Orally administered *Bifidobacterium* triggers immune responses following capture by CD11c⁺ cells in Peyer's patches and cecal patches

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Abstract We have investigated the immunomodulatory mechanisms of *Bifidobacterium pseudocatenulatum* JCM7041 (Bp) as model of probiotics following oral administration to mice. This study was conducted with the aim of clarifying the mechanism of immunomodulation induced by oral administration of probiotic bacteria through elucidation of the detailed mechanism of transfer of orally administered bacterial cells within the body and the interaction between bacterial cells and cells of the immune tissues. We observed the localization of Bp in mice following oral administration, showing that Bp was surrounded by CD11c⁺ cells in Peyer's patches (PP) and cecal patches (CP). These results indicated that Bp might induce CD11c⁺ cell-mediated immune responses directly. Furthermore, IL-10

and IL-12p40 production by Thy1.2⁺ cells, including CD11c⁺ cells, increased significantly. Production of IL-10 and IL-12p40 by bone marrow-derived dendritic cells (BMDC) was significantly increased by Bp stimulation. These results suggest that oral administration of Bp induces immune responses directly following capture by CD11c⁺ dendritic cells (DCs). Subsequently, we observed oral administration of Bp for 1 week induced IgA and IgA-associated cytokine production by CP and PP cells, suggesting that Bp induced DC-mediated immune responses on CP as well as PP.

Keywords Probiotics · *Bifidobacterium* · Dendritic cells

Abbreviations

Bp *Bifidobacterium pseudocatenulatum* JCM7041
BMDC Bone marrow-derived dendritic cells
BIM *Bifidobacterium* immunomodulator

Introduction

Our bodies have a unique intestinal immune system that recognizes orally ingested microorganisms bearing xenogeneic antigens, thereby allowing them to live symbiotically or eliminating them. Stimulation by intestinal bacteria is important in the development

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of the intestinal immune mechanism, and complex interactions are formed between them (Cebra 1999; Rhee et al. 2004). Some intestinal bacteria, such as *Bifidobacterium* and *Lactobacillus*, bringing beneficial effects for our health when orally ingested and are expected to act as probiotics.

In recent years, it has been reported that probiotic bacteria or bacterial components have allergy-improving and anti-infective immunomodulatory effects. Shida et al. (2000) reported that in ovalbumin-specific T cell receptor transgenic mice, an animal model that shows elevated blood IgE when fed an ovalbumin-containing diet, intraperitoneal administration of *Lactobacillus casei* strain Shirota suppressed the increased IgE, thereby suppressing the allergic reaction (Dohi et al. 1999). Also, Pessi et al. (2000) reported that administration of *L. rhamnosus* GG to patients with atopic dermatitis enhanced IL-10 production leading to an increased blood IL-10 level. Furthermore, the long-term administration of commercial fermented milk, containing probiotic bacteria, modulated the mucosal immune responses (IgA, cytokines etc.) (de Moreno de LeBlanc et al. 2008). We previously reported that when *Bifidobacterium*-derived immunomodulator (*Bifidobacterium* immunomodulator; BIM), prepared by ultrasonic disintegration of *Bifidobacterium pseudocatenulatum* 7041 derived from human intestinal bacteria, was administered orally to mice, IgA production in Peyer's patches (PPs) of the small intestine increased, thereby demonstrating that oral administration of bacterial cell components induced an intestinal immune response (Nakanishi et al. 2005). However, the immunomodulating activity of these probiotics induces different immune responses depending on the bacterial strain and the mechanism of the immunomodulating activity has yet to be elucidated. In particular, the intestinal localization of the uptake of the probiotic bacteria into the gut-associated lymphoid tissues after oral feeding has not yet been clarified.

This study was conducted with the aim of clarifying the mechanism of immunomodulation induced by oral administration of probiotic bacteria through elucidation of the detailed mechanism of transfer of orally administered bacterial cells within the body and the interaction between bacterial cells and cells of the immune tissues.

Materials and methods

Mice

BALB/c mice (CLEA Japan, Inc., Tokyo, Japan) were maintained in plastic cages at 23 ± 2 °C, at a relative humidity of $50 \pm 10\%$, and under 12-h light cycles (illuminated from 8:00–20:00). Seven-week-old female mice were used for this experiment. During the first 3 days of the acclimatization period, mice were allowed free access to solid chow MF (Oriental Yeast Co., Ltd., Tokyo, Japan) and ion-exchanged water. The animal experiments in this study were carried out according to the 'Guideline for the Care and Use of Laboratory Animals' by the College of Bioresource Sciences, Nihon University.

Bacteria

Bifidobacterium pseudocatenulatum JCM 7041 (Bp), selected through screening by Lee et al. (1993) was obtained from RIKEN (Bioresource, Japan collection of microorganisms, Tokyo, Japan). Bp was cultured in GAM broth (Nissui, Tokyo, Japan) at 37 °C for 18 h, washed three times in PBS, and suspended to a final cell density of 1×10^9 CFU (colony forming unit)/200 µl. Sonicated *B. pseudocatenulatum* (*Bifidobacterium* immunomodulator; BIM) was prepared according to the method previously reported by Hosono et al. (1997, 1998).

Analysis of bacterial migration

Seven mice were orally administered 5-(and 6-) carboxyfluorescein succinimidyl ester (CFSE) (Dojindo Laboratories, Kumamoto, Japan) fluorescent-labeled Bp (1×10^9 CFU/mouse) or CFSE solution, and were sacrificed over a time course (0, 1, 4, 6, 12, 18, 24 h after administration) to remove PP, mesenteric lymph node (MLN), liver, thymus, and spleen (SPL). After oral administration, the tissue samples were obtained at the following times; 0, 1, 4, 6, 12, 18, 24 h, and a cell suspension was prepared from each tissue. Cells of each tissue were suspended in sterilized PBS to a cell density of 5×10^5 cells/mL. The cell suspension thus obtained was analyzed by a flow cytometer [FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA, USA);

CellQuest program (BD Biosciences Immunocytometry Systems)].

Immunohistochemical analysis of intestinal tissue sections after one-shot oral administration of fluorescent-stained *B. pseudocatenulatum* 7041

Fluorescent-labeled *B. pseudocatenulatum* 7041 was prepared according to the method of Ueckert et al. (1997). Briefly, CFSE dissolved in PBS to a final concentration of 50 $\mu\text{L}/\text{mL}$ was added to Bp suspension at a cell density of 1×10^9 CFU/mL, and the mixture was allowed to react at 37 °C for 1 h under light-protected conditions. The reaction mixture was then washed with PBS three times and used as the bacterial cell sample for oral administration. The fluorescent-labeled bacterial cells or CFSE-PBS (control) were administered orally at a dose of 1×10^9 CFU/200 $\mu\text{L}/\text{mouse}$ to seven mice that had fasted for 24 h respectively. Mice were then sacrificed over a time course (0, 1, 4, 6, 12, 18 and 24 h) after administration to remove PP, MLN, and CP, and a frozen block of each intestinal tissue was prepared. Cryosections of 6 μm thickness were prepared using a cryostat HM505E (Microm International GmbH, Walldorf, Germany). The cryosections of each tissue were washed with PBS, then with TNT (0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20; pH 7.5). In order to inactivate endogenous peroxidase, 2 drops of Peroxidase Blocking Reagent (Dako JAPAN, Kyoto, Japan) was added to each section, and the section was left standing for 10 min, after which the section was washed with TNT twice. In order to suppress nonspecific binding, 100 μL of TNB [0.1 M Tris, 0.15 M NaCl, Blocking Reagent (supplied with TSA kit) (PerkinElmer Life Sciences, Boston, MA, USA) pH 7.5] was added to each section, and the section was left standing for 30 min. After washing with TNT, 100 μL of TNB-diluted hamster anti-CD11c mAb (Clone; N418, given by Dr. Hachimura) was added to each section, and the section was left standing for 60 min. After the section was washed three times, 100 μL of TNB-diluted HRP-labeled goat anti-hamster antibody (Southern Biotechnology Associates, Alabama, USA) was added to each section, and the section was left standing for 30 min. After the section was washed three times, 100 μL of Cy3-containing staining solution (supplied

with TSA kit) was added to each section, and the section was left standing for 5 min. The section was washed with TNT twice, then with PBS. The section was mounted using IMMUMOUNT (Thermo Shandon, Pittsburgh, PA, USA) containing 2% 1,4-diazabicyclo [2,2,2] octane (Sigma). In the immunohistochemical analysis of CP, we stained B cells with a B220-FITC mAb (Clone; RA3-6B2, BD-Pharmingen), and T cells with a CD3 ϵ -biotin mAb (Clone; 145-2C11, eBioscience) and Streptavidin, Alexa Fluor 647 conjugate (Molecular probes, Eugene, OR, USA). The immunostained cryosection was observed by a confocal laser scanning microscopy FV500 (Olympus, Tokyo, Japan) using a software to analyze, FLUOVIEW FV500 (Olympus).

General anesthesia, terminal ileum ligation, and injection of CFSE-labeled Bp

For general anesthesia, pentobarbital solution (pentobarbital sodium salt 5 mg/mL) was injected into the abdominal cavity of BALB/c mice at 100 $\mu\text{L}/10$ g body weight. We then dissected the abdominal cavity, and ligated the terminal ileum. After this treatment, we injected CFSE-labeled Bp (1×10^9 CFU/50 μL) or PBS (control) just beneath the ligated portion. At 3 h after injection of CFSE-labeled Bp, frozen blocks of CP were prepared and used for immunohistochemical analysis.

Immunological assay of mucosal lymphocytes following oral administration of Bifidobacterium for 7 consecutive days

Live Bp cells were suspended in PBS to a final concentration of 1×10^9 CFU/200 $\mu\text{L}/\text{mouse}$ ($n = 6$ mice per group) and orally administered by gavage using a feeding tube. Mice in the control group ($n = 6$) were administered the same amount of PBS only. After oral administration for 7 consecutive days, cells were prepared from each of CP and MLN, and amount of IgA, IL-5, IL-6, IL-12p40 and IFN- γ produced by these cells was measured by ELISA according to the previously reported methods (Nakanishi et al. 2005). The culture supernatant IgA (7 days culture) and cytokines (72 h culture) were measured by ELISA.

Measurement of IL-12p40 and IL-10 produced by Thy1.2⁺ cells from PP after single shot administration of Bp

Bp suspension, prepared to a cell density of 1×10^9 CFU/200 μ L PBS, was administered orally to 7-week-old female BALB/c mice ($n = 6$ per group) that had fasted for 24 h. They were sacrificed at 24 h after administration to remove PP. Thy1.2⁺ cells from PP were prepared. Thy1.2⁺ cells were negatively isolated by using anti-mouse Thy1.2 mAb conjugated magnetic microbeads and autoMACS^{TM} (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacture's protocol. Purity of Thy1.2⁺ cells was confirmed >90% by flow cytometry. Cell suspensions were prepared to a cell density of 2.5×10^6 cells/mL, and cultured for 24 and 72 h in the presence of 10–50 μ g/mL of BIM for the secondary stimulation. The supernatant of 24 h culture was collected and measured for the amount of IL-12 p40 produced using Opt EIA^{TM} Mouse IL-12 (p40) set (Pharmingen, San Diego, USA.). The supernatant of 72 h culture was collected and measured for the amount of IL-10 produced using Opt EIA^{TM} Mouse IL-10 set (Pharmingen, San Diego, USA).

Preparation of BMDC

Bone marrow-derived dendritic cells (BMDC) were obtained from bone marrow precursors isolated from the femurs of BALB/c mice as described by Lutz et al. (1996) with minor modification. Prepared leukocytes (2×10^5 cells/mL) were obtained from three mice and were cultured with GM-CSF (Pepro-Tech, Rocky Hill, NJ, USA) (200 U/mL). At day 3, another medium containing 200 U/mL GM-CSF was added, and at day 6 and 8, culture medium was exchanged to a fresh medium containing 200 U/mL GM-CSF. At day 10, a subcultured cell population was analyzed by flow cytometry using anti-CD11b, CD11c and MHC-II antibodies. At day 10, non-adherent cells were collected by centrifugation at 1,300 rpm for 6 min to obtain BMDC (the purity was >90% of CD11b⁺ CD11c⁺ cells). We then resuspended the BMDC with RPMI 1640 containing 10% FCS, 100 U/mL GM-CSF and Bp (1×10^7 CFU/mL) for 24 and 72 h. After incubation, culture supernatants and cells were collected for analysis of

cytokine production and cell surface antigen expression. The supernatant of 24 h culture was measured for the amount of IL-12 p40 concentration by using Opt EIA^{TM} Mouse IL-12 (p40) set (Pharmingen), and that of 72 h culture was measured for the amount of IL-10 by using Opt EIA^{TM} Mouse IL-10 set (Pharmingen).

Statistical analysis

Data are represented as mean $\pm$ SD. Statistical significance was calculated using the unpaired Student's *t* test. Differences were considered statistically significant for values of $p < 0.05$.

Results

Determination of time required for orally administered Bp cells to reach each lymphatic tissue in mice

The phenomenon of intestinal microorganisms being incorporated into the body and transported to the mesenteric lymph nodes is recognized as bacterial translocation. Intestinal bacteria have as their cell components teichoic acids and peptidoglycans which induce an immune response, and are therefore supposed to be capable of directly stimulating immune cells, leading to the activation of intestinal immunity. Probiotics and food antigens are also capable of activating immunity through their cell components and metabolites. These facts suggest the possibility that orally administered bacterial cells also directly affect the immune cells of the gut associated lymphoid tissue (GALT). However, details of the translocation of orally administered bacterial cells in the body remain unclear. To elucidate this, fluorescent-labeled bacterial cells were administered orally, and their distribution in the body was studied over time by detecting bacterial cell-specific fluorescence by means of flow cytometry, to validate the hypothesis that the administered bacterial cells are incorporated or transferred to PP, CP, MLN, SPL, thymus, and liver.

We confirmed that no fluorescent peak of CFSE was detected in the intestinal mucosal tissues derived from the administered mice of CFSE-PBS without Bp as a control (data not shown). At 1 h after oral

administration of the bacterial cells, fluorescent peaks specific to bacterial cells were detected in PP (Fig. 1a). Also, the bacterial cell-specific fluorescent peaks were detected in MLN at 24 h after oral administration (Fig. 1b). In contrast, bacterial cells could not be identified in SPL (Fig. 1c), CP (data not shown) or in the thymus (data not shown) within 24 h of oral administration. In the liver, it was difficult for us to detect bacterial cell-specific fluorescence because of the autofluorescence of hepatocytes (data not shown). These results showed that orally ingested bacterial cells were incorporated directly into Peyer's patches 1 h after administration, followed by translocation to MLN within 24 h.

Incorporation of orally administered bacterial cells into intestinal tissues and their localization

Presence of enteric bacteria in the intestinal lymphoid tissues has been demonstrated by the culture of bacteria using tissue cell suspensions and other methods, but the localization of the bacteria in the tissues has remained unresolved. Localization of bacterial cells in tissues appears to have substantial effects on the immune response to be induced subsequently, judging from their interaction with immune cells. To clarify this ambiguity, fluorescent-labeled bacterial cells were administered orally, and their localization in frozen tissue sections was studied

Fig. 1 Peaks of Bp stained by CFSE (50 $\mu\text{g/mL}$) in cell fluid from PP (A), MLN (B) or SPL (C). Samples were taken after oral administration of Bp-CFSE respectively. Peaks were

detected by flow cytometric analysis. The results (at the time course of 0, 1, 6, 24 h after administration) shown are representative of two independent experiments

by confocal microscopy. As a result, in PP in the upper small intestine, Bp cell-specific fluorescence was detected in the upper dome of lymphoid follicles constituting PP in tissue samples taken 1 h after oral administration (Fig. 2a). The site of localization was identified as the site where CD11c⁺ cells (dendritic cells) reside as judged by the immuno-staining performed simultaneously using anti-CD11c antibody (Clone; N418). Some of the incorporated bacterial cells were localized at the same site as CD11c⁺ cells.

In CP, the fluorescence of CFSE-labeled Bp was observed in the tissue specimens obtained 4 h after oral administration (Fig. 2b). However, the fluorescent intensity of CFSE-labeled Bp was observed to be faint in CP. Therefore, to demonstrate whether Bp was taken up into CP from the intestinal luminal side, we ligated the terminal ileum and injected the CFSE-labeled Bp into the cecum directly. In CP, bacterial cell-specific fluorescence was observed in the tissue specimen obtained 3 h after injection (Fig. 2c). Some

of the incorporated bacterial cells were also localized at the same site as CD11c⁺ cells in CP as seen in PP. In MLN, bacterial cell-specific fluorescence was observed 20 h after oral administration (Fig. 2d). In this tissue, Bp was found not to be localized to the outer region of MLN but close to the central region, the site of CD11c⁺ cells.

These results suggest that orally administered bacterial cells were incorporated via PP and CP into the body, and phagocytosed by CD11c⁺ cells. Furthermore, they suggest that, after being incorporated into the intestinal region, Bp captured cells migrated to MLN via lymph ducts, etc.

Change in IL-10 and IL-12p40 production by Thy1.2⁺ cells derived from PP after single shot of Bp

The above results showed that orally administered Bp cells were incorporated via PP and CP into the body.

Fig. 2 Location of CFSE-labeled Bp in PP (A), CP (B), and MLN (C) after single shot orally, and the location of Bp in CP in which Bp was injected into the ligated cecum directly. Each sample was obtained at the following times: PP (A); 1 h, and MLN (D); 20 h after oral administration and CP (B); 4 h after injection. Furthermore, the localization of Bp was observed in CP (C); 3 h after injection of CFSE-Bp into the cecum directly in the condition of the ligated terminal ileum. CD11c cells are stained by anti-CD11c Ab (Clone; N418) (blue). Bp stained by CFSE (red) was observed in the PP, MLN, and CP (arrow). Some of the Bp spots stained by CFSE were overlapped with those stained by anti-CD11c Ab. The results shown are representatives of two independent experiments

They suggest that Bp cells might be captured by phagocytes. These findings suggest the possibility that orally administered Bp directly induce innate immune responses by affecting the immune system through phagocytes such as dendritic cells and macrophages capturing Bp. To support this hypothesis, the amount of IL-10 and IL-12p40, cytokines produced by phagocytes, from Thy1.2⁺ cells including CD11c⁺ cells derived from PP cells were measured 24 h after single dose oral administration of Bp. IL-10 (Fig. 3a) and IL-12p40 (Fig. 3b) production was observed to be increased significantly. These results indicate that, in tissues to which bacterial cells can be easily incorporated, such as PP, the innate immune system is directly modulated by orally administered Bp.

Cytokine production by BMDC was significantly increased following Bp stimulation

To clarify the immunomodulatory effects of Bp on DC, we measured cytokine (IL-10 and IL-12p40)

Fig. 3 The concentration of IL-10 and IL-12p40 produced by Thy1.2⁺ cells derived from PP. PP Thy1.2⁺ cells were obtained and pooled for each experimental group ($n = 6$ mice per group) respectively after a single shot administration of PBS (control: white bar) or Bp (black bar). The cells were co-cultured with BIM (0, 10, 50 µg/mL) for 24 or 72 h. The amounts of IL-10 (A) and IL-12p40 (B) in the culture supernatant were measured by ELISA. *Significant difference from control group of the same BIM dose at $p < 0.05$ by Student's t test. The results shown are representative of three independent experiments

production by BMDC as a model of intestinal DC (Kelsall and Leon 2005). IL-10 production from BMDC was up-regulated by Bp stimulation (Fig. 4a). IL-12p40 production from BMDC was significantly increased by Bp stimulation compared with unstimulated BMDC (Fig. 4b).

Effect of 7 consecutive days repeated oral administration of Bp on IgA and cytokine production by CP cells

In this study, we demonstrated that Bp was taken up into the CP from the intraluminal side and captured by CD11c⁺ cells in CP 3 h after injection into the cecum. This result suggested that Bp might induce immune responses in CP cells directly. Therefore, the effect of repeated administration of Bp cells for 7 consecutive days on the amount of IgA and cytokines produced by CP cells was investigated. When Bp cells were administered orally for 1 week, the total IgA production in the culture supernatant of cells from CP increased significantly (Fig. 5a). As regards cytokine production, IL-5 also increased significantly (Fig. 5b). Increased IgA production was demonstrated in the culture supernatant of cells from MLN (data not shown). Both IL-6 (Fig. 5c) and IFN- γ (Fig. 5d) secretion by CP cells derived from Bp-administered mice was not changed when compared with the group administered PBS alone.

Discussion

Previously, we reported the characteristics of the immune response in PP observed following 1-week of repeated oral administration of BIM, Bp-derived cell components (Nakanishi et al. 2005), and showed that production of cytokines such as IL-5 and IFN- γ and BIM-specific IgA antibody increased in PP when BIM was repeatedly administered orally to mice for 1 week. Our study also showed that the amount of IL-6 and IFN- γ produced by CD4⁺ T cells in PP increased and that IL-12p40 production by Thy1.2⁺ cells as antigen presenting cells in PP increased, while production of IFN- γ , IL-5 and IL-6 in these cells was not affected by BIM administration. These results demonstrated that oral administration of BIM modulated the activity of CD4⁺ T cells in PP. However, our study did not elucidate the mechanism

Fig. 4 Production of IL-10 (for 72 h) (A) and of IL-12p40 (for 24 h) (B) by BMDC after stimulation by Bp. BMDC were generated by GM-CSF stimulation for 10 days. The cells were co-cultured with Bp (1×10^7 CFU/mL) (black bar) or none as control (white bar) for 24 or 72 h. The culture supernatant

IL-10 for 72 h (A) and IL-12p40 for 24 h (B) were measured by ELISA. *Significant difference from control (unstimulated) at $p < 0.05$ by Student's t test. The results shown are representative of three independent experiments

of the effect of BIM on PP cells in detail. Although similar immunomodulatory effects of orally ingested probiotic bacteria have also been reported, the mechanisms have not been clarified exactly. Thus, it was considered important to clarify the behavior of the orally administered probiotics in the body.

Using flow cytometric analysis, orally administered Bp cells were detected in PP and MLN but not in spleen or thymus. These results were in line with the report by Macpherson and Uhr that bacterial cells, upon entry into the body, reached MLN by bacterial translocation, where they were completely processed by dendritic cells and macrophages (Macpherson and Uhr 2004). Observation of tissue sections of the intestinal immune system by confocal laser microscopy showed that dietary Bp cells were incorporated in CP as well as PP and were localized at the upper site of the dome area surrounded by CD11c⁺ cells, as demonstrated by immunostaining with anti-CD11c antibody (Clone; N418). In addition, measurement of IL-10 and IL-12p40 produced by Thy1.2⁺ cells including CD11c⁺ cells from PP cells 24 h after a single dose oral administration showed Bp administration caused a significant increase in IL-10 and IL-12p40 production in the gut immune system.

Moreover, in this study, we demonstrated that Bp induced IL-10 and IL-12p40 production from CD11b⁺ CD11c⁺ BMDC. This is the predominant DC phenotype in the intestinal immune tissue (Kelsall and Leon 2005).

These results suggested that the orally administered Bp cells could be captured by CD11c⁺ cells (e.g., dendritic cells) in PP, thereby directly affecting antigen presenting cells and phagocytic cells, including dendritic cells. When Bp was consecutively administered orally for 1 week, the amount of IL-6, IFN- γ and IgA produced by PP cells (Hiramatsu et al. 2007), the amount of IL-5 and IgA produced by CP cells, and IgA produced by MLN cells increased significantly.

These results suggest that oral administration of Bp for 1 week can activate the mucosal immune response for IgA production in the gut. Previous reports have shown that 1-week oral administration of BIM, consisting of components of Bp cells, induced an increase in IgA production in PP, and that an effect on CD4⁺ T cells was observed as a characteristic feature of the effect of the administration (Nakanishi et al. 2005). Therefore, it is suggested that the immunomodulatory function of dietary Bp consists of

Fig. 5 Immunomodulatory effects of oral administration of Bp for 7 consecutive days on CP. Culture supernatant IgA (**A**), IL-5 (**B**), IL-6 (**C**) and IFN- γ (**D**) on CP after oral administration of PBS as control (white bar) or Bp (black bar) for 7 consecutive days ($n = 6$ mice per group). Cells from CP were obtained and pooled for each experimental group respectively. The cells were co-cultured with BIM 0, 10,

50 $\mu\text{g/mL}$ for 72 h or 7 days. The culture supernatant IgA (7 days culture) and cytokines (72 h culture) were measured by ELISA. The cells were co-cultured with BIM 0, 10, and 50 $\mu\text{g/mL}$. *Significant difference from control group at $p < 0.05$ by Student's t test. The results shown are representative of three independent experiments

capture by CD11c^+ cells such as dendritic cells in PP, thereby directly activating CD11c^+ cells. Subsequently, CD4^+ T cells receive the information of bacterial cells from dendritic cells, resulting in the immune activation of PP, ultimately leading to the induction of IgA producing response.

In the present study, we succeeded in observing Bp cells in CP 4 h after oral administration. Kweon et al. (2005) and Dohi et al. (1999) reported the presence of M cells in the epithelial layer covering colonic patches (Macpherson and Uhr 2004). Dohi et al. (1999) further demonstrated that the cell composition of CP and distribution of T and B cells resembled those of PP in the small intestine, which suggests the possibility that M cells are present in CP as well and that they incorporate orally administered Bp from the intestinal lumen. It is interesting to note that orally administered CFSE-fluorescent labeled Bp was also observed in CP, the cecal lymphoid follicles, demonstrating that orally administered Bp cell

components directly modulate the immune response not only in the small intestine but also in the cecum. Thus, it is expected that the bacterial cell components have reached CP by some route; through M cells on CP or captured by CD11c^+ cells in the CP, requiring future investigation to clarify the process of incorporation of orally ingested probiotic bacterial cells into the large intestinal region including the cecum and subsequent translocation within the body.

We could not observe Bp in the regions of cecum or colon other than CP within 6 h of oral administration, in contrast to successful observation of Bp in the villi of the small intestine simultaneously with the observation in PP (data not shown). This suggests that luminal antigens were not easily incorporated into the lamina propria of the cecum and the colon. In other words, orally ingested food antigens may be aggressively incorporated into the small intestine. van der Waagi et al. (2005) showed that the small intestine is covered with several thin ($\sim 10 \mu\text{m}$) viscous layers of

mucus whereas the large intestine is covered with a thick ($\sim 80\ \mu\text{m}$) mucosal layer. Failure in the present study to observe the incorporation of bacterial cells into the lamina propria of the large intestine, despite the presence in CP of villous M cells which serve as the site for incorporating luminal antigens (Kweon et al. 2005), may be due to the thick mucosal layer acting as the barrier.

In a study on the expression of Toll-like receptor (TLR)-2 in response to the stimulation by Gram-positive bacterial ligands in intestinal epithelial cell (IEC) lines, Melmed et al. (2003) demonstrated that TLR-2 expression in cells of intestinal tissues (e.g., dendritic cells, macrophages, lymphocytes and IECs) was inhibited by Toll-interacting protein (Tollip) expressed at a high level. These results suggest that IECs are not closely involved in the induction of intestinal immunity by bacterial cells or bacterial cell components (e.g., pathogen-associated molecular patterns). Also, van der Waaij et al. (2005) showed that, in the human large intestine, most intestinal bacteria exist in the luminal side of the mucosal layer or in the mucus, and further that intestinal bacteria and intestinal epithelial cells are not in direct contact with each other. These results suggest that, for induction of an immune response by bacterial cells and bacterial cell components, it is essential that they might be incorporated into tissues via M cells present in the small and large intestines, thereby directly stimulating cells of the immune system in lymphatic tissues.

In conclusion, in this study we demonstrated a detailed mechanism by which bacterial cells (or bacterial cell components) directly activate intestinal lymphatic tissues in the large and small intestine upon incorporation into the body following oral administration. It suggested that luminal bacterial components might be taken up into CP directly or might reach the CP via the vessel from PP. We believe that these results provide important information for the elucidation of the interaction between the host immune system and intestinal bacteria including probiotics.

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