzed for significance using a Student's *t*-test. Probability values of $p<0.05$ were considered to be significant.

RESULTS

Effect of Orally Administered BBG9-1 on the Production of Total IgE in Immunized Mice

When BBG9-1 was orally given to mice for 2 weeks from 1 week before the 1st immunization, time course of the total IgE serum levels in mice are shown in Fig. 2. In the control mice as OVA-immunized mice, total IgE levels were significantly elevated at 3 weeks after the 1st immunization and reached to approximately 1800 ng/ml further 1 week later. The treatment of BBG9-1 significantly and time-dependently reduced total IgE levels from 3 weeks after the 1st immunization, the treatment reduced the IgE to almost the same level of non-sensitized animals at the end of experiment. On the other hand, non-sensitized animals hardly changed total IgE levels for experimental period.

No remarkable differences were observed in the food intake, the body weight and the fecal form between the control and BBG9-1-administered groups.

Time Course of Serum OVA-Specific Immunoglobulin Levels in Immunized Mice and Effect of BBG9-1 on the Production of OVA-Specific Immunoglobulins

Figure 3 illustrates the time course of serum OVA-specific immunoglobulin levels in OVA-immunized mice. OVA-specific IgE levels were elevated time-dependently and reached to maximum level at 4 weeks after the 1st immunization, while OVA-specific IgG1 and IgG2a levels continued to elevate time-dependently for 5 weeks after the 1st immunization. From these results, we examined effect of BBG9-1 on this specific immunoglobulin level at 4 weeks after the 1st immunization.

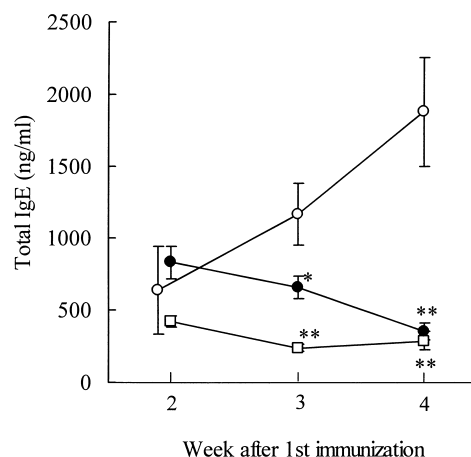


Fig. 2. Effect of Oral Administration of BBG9-1 on the Total IgE Serum Level in OVA-Immunized Mice

Mice were immunized by two separate intraperitoneal injections of OVA absorbed on alum at an interval of 1 week. BBG9-1 was orally given to the mice at 10^{10} cfu/0.2 ml/d/animal for 2 weeks from 1 week before the 1st immunization. Sera were collected at every week after the 2nd immunization, and then the total IgE level was determined by ELISA. Each point (open circle; control, closed circle; BBG9-1, open square; non-sensitized) represents the mean $\pm$ S.E. of 6 animals. *, ** Statistical significance of the difference from control at $p<0.05$, $p<0.01$, respectively.

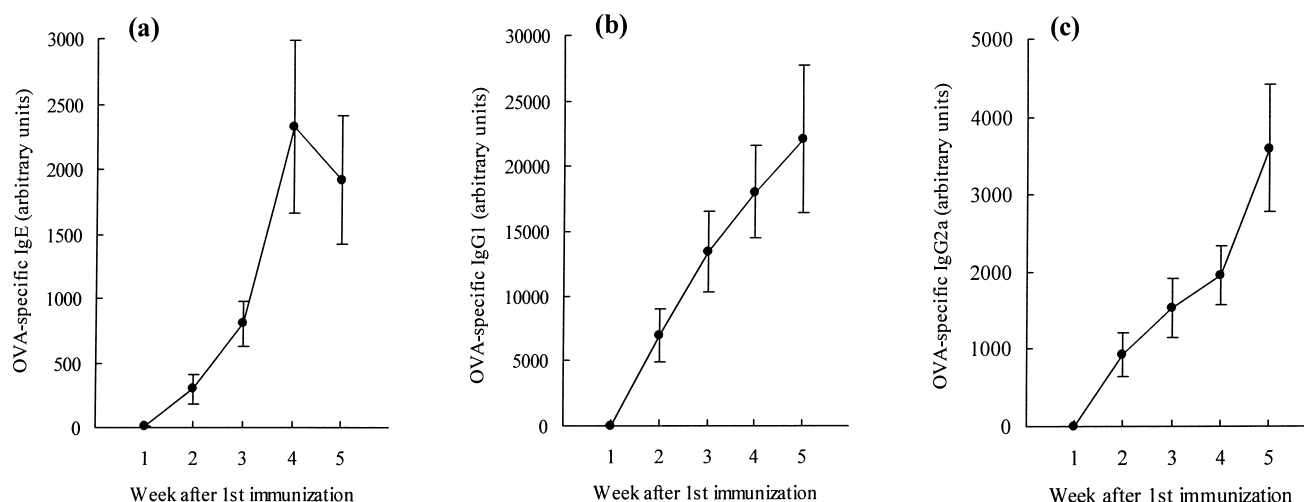


Fig. 3. Time Course of Serum OVA-Specific Immunoglobulin Levels in OVA-Immunized Mice

Mice were immunized by two intraperitoneal injections OVA absorbed alum at an interval of 1 week. Blood was collected from the carotid artery during deep anesthesia, and then the serum levels of OVA-specific-IgE (a), -IgG1 (b) and -IgG2a (c) were determined by ELISA. Each point represents the mean $\pm$ S.E. of 6 animals.

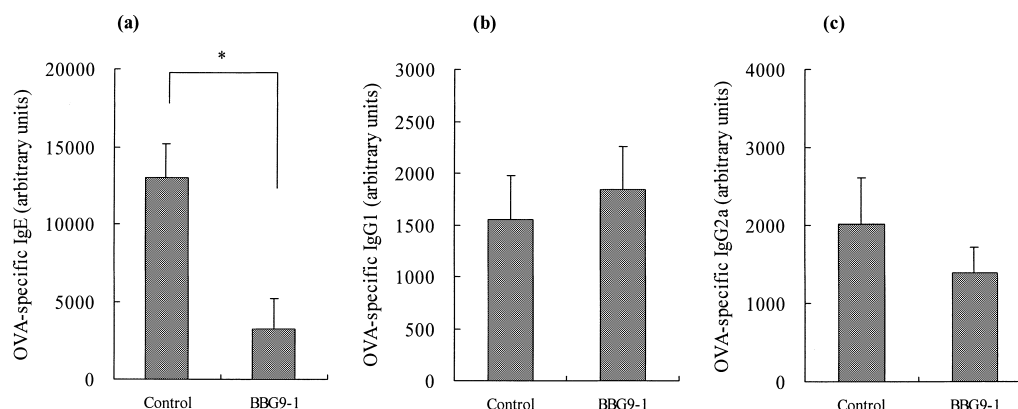


Fig. 4. Effect of Oral Administration of BBG9-1 on the Serum Levels of OVA-Specific-IgE (a), -IgG1 (b) and IgG2a (c) in OVA-Immunized Mice

Mice were immunized by two separate intraperitoneal injections of OVA absorbed on alum at an interval of 1 week. BBG9-1 was orally given to the mice at 10^{10} cfu/0.2 ml/d/animal for 2 weeks from 1 week before the 1st immunization. Sera were collected at 4 weeks after the 1st immunization, and then the serum levels of OVA-specific-IgE, -IgG1 and IgG2a were determined by ELISA. Each column represents the mean $\pm$ S.E. of 6 animals. * Statistical significance of the difference from control at $p < 0.05$.

With regard to OVA-specific IgE, a significant decrease was observed in mice administered BBG9-1 as compared to the control mice (Fig. 4). In contrast, the OVA-specific IgG1 production failed to be suppressed by the probiotic treatment. Moreover, a tendency to reduce OVA-specific IgG2a production required for the participation of Th1 cells was observed.

Production of IL-12 and IFN- γ from Splenocytes Stimulated with Heat-Killed Bacteria Isolated splenocytes were stimulated with the heat-killed bacteria. As shown in Fig. 5, heat-killed EFJCM8726 from 0.001 to 1 μ g/ml stimulated both productions of IL-12 and IFN- γ in a concentration-dependent manner from mouse splenocytes. On the other hand, BBG9-1 at 0.001 to 1 μ g/ml induced neither IL-12 nor IFN- γ from the cells (Fig. 5).

In Vitro OVA-Induced IFN- γ , IL-4 and IL-5 Production from Splenocytes of Mice Administered BBG9-1 As shown in Fig. 6, OVA-induced productions of cytokines (IFN- γ , IL-4 and IL-5) were augmented in splenocytes obtained from OVA-immunized mice. Every cytokine productions from the splenocytes of mice given BBG9-1 tended to be lower than those of the control mice (Fig. 6). We also

found the same results in another experiment (data not shown).

DISCUSSION

Some recent reports^{15,18–20} have suggested that some *lactobacilli* and other substances that presumably promote a Th1 type immunoresponse, can suppress the production of IgE as well as IgG1. In the present study, we clearly demonstrated that BBG9-1 significantly and powerfully reduced the total and antigen-specific IgE serum levels without lowering of antigen-specific IgG1 (required for participation of Th2 cells) and increasing of the specific IgG2a (required for participation of Th1 cells). In addition, BBG9-1 was found to induce hardly IFN- γ and IL-12 from splenocytes, and the oral administration of BBG9-1 led to reduce the production of not only IL-4 and IL-5 but also IFN- γ from the splenocytes by OVA stimulation *in vitro*. From these results, it is suggested that BBG9-1 suppressed antigen-specific IgE production *via* a different mechanism from that of Th1 cytokine inducible bacteria as some *lactobacilli*,^{11–14,16} presumably a

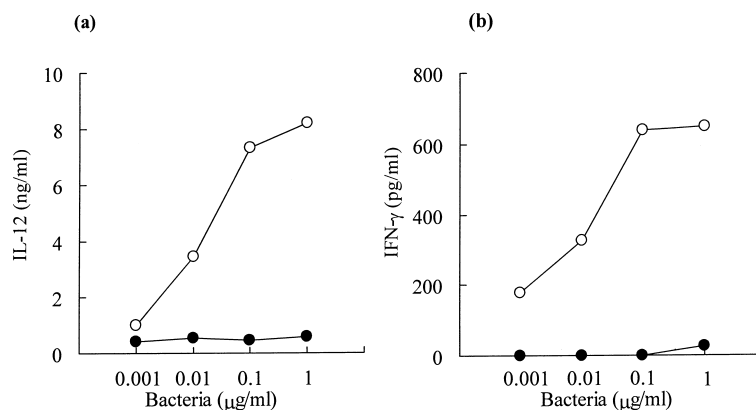


Fig. 5. BBG9-1- or EFJCM8726-Induced the Production of IL-12 (a) and IFN-γ (b) from Mouse Splenocytes

Mouse splenocytes (4×10^5 cells/well) were stimulated with heat-killed BBG9-1 (closed circle) or EFJCM8726 (open circle) for 24 h (IL-12) or 72 h (IFN-γ) at the concentrations indicated. IL-12 and IFN-γ in the supernatants were determined by ELISA. Amounts of spontaneous production of IL-12 and IFN-γ were 0.39 ng/ml and 27 pg/ml, respectively. Each point represents the mean of triplicate determinations.

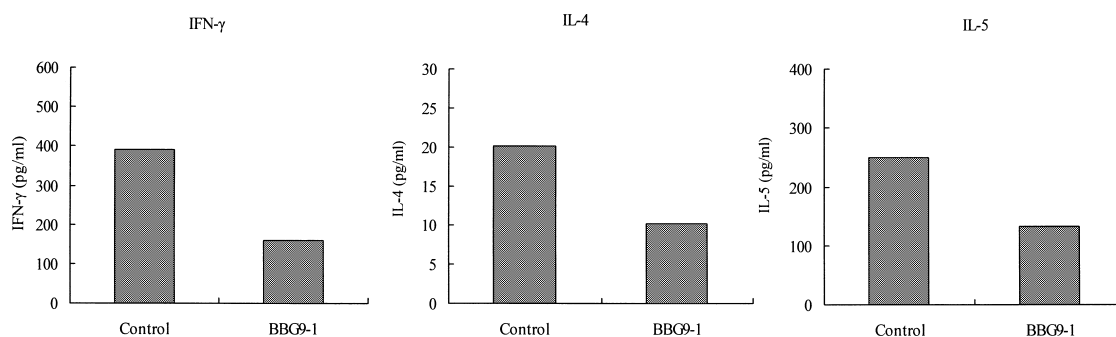


Fig. 6. Effect of BBG9-1 Administration of Cytokine Productions by Splenocytes *in Vitro*

Mice were immunized by two separate intraperitoneal injections of OVA absorbed on alum at an interval of 1 week. BBG9-1 was orally given to the mice at 10^{10} cfu/0.2 ml/d/animal for 2 weeks from 1 week before the 1st immunization. At 4 weeks after the 1st immunization, splenocytes were collected from 6 mice and mixed. The splenocytes (4×10^5 cells) were stimulated with OVA at a final concentration of 50 μg/ml, at 37 °C for 24 h (for IL-5) or 72 h (for IFN-γ and IL-4). The cytokine levels in the culture medium were determined by ELISA. Amount of spontaneous production of IFN-γ were 28 pg/ml, but those of IL-4 and IL-5 were not detectable.

mechanism without promoting a shift in the Th1/Th2 balance towards Th1-dominant immunity.

It has been reported that some bacterial antigens²¹⁾ or the toll-like receptor (TLR) 9 ligand for bacterial CpG DNA²²⁾ can also suppress IgE secretion by an IFN-γ independent mechanism. Some of the oral tolerance generated to orally administered antigens, have certain local and systemic immunoregulations linked to IFN-γ independent mechanisms. It is known that transforming growth factor (TGF)-β together with IL-10, are produced from Th3 cells and Tr1 cells, and are involved in the oral tolerance through active suppression.^{23–27)} Recently, Yamamoto *et al.*²⁸⁾ have reported that TGF-β suppresses IL-4 signaling, a prerequisite action for IgE and IgG1 production, in B cells. The suppressive mechanism of IgE production by BBG9-1 may be similar to one of the IFN-γ independent mechanisms as mentioned above. While the oral administration of BBG9-1, but not completely, inhibited IL-4 production from *in vitro* antigen-stimulated splenocytes, the antigen specific IgG1 production in the serum was hardly influenced. Two possibilities for this phenomenon can be proposed. BBG9-1 (1) did not inhibit IL-4 production to the level suppressing IgG1 secretion *in vivo* because IL-4 stimulated IgG1 secretion at lower concentration (about 1/100) than IgE secretion,²⁹⁾ (2) acted directly and selectively plasma cells secreting IgE. Further investigations are required to demonstrate whether the mechanism of

reducing IgE production by BBG9-1 is distinct from the IFN-γ independent mechanisms related to regulatory T cells or bacterial antigen as noted above.

Some of *Bifidobacterium* and lactic acid bacteria can be effectively used to improve symptoms resulting from changes in the intestinal flora.²⁾ Infants with food allergies have been reported to have a disturbed balance between beneficial and potentially harmful bacteria in the large intestine.^{30–33)} Indeed, there are data to suggest that an aberrant microbial composition in the gut such as inadequate bifidobacterial biota may deprive the developing immune system from counterregulatory signals against the Th2 mediated allergic responses.^{32–34)} Furthermore, Isolauri *et al.*³⁵⁾ have reported that serum total IgE concentrations correlated directly with *Escherichia coli* counts in allergic infants. In animal model, it has been reported that the serum IgE levels in BALB/c mice disturbed intestinal flora by the treatment of kanamycin elevated as compared to those in the normal mice, and orally administered probiotics decreased the elevating IgE level.³⁶⁾ These reports suggest that intestinal bacterial flora play a crucial role in preventing IgE production or allergic reactions. On the other hand, we previously have reported that BBG9-1 is useful in the regulation of disturbed intestinal flora, especially elevated *E. coli*.³⁷⁾ Taking the results obtained by other researchers and us together, the inhibitory effect of BBG9-1 on IgE production may, in part, be due to an

inhibitory action to *E. coli* or other bacteria composed of the intestinal flora. We need to investigate the relationship between regulation of intestinal flora and decrease of IgE production in BBG9-1-administered animals.

From the present results, we conclude that oral administration of BBG9-1 suppresses total and antigen specific IgE production in mice. It is suggested that BBG9-1 is useful for prophylactic treatment of IgE-dependent allergic diseases.

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