Oral Administration of *Bifidobacterium longum* Ameliorates Influenza Virus Infection in Mice

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We investigated whether the oral administration of *Bifidobacterium longum* BB536 could ameliorate influenza virus (IFV) infection in a mice model. Mice were orally administrated BB536 or saline for 2 weeks and then infected with IFV. Orally administrated BB536 significantly alleviated symptoms, reduced the loss of body weight, and inhibited viral proliferation in the lungs relative to the control group findings. Histopathological findings in the lungs were improved in the BB536 group compared to control group findings. There was no significant difference in the levels of interleukin-6 (IL-6), interferon-γ (IFN-γ), IL-10 and IL-12p40 in the lungs between the groups, but the levels of IL-6 and IFN-γ were lower \( (p=0.076, 0.103, \) respectively) in the BB536 group compared with those of control group. The levels of IL-6 and IL-10 correlated significantly with the values of weight loss, and the levels of IFN-γ correlated with the virus titers in the lungs. These results suggested the potential of the oral administration of BB536 in ameliorating IFV infection and the possible involvement of anti-inflammatory effects of BB536 in the anti-infection effects against IFV.

Key words  *Bifidobacterium; influenza virus; BB536; probiotics; infection*

Influenza is an acute viral respiratory disease caused by influenza virus (IFV) infection, which attacks the host respiratory tract mucosa. IFV infection occasionally causes lethal pneumonia in the elderly and encephalopathy in children, which results in high morbidity and significant mortality. To control influenza, inactivated vaccines against IFV have been administered parenterally to induce adaptive immunity that is protective against homologous virus infection. However, vaccination is effective in relieving symptom development but not in preventing viral infection; it is less effective against heterologous virus infection. In addition, the prevailing types of virus constantly change, and it is difficult to predict which type of new IFV will cause a pandemic in the future. On the other hand, it is known that both cellular and humoral immunity in the host are necessary for elimination of the virus and recovery from viral infection, and in particular, cellular immunity plays a crucial role in the first defense against infection. Cellular and humoral immunity in the host are believed to be cross-protective for any type of IFV. However, it has been reported that cellular immune responses such as natural killer (NK) cell activity tend to be low in the elderly and infants, as well as in people in poor health. Thus, as a strategy for the management of IFV infection, it is important to enhance host cellular immunity that is cross-protective against IFV infection.

Probiotics are live bacteria that when administrated in adequate amounts confer a health benefit to the host. Lactobacilli and bifidobacteria are commonly used as a probiotics. There is substantial interest in the preventive effects of probiotics against various infections. Several studies have demonstrated that some strains of probiotics are effective in protecting against IFV infection in a murine model and that their protective effects might be mediated by the augmentation of secreted immunoglobulin A production and the enhancement of cellular immunity in hosts. Leyer et al. reported that probiotics usage reduced the incidence and duration of cold and influenza-like symptoms in healthy children during the winter season. *Bifidobacterium longum* BB536 was originally isolated from a healthy infant, and it has been used in the dairy industry as a probiotic. Although intranasal administration of BB536 has been reported to improve the cumulative incidence and survival rate after IFV infection in mice, the effects of oral administration of BB536 on IFV infection are unknown. In a clinical trial with elderly subjects, it has been reported that BB536 intake reduced the incidence of influenza infection and fever; however, the effects of BB536 intake on the proliferation of IFV in the lung and the damage of the lung due to the IFV infection remain unclear. In this study, we investigated the effects of oral administration of BB536 on IFV infection in a mice model.

MATERIALS AND METHODS

**Animals** Specific-pathogen-free female BALB/c mice (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). They were fed a standard diet consisting of FR-2 pellets (Funabashi Farms, Chiba, Japan) and water *ad libitum* and were acclimatized for 6 d. All experimental protocols involving mice were performed according to the guidelines of the Prime Minister’s Office in Japan (No. 6, March 27, 1980), and the animal experiments were approved by the ethics committee of laboratory animals at Japan Biological Science Inc. (Gifu, Japan).

**Bacteria** A commercial material of lyophilized powder of BB536 (Morinaga Milk Industry Co., Ltd., Zama, Japan) was used in the animal experiments. Each gram of the lyophilized material contained approximately \( 4 \times 10^{11} \) live bacterial cells. The material was suspended in saline immediately before use.

**Virus** IFV A(PR/8/34(H1N1)) adapted to mice was stored at Japan Biological Science Inc., and the virus was grown in the allantoic cavity of 10-d-old chicken embryos for 2 d. Allantoic fluid was stored at \( -80 ^\circ C \) as a stock solution of the virus. Virus titers in the stock solution were determined to be \( 1.2 \times 10^7 \) plaque-forming units (pfu)/ml by the plaque assay described below.

**Influenza Virus Infection Model** Experimental proce-
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Mice were orally administered lyophilized BB536 daily beginning 2 weeks before IFV infection until 1 d before sacrifice at a dose of $2 \times 10^6$ colony-forming unit/0.2 ml/mouse (BB536 group; $n=10$). As a control, mice were given an equal volume of saline (Control group; $n=10$). All mice were infected intranasally with 50 $\mu$l of saline containing $5 \times 10^6$ pfu of the virus. Following infection, mice were monitored daily for infection symptoms in the eyes (extent of lid closure and eyelid reflex), fur, behavior (extent of locomotor activity), and breathing (extent of irregular respiration). Each condition was scored on a scale from 0 to 4 as follows: 0, normal; 1, mild; 2, moderate; 3, severe; 4, death. Symptom scores for each mouse were estimated from the average extent of these conditions. In this infection model, symptom scores and body weight loss would reach the maxima at approximately 6 d after the infection, and then the mice would recover from the infection. Therefore, we assessed body weight loss, viral titers in the lung and histopathological changes at 6 d after the infection. The weight loss due to infection was expressed as the ratio of body weight 6 d after the infection to body weight on the day of infection. Six days after the infection, mice were sacrificed under diethylether anesthesia, and the lungs were extracted. The right lobes of the lungs were weighed and homogenized in saline, and the viral titers of the lung homogenate were determined using a plaque assay. The left lobes of the lungs were used for histopathological examination.

**Plaque Assay** The viral titers of the lung homogenate were determined using a plaque assay as described elsewhere [17] with some modifications. Monolayers of Madin–Darby canine kidney cells in 12-well plates were incubated with 0.1 ml of the dilutions for 1 h, and the cells were overlaid with 1.5 ml of agar medium. The plates were maintained in a humidified atmosphere containing 5% CO$_2$ for 2 d, and the plaques in wells were counted. The viral titers of the lungs were expressed as the number of pfu per unit weight of lung.

**Histopathology of the Lung** The left lobes of lungs were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. Histopathological scores were established on the basis of the histopathological findings including hypertrophy, hyperplasia, abrasion and necrosis of the bronchial epithelium, infiltration of inflammatory cells in bronchial submucosa and alveolar septa, exudation of inflammatory cells in the alveolus, atelectasis, edema, and hemorrhage in alveolus. Each histopathological finding was scored as follows: 0, normal; 1, mild; 2, moderate; 3, severe. Histopathological scores were estimated from the average of the extent of these findings.

### Determination of Cytokines

Interferon-γ (IFN-γ), interleukin-6 (IL-6), IL-10 and IL-12p40 in the lung homogenate were quantified using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, U.S.A.) in accordance with the manufacturer’s instructions. The cytokine levels in lungs were determined as the amount of cytokine per unit weight of lung. To estimate basal levels of the cytokines, the cytokine levels of age-matched mice without IFV infection and BB536 administration were determined.

**Statistical Analysis** Data are expressed as means±S.D. Mann–Whitney U tests were used to analyze between-group differences, and Spearman’s correlation tests were applied for correlation analyses. Values of $p<0.05$ were considered statically significant.

### RESULTS

#### Effects of BB536 on Influenza Virus Infection in Mice

One mouse in the control group died 5 d after the infection, whereas all mice in the BB536 group survived until 6 d after the infection. Symptom scores were significantly lower in the BB536 group than in the control group from 2 d after the infection (Fig. 2a). Oral administration of BB536 significantly reduced the loss of body weight and inhibited viral proliferation in the lungs compared with the control group findings (Figs. 2b, c).

Figures 3a and b show representative histopathological images of the lungs for each group. In the control group, markedly increased necrosis and abrasion of the bronchial...
epithelium and pulmonary atelectasis were observed in comparison with their incidence in the BB536 group. Histopathological scores were significantly lower in the BB536 group than in the control group (Fig. 3c). The histopathological scores were significantly correlated with the symptom scores and the lost weights (data not shown).

Cytokine Levels in the Lungs The levels of IFN-γ and IL-6 markedly increased in the control group as compared with those of not-treated group. The levels of IFN-γ and IL-6 in the lungs showed values decreased by 55% (p = 0.076) and 63% (p = 0.103), respectively, as compared with those of control group (Fig. 4). No marked difference was found on the levels of IL-10 and IL-12 between the two groups (Fig. 4).

The levels of IL-6 (p = 0.02) and IL-10 (p = 0.006) correlated significantly with the values of weight loss. A significant positive correlation was observed between the levels of IFN-γ and the virus titers (p = 0.01).

DISCUSSION

Some studies have suggested that orally administered probiotics activated host cellular immunity and that the protective effect against IFV infection is caused via the activation of cellular immunity.1–8 We have reported the effects of intranasally administered BB536 on host cellular immunity and in the prevention of IFV infection in mice. It was found that 3 d of consecutive intranasal administration of BB536 enhanced the production of Th1 cytokines by cells from mediastinal lymph nodes and nasal-associated lymphoid tissue and improved the cumulative incidence and survival rate after IFV infection.15 BB536 has been reported to suppress the decline of plasma IFN-γ levels in patients with Japanese cedar pollinosis in the pollen seasons.18,19 In a clinical trial with elderly subjects, it was reported that BB536 reduced the incidence of influenza infection and fever and enhanced cellular immunity, such as NK cell activity and neutrophil bactericidal activity.20 These findings suggest the potential of BB536 in maintaining or activating cellular immunity. However, the enhanced production of IFN-γ in the lungs by oral administration of BB536 was not observed in this study. In contrast, the levels of IFN-γ in the lungs were correlated significantly with the virus titers in the lungs, but not with the body weight loss. It has been suggested that a large number of NK cells would be recruited to the lung from the blood and activated within days after viral infection and that they would be activated by interaction with virally infected epithelial cells, monocytes and dendritic cells via class I major histocompatibility complex molecules and cytokines such as IL-12 and IFN-α.21 Activated NK cells are a potent source of IFN-γ and contribute to protection against influenza, limiting early viral replication.22 Thus, IFN-γ levels would be greatly influenced by the amount of IFV in the lungs, which were supported by the results that the IFN-γ levels markedly increased in the control group as compared with those of not-treated mice and there was a positive correlation between the IFN-γ levels and the virus titers. Oral administration of BB536 may induce an enhancement of IFN-γ at the early stage of viral infection; further investigations such as time course analysis are needed.

In this study, although no significant difference between the groups was observed, the levels of IL-6 in the lungs in BB536 group showed a value decreased by 55% as compared with those of control group (Fig. 4). Further, the values of weight loss correlated significantly with the levels of IL-6 and IL-10 in the lungs. It has been reported that the symptoms such as fever and weight loss due to IFV infection were slightly reduced in IL-6-deficient mice as compared with wild-type mice.23 The decreased levels of IL-6 might contribute to the reduction of symptom in BB536 group; however, further studies are needed to elucidate the mechanisms involved for the effects of BB536.

Our results demonstrated that the oral administration of BB536 ameliorated the symptom of IFV infection in mice. It was found that the viral titers in the lungs were suppressed, and the histopathological findings were improved. Further, our results suggested the possible involvement of anti-inflammatory effects of BB536 in the anti-infection effects against IFV. As the effect of probiotics appears to be cross-protective against various types of IFV, the use of probiotics might become a valuable means of ameliorating IFV infection.

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