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Effects of *Bifidobacterium longum* BB536 Administration on Influenza Infection, Influenza Vaccine Antibody Titer, and Cell-Mediated Immunity in the Elderly

Kazuyoshi NAMBA,[1,†] Michiko HATANO,[1] Tomoko YAESHIMA,[1] Mitsunori TAKASE,[1] and Kunihiko SUZUKI[2]

[1]*Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd., 5-1-83 Higashihara, Zama, Kanagawa 252-8583, Japan*
[2]*Hakujinkai Shimura-Omiya Hospital, 313 Kamicho, Hitachiomiya, Ibaraki 319-2261, Japan*

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Twenty-seven elderly subjects (mean age 86.7 ± 6.6 years) were pre-administered a test food containing 1×10^{11} cfu of BB536 daily for 5 weeks (P1), during which they also received influenza vaccination at week 3. The subjects were then randomized to a BB536 group and a placebo group for 14 weeks (P2). The proportion of subjects who contracted influenza was significantly lower in BB536 group than in the to placebo group. The proportion of subjects with fever was also significantly lower in the BB536 group than in the placebo group. In the P1 period, the NK cell activity and the bactericidal activity of the neutrophils were significantly higher at week 5 than to before BB536 administration. In the P2 period, although NK cell activity and neutrophilic activities declined at the end of the study in both the placebo and the BB536 group, neutrophil phagocytic activity and NK cell activity tended to maintain slightly higher levels in the BB536 group than in the placebo group. These results suggest that continuous ingestion of BB536 reduces the incidence of influenza and fever, probably by potentiating innate immunity.

Key words: influenza; *Bifidobacterium longum*; innate immunity; NK cell; neutrophils

Influenza is an infection that affects all age groups, but severity and mortality are especially high when influenza infection occurs in the elderly population, aged 65 years and older. Influenza infection in group residences such as health care facilities for the elderly and special nursing homes for them has become an important issue and has been highlighted as a public concern. As a result, the rate of influenza vaccination among the elderly in Japan has increased as a preventive measure against influenza infection. However, reports have indicated that in the elderly, antibody titers to the vaccinated influenza strains decrease during the influenza epidemic season and adequate immunity might not be maintained. Also, NK cell activity and the acquired immunity represented by T cells are reduced by half as compared to middle-aged adults.[1–3] In these high-risk groups, lowered immunity triggers influenza infection. Therefore investigation of methods to maintain or augment immune capacity is required.

Recent research on probiotics, including bifidobacteria and lactic acid bacteria, has indicated that when ingested, these beneficial bacteria activate immunocompetent cells in the intestinal tract, promote the production of secretory IgA, and increase the bactericidal activity of neutrophils[4] and cell-mediated immunity such as NK cell activity.[5] As a result, the incidence of influenza and other infections is reduced and antibody titers to the influenza vaccine are maintained at high levels. These findings highlight the importance of probiotics in intestinal immunity.

Regarding cell-mediated immunity, studies using mouse spleen cells have demonstrated that bifidobacteria stimulate mitogen-induced lymphocyte proliferation, and cultured spleen cells from rats fed *Bifidobacterium longum* BB536 (*B. longum* BB536) showed an increase in interferon-γ (IFN-γ) production.[6] Enhanced IgA secretion in intestinal contents was observed in gnotobiotes mono-associated with *B. longum* BB536.[7] These findings indicate that oral administration of *B. longum* BB536 augments cell-mediated immunity and increases immunoglobulin production.

The present study was conducted in the winter, when influenza is prevalent. Elderly subjects were pre-administered a test food containing *B. longum* BB536 for 5 weeks, during which they were given influenza vaccination (the P1 period). Then a randomized double-blind controlled study was conducted, in which the subjects were randomized to continue consumption of the *B. longum* BB536-containing food (the BB536 group) or to consume a placebo-containing food (the placebo group) for 14 weeks (the P2 period), during which the rate of influenza and other infections as well as changes in antibody titers to influenza vaccine and cell-mediated immunity were evaluated.

Materials and Methods

Test food. Test food was provided by Morinaga Milk Industry (Kanagawa, Japan). The test food was a powder packaged in sachet, containing 1×10^{11} cfu of *B. longum* BB536 per sachets. The placebo food contained 2 g of dextrin similarly packaged in sachets. One sachet of the test or the placebo food was ingested once daily after lunch with cold or warm water.

† To whom correspondence should be addressed. Tel: +81-46-252-3057; Fax: +81-46-252-3077; E-mail: k_nanba@morinagamilk.co.jp

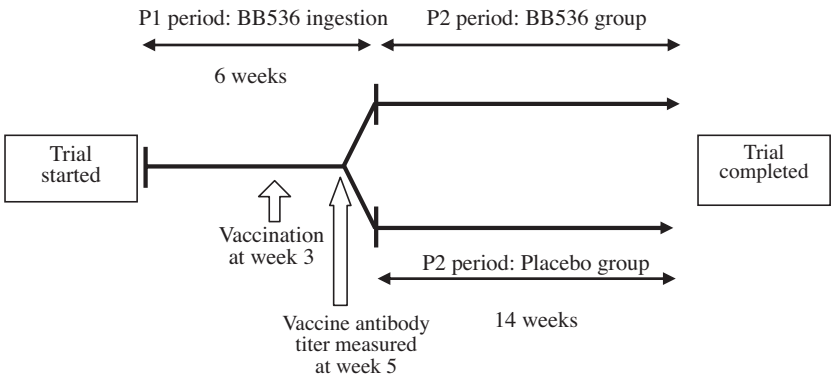

Fig. 1. Clinical Trial Schedule.

A total of 27 subjects (mean age 86.7 ± 6.6 years) comprising 13 in the BB536 group (Two males and 11 females, mean age 86.2 ± 5.4 years) and 14 in the placebo group (one male and 13 females, mean age 87.3 ± 7.8 years) completed the study. At week 3 of the study, each subject was vaccinated with influenza HA vaccine by subcutaneous injection of 0.5 ml in the upper arm. At week 5, the blood antibody titers against influenza vaccine, bactericidal activity of neutrophils, phagocytic activity of neutrophils and NK cell activity were measured (the P1 period). At week 6, the subjects were stratified by sex and H3N2 influenza vaccine antibody titer, and then randomized into the BB536 group or the placebo group for 14 weeks (the P2 period). During the P2 period, the status of drug-taking, including antibiotics and antifungal agents, as well as the occurrence of infection and fever, were recorded every day. Blood cell counts, blood chemistry, other blood tests, including IgG, IgM, IgA, antibody titers to influenza vaccine, and cell-mediated immunity, bactericidal activity of neutrophils, phagocytic activity of neutrophils, and NK cell activity were again examined at weeks 10, 15, and 20.

Subjects. Elderly residents aged 65 years or older in a health care facility for the elderly (Omiya Freudheim, an affiliated facility of the Hakujinkai Shimura-Omiya Hospital) in Ibaraki Prefecture, Japan who were scheduled to receive influenza vaccinations were recruited as subjects. Persons with intestinal functional disorders (including ileus and intestinal bleeding); persons with serious liver or renal dysfunction, serious concomitant diseases, autoimmune disease, a history of milk allergy, or dementia; and persons judged by the investigator to be inappropriate as subjects were excluded from the study.

The protocol of the study was approved by the Ethics Committees of Shimura-Omiya Hospital and Morinaga Milk Industry. Written informed consent to participate in the study was obtained from all the subjects after they were given an explanation of the study.

A total of 37 subjects, 18 in the BB536 group and 19 in the placebo group, were enrolled. After the study started, five subjects in the BB536 group and five subjects in the placebo group discontinued due to hospitalization due to aggravated medical conditions or departure from the facility for personal reasons. The cause of discontinuation was not in any case related to adverse reactions to the test food. Eventually, a total of 27 subjects (mean age 86.7 ± 6.6 years), 13 in the BB536 group (two males and 11 females, mean age 86.2 ± 5.4 years) and 14 in the placebo group (one male and 13 females, mean age 87.3 ± 7.8 years) completed the study. The study period ran from November 1, 2004 to March 31, 2005.

Study methods. The study included of two periods, P1 and P2 (Fig. 1). The P2 period was a randomized, placebo-controlled, double-blind trial (RCT). Initially, all 37 subjects were given the test food containing *B. longum* BB536. At week 3, each subject was vaccinated with influenza HA vaccine seiken (Denka Seiken, Tokyo, Japan, containing 30 μg/ml of each type of HA) by subcutaneous injection of 0.5 ml in the upper arm. At 2 weeks after vaccination (week 5 of the study), blood cell counts, blood chemistry, other blood tests, including IgG, IgM, IgA, blood antibody titers against the influenza vaccine, the bactericidal activity of the neutrophils, the phagocytic activity of neutrophils, and NK cell activity were done. The first 5 weeks of the study was designated the P1 period. One week later (week 6 of the study), the subjects were stratified by sex and by H3N2 influenza vaccine antibody titer, and then randomized to the BB536 group (continued ingestion of *B. longum* BB536) and the placebo group (ingestion of placebo). Then RCT was conducted for 14 weeks (the P2 period). During the P2 period, the status of drug-taking, including antibiotics and antifungal agents, as well as the occurrence of infection and fever, were recorded every day. Antibody titers to influenza vaccine and cell-mediated immunity, bactericidal activity of neutrophils, phagocytic activity of neutrophils, and NK cell activity were examined again at weeks 10, 15, and 20 of the study. Neutrophil phagocytic activity and neutrophil bactericidal activity were measured by flow cytometry.[8] NK cell activity was assayed using the K-562 cell line labeled with ^{51}Cr as target cells and peripheral blood mononuclear cell (PBMC) isolated from the blood as effector cells. The effector cells and target cells were incubated at a ratio of 20:1. The amount of ^{51}Cr released due to cytotoxicity to the target cells was measured, and NK cell activity was calculated. All measurements were performed by SRL (Tokyo, Japan).

Antibody titers to influenza vaccine were measured using the 2004–2005 vaccine strains A/New Caledonia/20/99 (H1N1), A/Wyoming/3/2003 (H3N2), and B/Shanghai/361/2002. When influenza was suspected, a rapid test for influenza infection was conducted at the bedside by testing a throat swab specimen with the Quick-S Influ A/B seiken kit (Denka Seiken).

Statistical analysis. Intra-group changes in antibody titers to influenza vaccine from week 5 of the study to the end were analyzed by multiple comparison by Scheffe's method. The inter-group difference in antibody titer at a given time was evaluated by unpaired *t*-test. The numbers of patients who contracted influenza from week 6 to the end of the study in the two groups were compared by Fisher's direct probability test. The numbers of fever episodes (38 °C or above) observed from week 6 to the end of the study in the two groups were compared by Fisher's direct probability test. NK activity, neutrophil phagocytic activity, and neutrophil bactericidal activity before the start of the study and at week 5 were compared by two-tailed paired *t*-test. Changes in the above parameters from week 5 to the end of the study were analyzed: intra-group differences by multiple comparison by Scheffe's method, and inter-group differences at a given time by unpaired *t*-test. A p value of less than 0.05 was considered significant. All data analyses were performed using SPSS (version 10) software.

Results

Changes in hematological values during the P1 period

As for the hematological data before BB536 and at week 5 of BB536 administration in the P1 period (Table 1), all the values were within clinically normal ranges, with no abnormalities. However, the erythrocytic and megakaryocytic parameters represented by the red blood cell count, and the hemoglobin and platelet counts were significantly increased at week 5. This suggests that oral administration of BB536 promotes hematopoiesis in elderly persons.

Table 1. Blood Test Results before Ingestion of BB536 and at Week 5 of Ingestion

Item	Unit	Before ingestion	Week 5 of study	Significant difference
Red blood cell (RBC)	($\times 10^6$/μl)	3.84 ± 0.40	3.97 ± 0.41	**
Hemoglobin (Hb)	(g/dl)	11.7 ± 1.3	12.2 ± 1.4	**
Hematocrit (Ht)	(%)	37.1 ± 4.2	38.1 ± 4.0	*
Mean Corpuscular Volume (MCV)	(fl)	96.7 ± 4.6	96.1 ± 4.8	*
Mean Corpuscular Hemoglobin (MCH)	(pg)	30.5 ± 1.9	30.8 ± 1.8	**
Mean Corpuscular Hemoglobin Concentration (MCHC)	(%)	31.5 ± 1.2	32.0 ± 0.8	**
White blood cell (WBC)	($\times 10^3$/μl)	5.1 ± 1.2	5.1 ± 1.0	
Basophil	(%)	0.5 ± 0.3	0.4 ± 0.2	
Eosinophil	(%)	3.2 ± 1.6	2.9 ± 1.9	
Neutrophil	(%)	61.4 ± 9.8	61.5 ± 9.0	
Lymphocyte	(%)	29.5 ± 8.9	29.5 ± 8.4	
Monocyte	(%)	5.5 ± 1.9	5.7 ± 1.7	
Platelet (Plt)	($\times 10^4$/μl)	21.6 ± 6.0	22.6 ± 6.2	*
IgA	(mg/dl)	419 ± 231	418 ± 214	
IgG	(mg/dl)	1630 ± 384	1620 ± 377	
IgM	(mg/dl)	88 ± 36	89 ± 37	
Neutrophil phagocytic activity	(%)	92.9 ± 3.4	93.6 ± 2.4	
Neutrophil bactericidal activity	(%)	88.9 ± 6.4	92.7 ± 5.8	**
NK cell activity	(%)	26 ± 11	37 ± 13	**
A/H1N1 antibody titer	(log)	0.8 ± 0.7	1.3 ± 0.5	*
A/H3N2 antibody titer	(log)	1.5 ± 0.8	2.1 ± 0.6	**
B antibody titer	(log)	0.5 ± 0.6	1.1 ± 0.6	

Values are presented as mean ± SD for 27 subjects.
Data were evaluated by two-tailed paired *t*-test for the start of the study *versus* week 5 of the study.
*, $p < 0.05$; **, $p < 0.01$.

Effects of maintenance of antibody titers on influenza vaccine

Tables 1 and 2 show the results for antibodies to influenza vaccine. The proportion of subjects possessing a titer of 40 or above (WHO method, considered to be an effective antibody titer) against A/New Caledonia/20/99 (H1N1) increased from 25.9% (7 of 27 subjects) before vaccination to 40.7% (11 of 27 subjects) at 2 weeks after vaccination (week 5 of the study). At randomization, the proportion of subjects with effective titer was 53.8% in the BB536 group and 28.6% in the placebo group, and was higher in the BB536 group. The proportions during the subsequent period ranged from 23 to 25% in the BB536 group, and the proportion at week 10 of the study returned to the pre-vaccination level. In the placebo group, the proportion of subjects with effective titer ranged from 29 to 36%. On the other hand, the proportions of subjects possessing a titer of 40 or above against A/Wyoming/3/2003 (H3N2) after vaccination did not decrease over time, but remained at constant rates of 77 to 86% in the placebo group and 83 to 92% in the BB536 group. The proportions of subjects possessing a titer of 40 or above against type B influenza remained at 25 to 46% after vaccination.

In Table 2, the numbers in parentheses are the mean antibody titers (logarithmic values) for all subjects in the various groups. At week 5 of the study, the titers for A/H1N1 were not different as between the BB536 group (1.2 ± 0.6) and the placebo group (1.3 ± 0.3). Similarly, the titers for A/H3N2 and B were not different as between the BB536 and placebo groups. There were no significant changes with time within the two groups, and no significant differences between the BB536 and placebo groups throughout the study period.

These findings suggest that oral administration of *B. longum* BB536 has no effect on the maintenance of antibody titers against influenza vaccine given at the same time.

Incidence of influenza

On March 22–23, 2005, toward the end of the study, an outbreak of acute fever occurred in the facility, suggesting influenza infection. All the subjects were examined clinically and tested for influenza infection by the rapid test. Consequently, influenza infection was confirmed in five subjects in control group, and oseltamivir phosphate (Tamiflu) was administered. On the other hand, influenza infection was not found in the BB536 group (Table 3). The incidence of influenza was significantly lower ($p = 0.041$) in the BB536 group than in the placebo group.

Incidence of fever

Fever of 38 °C or above was recorded and the episodes of fever were analyzed. As shown in Table 3 and Fig. 2, a total of two subjects in the BB536 group developed fever during the 14-week period. In comparison, a total of eight subjects in the placebo group developed fever, five due to influenza and three due to fever of unknown origin. The number of subjects with fever was significantly smaller in the BB536 group than in the placebo group ($p = 0.046$).

Use of antibiotics

In this study, a positive result for influenza infection alone was not an indicator for antibiotic treatment, but fever of 38 °C or higher, suggesting microbial infection, was treated with antibiotics. During the 14-week period, two subjects in the BB536 group as compared with seven subjects in the placebo group were administered antibiotics. The number of subjects administered antibiotics tended to be smaller in the BB536 group than in the placebo group ($p = 0.103$).

Table 2. Changes in Proportions of Subjects Possessing Effective Antibody Titers to Influenza Vaccine during the Study Period

Antigen	Group	Before BB536 ingestion	Week 5 of study (2 wk after vaccination)	Week 10 of study	Week 15 of study	Week 20 of study
A/H1N1	BB536	25.9%	53.8% (1.2 ± 0.6)	23.1% (1.0 ± 0.7)	25.0% (1.0 ± 0.6)	23.1% (0.9 ± 0.7)
	Placebo		28.6% (1.3 ± 0.3)	30.8% (1.3 ± 0.4)	35.7% (1.2 ± 0.6)	28.6% (1.0 ± 0.7)
A/H3N2	BB536	48.1%	92.3% (2.2 ± 0.7)	84.6% (2.0 ± 0.7)	83.3% (1.9 ± 0.6)	84.6% (2.0 ± 0.7)
	Placebo		85.7% (2.0 ± 0.5)	76.9% (1.8 ± 0.4)	78.6% (1.7 ± 0.6)	85.7% (1.9 ± 0.4)
B	BB536	3.7%	38.5% (1.0 ± 0.7)	30.8% (1.1 ± 0.6)	25.0% (1.0 ± 0.8)	38.5% (0.9 ± 0.8)
	Placebo		35.7% (1.2 ± 0.6)	46.2% (1.2 ± 0.6)	35.7% (0.9 ± 0.8)	28.6% (0.9 ± 0.7)

Values are presented as percentages, or mean ± SD, for 13 subjects in the BB536 group and 14 subjects in the placebo group. The upper figure is number of subjects with effective antibody titer (40 or above), and the lower figure is mean ± SD of log antibody titers to influenza vaccine. Changes in the above parameters from week 5 to the end of the study were analyzed: intra-group difference by multiple comparison using Scheffe's method, and inter-group difference at a given time point by *t*-test. There were no significant changes with time in the BB536 group or the placebo group, and no significant differences between the BB536 group and the placebo group throughout the study period.

Table 3. Comparison of the Number of Subjects Who Contracted Influenza or Had Fever during the P2 Period in the BB536 Group and the Placebo Group

	BB536 group	Placebo group	Statistical analysis*
No. of subjects with influenza	0	5	$p = 0.041$
No. of subjects with fever**	2	8	$p = 0.046$
No. of subjects administered antibiotics	2	7	$p = 0.103$

*, Changes in the above parameters from week 5 to the end of the study were analyzed by Fisher's direct probability test.
**, Fevers of 38 °C or above were recorded. The number of subjects with influenza and fever were significantly smaller in BB536 group than in the placebo group.

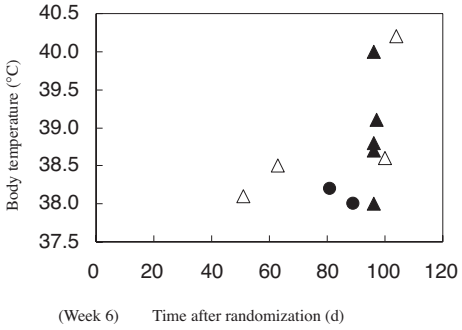

Fig. 2. Episodes of Fever over Time.
Fever of 38 °C or above was recorded, and the episodes of fever were analyzed. A total of two subjects in the BB536 group developed fever during the 14-week period. In comparison, a total of eight subjects (a total nine incidents) in the placebo group developed fever, five due to influenza and three due to fever of unknown origin. ●, BB536 group, fever episode; △, placebo group, fever episode; ▲, placebo group, fever episode due to influenza onset.

Changes in NK cell activity, neutrophil bactericidal activity, and neutrophil phagocytic activity

During the P1 period, NK cell activity increased from 26 ± 11% before *B. longum* BB536 ingestion to 37 ± 13% at week 5, a significant increase. Moreover, neutrophil bactericidal activity increased significantly, from 88.9 ± 6.4% before *B. longum* BB536 ingestion to 92.7 ± 5.8% at week 5. On the other hand, neutrophil phagocytic activity showed no increase at week 5 as compared to before ingestion, and a normal level was maintained (Table 1).

Table 4 shows changes in cell-mediated immunity during the P2 period. In the BB536 group, the mean NK cell activity apparently decreased at week 20 of the study, although there were no significant differences in NK cell activity throughout the P2 period. In the placebo group, however, NK cell activity decreased significantly from week 10 to week 20 ($p < 0.01$). Thus, at week 20, the last time point of the P2 period, while NK cell activity decreased in both the BB536 and the placebo group, activity was remained at a slightly higher level in the BB536 group than in the placebo group. Neutrophil bactericidal activity showed a trend similar to NK cell activity: no significant differences were observed within the BB536 group throughout the P2 period, but significant decreases were found in the placebo group between week 5 and week 20 ($p < 0.05$), and between week 10 and week 20 ($p < 0.01$). Thus neutrophil bactericidal activity declined at week 20 in both the BB536 and the placebo group, to the pre-administration level in the P1 period. For neutrophil phagocytic activity, the normal level was maintained in the BB536 group, whereas the level was significantly reduced at week 20 ($p < 0.05$) as compared to week 5 (the end of the P1 period) in the placebo group.

Figure 3 shows the magnitudes of changes in NK cell activity, neutrophil bactericidal activity, and neutrophil phagocytic activity in the P2 period, taking the activity at week 5 as baseline (0). For NK cell activity, the change in activity at week 10 (activity at week 10–activity at week 5) was significantly greater in the placebo group than in the BB536 group ($p = 0.019$), but the decreases at week 20 were comparable in the two groups. On the other hand, the changes in neutrophil bactericidal activity were not different between the two groups at any time point, and similar decreases were observed at week 20 in the two groups. However, neutrophil phagocytic activity at week 20 tended to be lower in the placebo group than in the BB536 group ($p = 0.099$).

Table 4. Changes in Cell-Mediated Immunity during the P2 Period in the BB536 Group and the Placebo Group

	Group	Week 5 of study (2 wk after vaccination) (a)	Week 10 of study (b)	Week 20 of study (c)	Intra-group difference
NK cell activity (%)	BB536	41 ± 12	40 ± 10	34 ± 15	NS
	Placebo	33 ± 12	38 ± 11	28 ± 16	†(b) *vs.* (c)**
Neutrophil bactericidal activity (%)	BB536	92.1 ± 7.0	91.2 ± 7.2	86.4 ± 14.9	NS
	Placebo	93.3 ± 4.7	94.8 ± 3.2	87.8 ± 8.4	†(a) *vs.* (c)*, (b) *vs.* (c)**
Neutrophil phagocytic activity (%)	BB536	93.3 ± 2.1	92.0 ± 4.0	90.7 ± 5.0	NS
	Placebo	93.4 ± 2.7	92.9 ± 3.2	86.9 ± 12.0	†(a) *vs.* (c)*

Values are presented as mean ± SD for 27 subjects. Changes in the above parameters from week 5 to the end of the study were analyzed: intra-group difference (†) by multiple comparison by Scheffe's method.

†, Significant intra-group differences were observed only in the placebo group during the entire P2 period.

*, $p < 0.05$; **, $p < 0.01$.

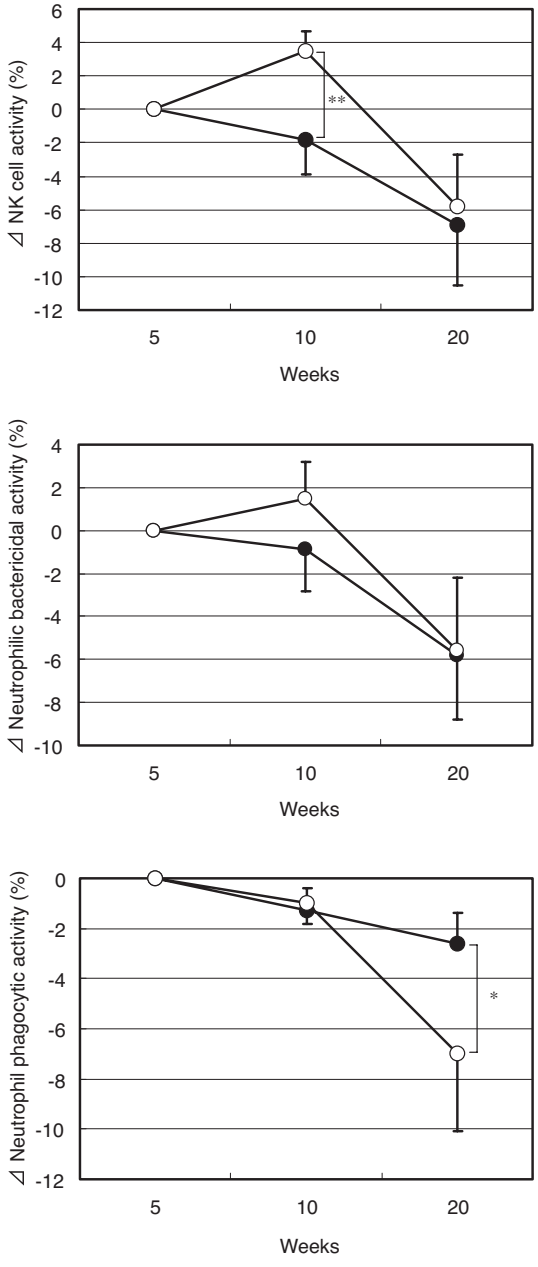

Fig. 3. Changes in Cell-Mediated Immunity over Time during the P2 Period for the BB536 Group and the Placebo Group, Taking the Level at Week 5 as Baseline (0).

Values are presented as mean ± SE for 13 subjects in BB536 group and 14 subjects in the placebo group. ●, BB536 group; ○, placebo group. *, $p = 0.099$; **, $p = 0.019$.

Discussion

When an influenza epidemic occurs, an increase in the number of deaths (excess deaths), mainly in elderly persons, is observed as compared to non-epidemic years. Consequently, active vaccination of elderly persons is conducted worldwide as a preventive measure against influenza. According to the recommendations of the US Advisory Committee on Immunization Practices (ACIP),[9] when the vaccine strain matches the epidemic strain, influenza vaccine is 70–90% effective in preventing influenza in healthy adults aged 65 years or below. Although the preventive effect decreases to 30–40% in elderly persons living in institutions, the vaccine is 50–60% effective in preventing hospitalization and pneumonia, and 80% effective in preventing death. On the other hand, in the case of elderly persons living at home, vaccination is about 58% effective in preventing influenza in persons aged 60 years or above, and is yet lower in those aged 70 years or above. Furthermore, a study on influenza vaccine antibody titers in the elderly reported approximately 7% low responders, and the immunogenetic background of these subjects was investigated.[10] In these subjects, low response was associated with MHC class II haplotypes which are receptors of the antigen peptides presented by antigen-presenting cells to T cells. Subjects possessing the HLA-DR2 haplotype had a relative risk of 13.9 of being low responder (low antibody production).

The above findings demonstrate the difficulty of preventing influenza by vaccination alone. Therefore, further strategies to prevent influenza in the elderly are required, such as: (i) augmentation of specific influenza antibody, (ii) maintenance of good nutrition status to enhance the antibody production stimulated by influenza vaccination, and (iii) improvement of immune capacity which, falls in old age.

The effects of ingestion of probiotics on antibody titers in blood following influenza vaccination have been studied. Yasui *et al.*[11] reported that administration of *Bifidobacterium breve* strain YIT4064 to influenza-infected rats increased influenza-specific IgG antibody production. In a human clinical study on the vaccine adjuvant effect of chlorella in subjects orally administered chlorella before influenza vaccination, the proportion of subjects showing an increase in antibody titers of 4 or above at week 4 after vaccination as compared to week 1 did not differ from the placebo group, and showed no dose dependence.[12]

In the present study, we examined changes over time in the antibody titers to the influenza type A and type B strains used in the 2004–2005 influenza vaccine. We were not able to estimate the adjuvant effect of BB536 administration, because the effective rate of influenza vaccination for the year of this study, which will serve as a historical control, has not been published. However, we found no significant changes in serum antibody titers against the influenza vaccine strains in the BB536 group, suggesting that continuous administration of *B. longum* BB536 has no effect on the maintenance of antibody titers against influenza vaccine.

On the other hand, several studies have indicated that ingestion of probiotics enhances innate immunity in elderly persons. Arunachalam *et al.*[13] investigated cell-mediated immunity in healthy elderly persons (mean age 69 years) administered *Bifidobacterium lactis* strain HN019, and observed increased IFN-α production by peripheral blood mononuclear cells (PBMCs) and augmented innate immunity, including PMN phagocytic activity and bactericidal activity, at week 6 of ingestion. Chiang *et al.*[14] investigated PMN and peripheral blood NK cells in Taiwanese adults administered *Bifidobacterium lactis* strain HN019, and reported increased PMN phagocytic activity and bactericidal activity as well as augmented NK cell activity from week 3 of ingestion. Gill *et al.*[15] examined the effects of *Bifidobacterium lactis* strain HN019 in enhancing NK cell activity, and observed increases in CD4- and CD25-positive cells the markers of activated T cells, as well as increases in the NK activity of CD56-positive cells. Morimoto *et al.*[16] studied NK cell activity in healthy normal adults (aged 20 to 50 years) with a smoking habit who were drinking a fermented milk containing *Lactobacillus casei* strain shirota, and found increased NK cell activity in the smokers as compared to non-smokers. The P2 period of the present study was a randomized double-blind controlled study aiming to examine the effects of ingesting a relatively high concentration (1×10^{11} cfu) of *B. longum* BB536 on the effect of influenza vaccination as compared to a placebo, in a group of elderly persons having the same living environment as well as dietary content and receiving influenza vaccination. NK cell activity and neutrophil bactericidal activity were found to be enhanced after taking *B. longum* BB536 for 5 weeks as compared to before *B. longum* BB536 ingestion in the P1 period.

When BALB/c mouse peritoneal cells were cultured in the presence of cell wall preparation (WPG) of *Bifidobacterium infantis* (now classified as *Bifidobacterium longum*), IFN-α mRNA expression was induced.[17] IFN-α/β is produced by virus-infected cells and dendritic cells, and is known to enhance NK cell activity.[18,19] In plasmacytoid (lymphoid) dendritic cells, IFN-α/β is produced by recognition of the bacterial body *via* Toll like receptor (TLR).[20] Based on these findings, we speculate that *B. longum* BB536 is recognized by lymphoid dendritic cells *via* TLR-2 and/or TLR-9,[21–23] resulting in the release of IFN-α/β and subsequently activation of NK cells. In addition, since neutrophils also constantly secret IFN-α/β, which exerts autocrine action on the neutrophils themselves, we speculate that the increase in neutrophilic function (bactericidal activity) is also an immunostimulatory effect induced by *B. longum* BB536.

Since ingestion of probiotics has been reported to increase the production of immunoglobulins against the injected influenza vaccine, we hypothesized initially that an increase in antibody titers stimulated by influenza vaccination is the most important factor affecting influenza virus infection. Hence we pre-administered probiotics to all subjects to equalize their effects in the two groups. After randomization, *B. longum* BB536 administration was continued in the BB536 group, whereas it was terminated in the placebo group. There was no difference between the two groups with respect to the maintenance of effective antibody titers against the influenza vaccine. Although not examined in the present study, the mucosal surface of the respiratory tract is an important defense mechanism against influenza infection. It is possible that continuous oral administration of BB536 maintains the mucosal secretion of IgA antibodies specific for influenza antigens, reducing the infection rate. This important aspect must be examined in further studies.

Xiao *et al.* examined the effects of a BB536-containing yogurt in relieving clinical symptoms of cedar pollinosis.[24] They measured serum IFN-γ levels during the study period, and reported that IFN-γ levels were reduced to the greatest extent in March to April as compared to January when the study was started, irrespective of whether BB536-containing yogurt was ingested. In the present study, week 20 of the RCT period fell at the end of March. It is possible that neutrophil bactericidal activity fell at week 20 due to a decrease in IFN-γ, a Th1 cytokine that enhances bactericidal activity, and the opsonin effect for phagocytes such as neutrophils and macrophages.[25] Indeed when we analyzed the changes in cell-mediated immunity during the P2 period, we found that NK cell and neutrophil bactericidal activities declined toward week 20 in both the BB536 and the placebo group, with comparable magnitudes (Fig. 3). This probably reflected common seasonal variations in all the subjects. However, despite the decline, NK activity remained at a slightly higher level at week 20 in the BB536 group than in the placebo group. On the other hand, the decrease in neutrophil phagocytic activity at week 20 tended to be greater in the placebo group than in the BB536 group, and consequently phagocytic activity remained at a slightly higher level in the BB536 group. Some of the immunological data obtained in the present study were weak statistically, probably because of the small sample size and also the mild biological modulating effects of probiotics. A large-scale study is required to obtain statistically significant data.

In the present study, we found that NK cell and neutrophil bactericidal activities were significantly augmented by ingestion of BB536. Despite declines in activity at week 20 of the randomized study in both the BB536 and the placebo group, NK cell and neutrophil phagocytic activities tended to remain at slightly higher levels in the BB536 group. Among the human immune defense mechanisms, while innate immunity, comprising NK cell activity and neutrophil/macrophage activation, plays important roles in defense against infection, acquired immunity, including mucosa secretion of specific secretary IgA and IgG, is equally important in preventing infection. Although these aspects were not

adequately investigated in the present study, we speculate that continuous ingestion of BB536 potentiates these functions, which act in an integrated manner to reduce the incidence of influenza and fever in elderly subjects.

Further large-scale clinical studies are warranted to examine the effects of probiotic administration on a more comprehensive profile of cell-mediated immunity as well as humoral immunity, including mucosal immunity and specific immunoglobulin production, together with correlations with protection against infections such as influenza.

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