2. MATERIALS AND METHODS

2.1 Mice

Germ-free TCR-Tg mice were separated into 2 groups for microflora conditioning - control and BIF. The control group was associated with segmented filamentous bacteria (SFB) and 46 strains of clostridia capable of inducing the development of the intestinal tissues. The BIF group was associated with *Bifidobacterium paseudocatenulatum* 7041 and SFB and clostridia.

2.2 Preparation of the cells from spleen (SP), PP and lamina propria (LP)

Murine small intestines and spleens were excised, and PPs were removed carefully from the intestines. Each single cell suspension from SP or PP was obtained by crushing the respective organ. LP lymphocytes were isolated from the small intestines by means of the following method. We excised PPs from the small intestines, after the intestines were turned inside-out using polyethylene tubing, and wiped carefully a few times with paper toweling. The intestines were cut into approximately 4 cm pieces and- washed with HBSS (-) (Hanks' balanced salt solution, Ca, Mg free, containing 5% FCS) with shaking at 37° C for 30 min three times. The supernatant was discarded following filtration with gauze and the intestines were then minced into 5 mm pieces and treated with collagenase (1 mg/ml in HBSS (+) (containing Ca, Mg and 5% FCS)) in a 100 ml-flask with gentle stirring at 37° Cfor 30 min. After collagenase treatment, the preparation was filtered with gauze and the cells were washed with HBSS (+) followed by centrifugation at 4° Ç1,300 rpm for 5 min. The supernatant was then removed. Furthermore, we added 3 ml of 100% Percoll (Amersham Biosciences AB, Uppsala, Sweden) to the cell pellets and added HBSS to 10ml (30% Percoll conc.). The cell suspension was then mixed gently and centrifuged at 20°C, 1,800 rpm for 20 min, after which the supernatant was removed, leaving 1ml of cells in suspension. Subsequently, we added 4.1 ml of 100% Percoll, and topped this up to 10 ml with 10% FCS-RPMI (44% Percoll conc.) and mixed. We then injected 2 ml of 70% Percoll into the bottom of the tube and centrifuged at 20°C, 1,800 rpm for 20 min. Finally, the cells located at the interface between the 44% and 70% Percoll fractions were collected as LP cells and washed with RPMI.

2.3 Cell culture for cytokine production

SP or PP cells (5×10^5 cells) and LP cells (1×10^5 cells) were cultured in 200 µl of RPMI medium in 96-well culture plates

containing 5%FCS and 0-500 μg/ml of OVA. Supernatants were collected after 48 hours. Spleen cells isolated from Balb/c mice were treated with mitomycin C, and then added (4×10^5 cells) as antigen presenting cells in LP cell cultures.

2.4 ELISA for cytokines

The culture supernatants were assayed for interferon (IFN)-γ, interleukin (IL)-4, IL-5 and IL-6. The amounts of cytokines in the supernatants were measured by a sandwich ELISA method. Rat anti-mouse IFN-γ, IL-4, IL-5 and IL-6 monoclonal antibodies were used as the capture antibody, with biotinylated rat anti-mouse IFN-γ, IL-4, IL-5 and IL-6 monoclonal antibodies, respectively, as the detection antibodies.

3. RESULTS AND DISCUSSION

We examined the effects of *Bifidobacterium* on the responsiveness of gut immune systems to a specific antigen, using gnotobiotic TCR-Tg mice. We did not think that it would be better to compare *Bifidobacterium*-monoassociated mice with germ-free mice in this investigation of intestinal immune responses, because the intestinal tissues of germ-free mice are undeveloped. Consequently, mice associated with SFB and clostridia were set up to be the control group. It has previously been reported that SFB and clostridia promote the development of both intestinal intraepithelial lymphocytes (IEL) and IgA producing cells in the small intestine and of IEL only in the large intestine (7, 8). We confirmed that the shape of PP and the number of PP cells in the mice associated with SFB and clostridia were very similar to that of conventional mice(6). We selected and used *Bifidobacterium pseudocatenulatum* 7041 in this study and the mice associated with *B. pseudocatenulatum* 7041 and SFB and clostridia were defined as the BIF group. It has been reported that *B. pseudocatenulatum* 7041 have a bacterial component which exhibits very strong mitogenic activity for murine splenocytes and PP cells (9). Thus, this *Bifidobacterium* strain enables us to easily evaluate the effects of *Bifidobacterium* on intestinal immune systems.

To examine the effects of *Bifidobacterium* on the antigen-specific cytokine responses in intestinal immune systems, SP, PP and LP cells from control and BIF mice were cultured with OVA and cytokine secretion was measured by ELISA.

Table 1. Production of IFN-γ and IL-6 by PP cells co-cultured with OVA

OVA stimulation	IFN-γ secretion		IL-6 secretion	
(μg/ml)	cont	BIF	cont	BIF
0	N.D	N.D	N.D	N.D
50	+	N.D	+	N.D
500	+++	+	+++	+

PP cells from control or BIF mice were co-cultured with 0, 50, or 500 μg/ml OVA. IFN-γ and IL-6 secreted in the culture supernatants were measured by ELISA. Secretion of cytokines was judged based on the results of two or more individual experiments.

In SP cells as the tissue of the systemic immune system, BIF mice produced IFN-γ and IL-6 levels as high as those seen in control mice. PPs are supposed to be the inductive site of the intestinal immune system, and the LP is the effector site. PP cells in BIF mice produced lower levels of IFN-γ and IL-6 than those of control mice. However, IL-4 and IL-5 production by PP cells in both control and BIF mice was not detected. LP cells in BIF mice also produced lower levels of IFN-γ and IL-6 compared with those of control mice. We previously confirmed that PP and LP cells of GF mice responded to OVA with higher IFN-γ and IL-6 production than those of CV mice(6). Thus, we found that the patterns of IFN-γ and IL-6 production in BIF mice were very similar to those of CV mice when compared with GF mice.

The difference in responsiveness to OVA between CV mice and GF mice implies that CV mice have a higher ability to regulate their responses to specific antigens compared with GF mice. In our results, PP and LP cells in BIF mice responded to OVA with lower cytokine production compared with control mice, and cytokine responses to OVA in BIF mice were very similar to CV mice. These results thus suggest that colonization by *Bifidobacterium* may contribute to the functional development of intestinal immune systems, and may have a role in the downregulation of hyperresponsiveness to specific antigens in intestinal immunity, although SFB and clostridia may affect the development of intestinal immune tissues.

Table 2. Production of IFN-γ and IL-6 by LP cells co-cultured with OVA

OVA stimulation	IFN-γ secretion		IL-6 secretion	
(μg/ml)	cont	BIF	cont	BIF
0	N.D	N.D	++	+
50	+	+	++	+
500	+++	++	+++	+

LP cells from control and BIF mice were co-cultured with 0, 50, or 500 μg/ml OVA. IFN-γ and IL-6 secreted in the culture supernatants were measured by ELISA. The level of secretion of cytokines was judged based on the results of two or more separate experiments.

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5. REFERENCES

1. Qiao, H., L. C. Duffy, E. Griffiths, D. Dryja, A. Leavens, J. Rossman, G. Rich, M. Riepenhoff-Talty, and M. Locniskar. 2002. Immune responses in rhesus rotavirus-challenged BALB/c mice treated with bifidobacteria and prebiotic supplements. *Pediatr Res 51:750.*
2. Shu, Q., and H. S. Gill. 2001. A dietary probiotic (Bifidobacterium lactis HN019) reduces the severity of Escherichia coli O157:H7 infection in mice. *Med Microbiol Immunol (Berl) 189:147.*
3. Yasui, H., J. Kiyoshima, and H. Ushijima. 1995. Passive protection against rotavirus-induced diarrhea of mouse pups born to and nursed by dams fed Bifidobacterium breve YIT4064. *J Infect Dis 172:403.*
4. Bjorksten, B., P. Naaber, E. Sepp, and M. Mikelsaar. 1999. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy 29:342.*
5. Sudo, N., S. Sawamura, K. Tanaka, Y. Aiba, C. Kubo, and Y. Koga. 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol 159:1739.*
6. M. Fujioka, S. Hachimura, A. Hosono, R. Nakamura, K. Hirayama, K. Itoh, and S. Kaminogawa. 2002. Establishment and analysis of germfree T cell receptor transgenic mice. *Animal Cell Technology: Basic & Applied Aspects 13:243.*

7. Umesaki, Y., H. Setoyama, S. Matsumoto, A. Imaoka, and K. Itoh. 1999. Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system. *Infect Immun 67:3504.*
8. Umesaki, Y., Y. Okada, S. Matsumoto, A. Imaoka, and H. Setoyama. 1995. Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol Immunol 39:555.*
9. Hosono, A., J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, and S. Kaminogawa. 1997. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from Bifidobacterium adolescentis M101-4. *Biosci Biotechnol Biochem 61:312.*

THE ROLE OF $CD4^+$ T CELLS IN IGA PRODUCTION IN MURINE PEYER'S PATCHES FOLLOWING ORAL FEEDING OF *BIFIDOBACTERIUM* COMPONENTS

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Abstract: Immunomodulatory effects, especially in murine Peyer's patch (PP) cells, were demonstrated following oral administration of *Bifidobacterium* immunomodulators (BIM) to BALB/c mice for 7 consecutive days. The BIM was derived from sonicated *B. pseudocatenulatum* 7041. We previously demonstrated that BIM administration augmented total IgA production including BIM-specific IgA by PP cells and enhanced the secretion of interferon-γ (IFN-γ), interleukin-5 (IL-5), IL-6 and IL-12 by the PP cells. In the present study, the immunoresponses of $CD4^+$ PP T-cells have been characterized to elucidate the influences of oral feeding of BIM. Also, we examined how $CD4^+$ PP T cells induced IgA production following oral BIM administration. After 7 days of oral administration of BIM, PP cells were obtained from each experimental mouse and $CD4^+$ T-cells were isolated using a magnetically activated cell sorting system. The expression of cell-surface antigens on $CD4^+$ PP cells was analyzed by flow cytometry. $CD4^+$ T cells were co-cultured with BIM in the presence of antigen-presenting cells and the cytokine secretion and IgA production were then measured by ELISA. On $CD4^+$ PP T-cells from BIM-fed mice, the expression of $CD45RB^{high}$, a naïve marker, showed significant enhancement compared with controls. On the other hands, the secretion of cytokines (IFN-γ, IL-6) by $CD4^+$ PP T-cells was increased by BIM administration. In addition, these cells increased BIM-specific IgA production but did not induce an increase in the total IgA production.

Key words: *Bifidobacterium*; Peyer's patch; IgA; $CD4^+$ T cells.

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1. INTRODUCTION

The intestinal microflora is composed of a large number of anaerobic and aerobic bacteria, the makeup of which is changed by aging, diet, stress, and other factors. The intestinal bacteria are supposed to play a crucial role in homeostasis and host defenses through their action as immunomodulators of innate and acquired immune responses. These are harmful bacteria for human health. Recently, "probiotic" bacteria have attracted considerable attention. They are defined as live microbes ingested as food ingredients with the expectation of beneficial effects on our health. Some probiotic bacteria have been reported to have immunopotentiating activity, anti-tumor effects, or anti-allergic effects. In addition, live Gram-positive bacteria such as members of the *Bifidobacterium* or *Lactobacillus* families, as well as some components derived from these bacteria, have been shown to be effective in the prevention of allergy and cancer. However, the immunological mechanisms responsible for the actions of probiotic bacteria have not been clarified in detail. In this study, we have investigated the influence of oral administration of *Bifidobacterium* immunomodulator (BIM) on mucosal immune responses in the intestine. BIM was prepared by sonication of *Bifidobacterium pseudocatenulatum* 7041 derived from human intestinal microflora. We have previously confirmed its strong mitogenic activity on murine lymphocytes [1]. This activity was increased by disruption of the cells, which is perhaps due to the fact that this strain contains water-soluble immunoactive polysaccharides [2, 3]. Therefore, BIM was used to investigate the immunomodulatory effect on mucosal immune responses following oral administration of *Bifidobacterium* components.

2. MATERIALS AND METHODS

Animals. Female 6-week-old BALB/c mice were obtained from Clea Japan (Tokyo, Japan) and were housed in a room, with a 12h light-dark cycle. The mice were naturalized and given MF diet (Oriental Yeast, Tokyo, Japan) before experiments for 3 days. All mice were kept in accordance with the Nihon University guidelines for care of laboratory animals.

Preparation of Bifidobacterium *immunnomodulator (BIM) derived from sonicated* B. pseudocatenulatum *7041, and oral administration of BIM.* Sonicated *B. pseudo catenulatum* 7041 was prepared by the method described in the previous report [4]. Mice were orally administered a dose of 10 mg/day of BIM in saline by using a feeding-tube for 7 days. The mice of control group were given saline by the same feeding of tubing. Mice were allowed free access to a pelleted MF diet and sterile deionized water throughout the experimental period.

Preparation of $CD4^+$ T cells from PP, and APC from splenocytes. After 7 days oral administration of BIM, PP cells were obtained from each experimental group, and $CD4^+$

T cells were isolated by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-mouse CD4-conjugated magnetic microbeads and an LS column following the manufacturer's instructions. APC derived from splenocytes of BALB/c mice, which had no experimental feeding. The cell suspensions of splenocytes were treated with 50 μg/ml mitomycin C (Sigma, St. Louis, MO).

Flow cytometric Analysis. Flow cytometric analysis of $CD4^+$ cells was performed using FACScalibur flow cytomerter (Becton Dickinson, San Jose, CA) and staining with anti-TCRαβ-biotin, anti-CD4-FITC, and either PE-, anti-CD45RB, anti-CD44, or anti-CD69. Fc receptors (FcγRIII/II) were blocked using anti-mouse CD16/CD32. The above Abs purchased from BD PharMingen. Isotype-matched negative controls were included in the analysis of the cells suspensions. Analysis was done on the Cellquest software. Purity $CD4^+$ cells from PP were >93% pure (data not shown).

Preparation of insensitive Thy1.2$^-$ PP cells. Thy1.2 (CD90) negative PP cells were isolated by MACS with anti-mouse CD90-conjugated magnetic microbeads and LS column. Insensitive Thy1.2$^-$ PP cells derived from PP of BALB/c mice, which had not been given the experimental diet. The isolation technique were as identified above. We checked freedom from CD90 expression cells in Thy1.2$^-$ PP cells by FACS (data not shown).

Measurement of total IgA. Total IgA in the culture supernatant was measured by sandwich ELISA. PP cells given the experimental groups were plated on a 48- well plate at 2.5×10^6 cells, and co-culture with 0, 10, 50 μg/ml BIM in RPMI 1640 medium containing 5% fetal bovine serum. $CD4^+$ T cells from PP from experimental mice (1×10^6 cells/well) in a total volume of 1 ml were cultured with similar dose of BIM in the presence of insensitive Thy1.2$^-$ PP (4×10^6 cells/well) cells from not experimental mice in a 48-well plate. The culture supernatants were collected after 5-7days for measurement of total IgA. The amounts of total IgA in the supernatants were measured by means of a sandwich ELISA method described as a previous report [4].

Measurement of BIM-specific IgA. BIM-specific IgA was measured by ELISA. Plates were coated with 50 μl of 100 μg/ml BIM. Subsequence handling followed as described above. BIM-specific IgA was qualitatively determined by absorbance at 405 nm.

Culture and cytokine determinants. $CD4^+$ T cells from PP cells from experimental mice (2×10^6 cells/well) in a total volume of 1 ml were cultured with 0, 10, and 50 μg/ml BIM in the presence of APC derived splenocytes (8×10^6 cells/well) in RPMI 1640 medium containing 5% FCS in a 48-well plate. The culture supernatants were collected after 24 or 72h and assayed for IL-12 p40 and for IL-5, IL-6, and IFN-γ, respectively. The amounts of IL-5, IL-6, and IFN-γ in the supernatants were measured by means of a sandwich ELISA method described as a previous report [4]. IL-12 measured using OptEIA mouse IL-12 (p40) set (BD PharMingen).

Statistical analysis. Data are expressed as means±SD. Differences were examined by one-way analysis of variance (ANOVA), and significant differences found between groups were further evaluated by Tukey's test (SPSS Ver. 10.0, Chicago, IL, USA). Differences were considered significant at $P<0.05$.

3. RESULTS

Total IgA production and BIM-specific IgA of PP cells after BIM administration

There was no significant increase body weight gain among the experimental groups (data not shown). We examined total IgA production by PP cells derived from the experimental mice during a 7-days primary cells culture with different dose of BIM. PP cells had been prepared from the experimental mice. The total IgA production by PP cells of BIM-fed groups was higher than that of control groups all of dose of BIM in vitro. In addition, BIM-specific IgA production by PP cells was enhanced by oral administration of BIM.

Table 1. Effects of oral administration of BIM on total IgA production from murine PP.

BIM stimulation (μg/ml)	Cont.	BIM-fed.
	Total IgA production	
0	±	++
10	+	++
50	+	++
	BIM-spe IgA production	
0	±	±
10	+	++
50	+	++

PP cells were obtained and pooled for each experimental group respectively, and then the cells were cultured with 0-50 μg/ml of BIM for 7 days. Total IgA in the culture supernatants was measured by ELISA. BIM-specific IgA in the culture supernatants was qualitatively measured by ELISA. ++,+, ± was judged based on the results on more than 3 experiments.

PP cells were obtained and pooled for each experimental group respectively, and then the cells were cultured with 0-50 μg/ml of BIM for 7 days. Total IgA in the culture supernatants was measured by ELISA. BIM-specific IgA in the culture supernatants was qualitatively measured by ELISA. ++, + , ± was judged based on the results on more than 3 experiments.

Characteristic $CD4^+$ T cells induced by oral administration of BIM.

We examined the cytokine production patterns of $CD4^+$ T cells derived from PP cells, which were obtained from the oral administrated mice with or without BIM. The prepared $CD4^+$ T cells were cultured with BIM in the presence of APC from naïve mice splenocytes in vitro, and the amounts of cytokine in the supernatants were measured. Both of IFN-γ and IL-6 secretion enhanced by administered BIM orally (data not shown). But, the secretion of IL-4 and IL-5 were not detected. To examine the effects of BIM feeding on the state of $CD4^+$ PP T cells derived from experimental mice, we analyzed

purified $CD4^+$ PP cells by flow cytometic analysis. Oral administration of BIM increased naïve-marker $CD4^+CD45RB^{high}$ expression cells (cont. 49.6±2.4, BIM-fed. 57.9±6.1). In contrast, activated-marker CD44 or CD69 were not significant difference from control (data not shown).

BIM-affected $CD4^+$ T cells enhanced BIM-specific IgA but not induced total IgA.

We tried to demonstrate whether PP $CD4^+$ T cells, which derived from BIM administered mice, induced IgA production in PP cells or not. Then, we prepared $CD4^+$ T cells derived from BIM or control diet group, and the $CD4^+$ T cells were cultured with insensitive $Thy1.2^-$ PP cells from non-experimental mice in the presence of BIM respectively. Total IgA production of the culture soup showed not difference from control group. While on the other hand, BIM-specific IgA level in culture from BIM-fed group was higher than that from control group.

Table 2. Effects of activated $CD4^+$ T cells by BIM administered on IgA production.

BIM stimulation (μg/ml)	Cont.	BIM-fed.
	Total IgA	
0	±	±
10	+	+
50	±	±
	BIM-spe IgA	
0	±	±
10	+	+++
50	±	++

PP CD4 T^+cells were obtained and pooled for each experimental group respectively, then the cells were co-cultured with 0-50 μg/ml BIM in the presence of insensitive Thy1.2 PP cells from not experimental mice for 7 days. Total IgA in the culture supernatants was measured by ELISA. BIM-specific IgA in the culture supernatants was qualitatively measured by ELISA. ++, +, ± was judged based on the results on more than 3 experiments.

4. DISCUSSION

We examined whether sonicated Bifidus components that have strong mitogenic activity influenced the ability of PP cells to produce IgA. We elucidate BIM administration up-regulated proliferation activity of PP cells *in vitro* (data not shown). These results indicate that orally administered BIM was taken up by M cells on the PP and then activated PP cells. We demonstrated that BIM-feeding enhanced cytokine

production, such as IFN-γ, IL-5, IL-6, and IL-12, in the PP cells (data not shown). We also observed that both total IgA and BIM-specific IgA production in the PP derived from BIM-fed animals was higher than that of control group. Additionally, our results show that secretion of both IFN-γ and IL-6 by $CD4^+$ T cells were increased by BIM administration. Our results suggest that BIM-feeding induce increased cytokine production in PP cells including $CD4^+$ T cells, which enhanced both total IgA and BIM-specific IgA production in the PP. Consequently, when BIM-affected $CD4^+$ T cells cultured with insensitive $Thy1.2^-$ PP cells from not experimental mice, BIM-specific IgA was increased. These results suggest that $CD4^+$ T cells play an important role in induction of IgA production by BIM-feeding in the PP.

5. REFERENCES

[1] J. Lee, A. Ametani, A. Enomoto, Y. Sato, H. Motosima, F. Ike, S Kaminogawa, Screening for the immunopotentiating activity of the immune response by Bifidobacterium adolescentis M101-4, Biosci. Biotech. Ciochem. 57 (1993) 2127-2132.

[2] A. Hosono, J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, S. Kaminogawa, Comparison of the Immunopotentiating Activity with Structural Characteristics among Water-soluble Polysaccharides Isolated from the Genus Bifidobacterium, Bioscience Microflora. 17 (1998) 97-104.

[3] A. Hosono, J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, S. Kaminogawa, Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from Bifidobacterium adolescentis M101-4, Biosci Biotechnol Biochem. 61 (1997) 312-316.

[4] A. Hosono, A. Ozawa, R. Kato, Y. Ohnishi, Y. Nakanishi, T. Kimura, R. Nakamura, Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells, Biosci Biotechnol Biochem. 67 (2003) 758-764.

MODULATION OF CYTOKINE AND IMMUNOGLOBLIN A RELEASE BY BETA-(1,3-1,6)-GLUCAN FROM *AUREOBASIDIUM PULLULANS* STRAIN 1A1

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Abstract: Beta-glucan, derived from mushroom or yeast extracts, is well known for its various immunopharmacological effects such as anti-microbial properties and anti-tumor activities for host defense. We isolated β-glucans (DS-βG), secreted by the fungus body, from the culture medium of *Aureobasidium pullulans* strain 1A1. DS-βG form soluble microparticles and we prepared DS-βG to 85-95% purity. The structure of DS-βG is based on a backbone of β-(1,3)-linked β-D-glucopyranosyl units, with β-(1,6) linked side-chains of varying distribution. Their molecular weight range is 50,000-300,000, with the average estimated to be 100,000. In this study, we investigated the immunomodulatory effects of DS-βG, especially its *in vitro* and *in vivo* effects on the intestinal immune system. Peyer's patches (PP) cells from BALB/cA mice were cultured with DS-βG (0~200μg/ml) and IgA and cytokine levels in culture supernatant measured by ELISA. The addition of DS-βG induced IgA production in a dose-dependent manner. Both the levels of interleukin-5 (IL-5) and interleukin-6 (IL-6), cytokines known to enhance IgA production, were also elevated. Oral administration of DS-βG (400μg/mouse/day) for 7 consecutive days induced IgA production by PP cells. These results demonstrate that DS-βG acts on the gut immune system and increase IgA production that is vitally important for defense against infection.

Key words: β-(1,3-1,6)-glucan; *Aureobasidium pullulans*; immunomodulation

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1. INTRODUCTION

Many kinds of fungi, yeasts and plants have immunomodulatory effects such as anti-microbial and anti-tumor activities important for host defense. These have been used clinically, as in the cases of lentinan (from *Lentinus edodes*), shyzophillan (from *Schizophyllum commune*) and Krestin (from *Coriolus versicolor*) for example (Adachi et al., 1994). Schizophyllan shows anti-tumor activity against sarcoma180, sarcoma37 etc (Hobbs, 1995). Lentinan significantly increased macrophage cytotoxicity *in vivo* when injected subcutaneously or intraperitoneally (Hamuro et al., 1980, Ladányi et al., 1993). β-glucan is known to be the major active constituent responsible for eliciting the immune responses of these molecules. It is an indigestive polysaccharide in humans. In light of these facts, it is speculated that β-glucans may modulate mucosal immunity in the intestinal tract.

Recent analysis of the response of leukocytes to β-glucan has shown that the integrin CR3 or dectin-1 receptor is required for its binding to cells (Xia et al., 1999, Brown et al., 2001, Thornton et al., 1996). β-glucans are known to act as immunostimulants, enhancing the activities of leukocytes, especially macrophages and natural killer cells (Konopski et al., 1991; Onderdonk et al., 1992). However, the mechanism of their immunomodulatory activities is not completely understood. The various physiological functions of different β-glucans have not been well clarified because of differences in their origin, the bonding pattern, the molecular weight and/or the size of the particles that contain the β-glucans (Janelle et al., 1999). In addition, the purification of β-glucan is difficult and few studies have investigated immune responses to purified β-glucan.

In this study, we isolated the original and purified β-glucan (DS-βG) from the culture of *Aureobasidium pullulans* strain 1A1 by fermentation methods, and determined the possibility to supplying them in large amounts (through Daiso, Osaka, Japan). DS-βG is a soluble microparticle glucan of low-molecular mass and with 50-80% (β-1,6/β-1,3) branches. Additionally, we prepared DS-βG to around 85-95% purity. We investigated cellular immune responses to DS-βG, including cytokine and immunoglobulin A production in the mouse intestinal tract immune system.

2. MATERIALS AND METHODS

2.1 Beta-glucan

Beta-(1,3-1,6)-linked glucan (DS-βG) was received from DAISO CO., LTD. (Osaka, Japan). The β-glucan was prepared by Daiso Co., Ltd. as follows: The glucan was supplied as a soluble microparticle glucan obtained from the culture medium of *Aureobasidium pullulans* strain 1A1, grown in Czapek medium containing 3% sucrose as the sole carbon source and 0.3% ascorbic acid, sodium salt, under aerobic fermentation conditions for 3-7 days at 27°C. The highly purified β-glucan (85-95% in purity) was prepared by treatments that reduced viscosity by under-stirring with sodium hydrate (pH 12). The cell filtrate from the alkali-treated culture was filter pressed and low molecular weight substances and salts removed by ultrafiltration (UF membrane, NITTO DENKO CORPORATION). The ultrafiltered supernatant was adjusted to pH 3.5 with citric acid and filtered through a 0.8μm membrane. The resulting solution was used as the purified β-glucan sample. In some case, the β-glucan was precipitated with ethanol (more than 70%), freeze-dried, and dissolved in sterile saline.

The amount of total saccharide was measured via the phenol-sulfuric acid method (Hodge, J. E. et al. 1962). The amount of poly-saccharide was determined after the recovery of polysaccharides by ethanol precipitation, as described above. The molecular weight was measured by gel filtration chromatography at pH 12 (TOYO PEARL HW-650; TOSOH CORPORATION, Tokyo, Japan), with pullulan (β-1,3-glucan) as a marker of molecular mass (5,900-1,600,000). The structure was determined by C-Hcosy-NMR spectra and the analysis of reaction products by β-1,3-glucanase (kitalase, K-I CHEMICAL INDUSTRY CO., LTD., Shizuoka, Japan) processing (Hamada et al. 1983). The size of the β-glucan microparticles was estimated by the measurement of particle size distribution by light scattering.

2.2 Animals

BALB/cA (6-8 weeks old) mice were obtained from Clea Japan (Tokyo, Japan) and housed in a room at 23-25°C in a humidified atmosphere with a 12 h light-dark cycle. Mice were fed ad libitum on a routine pelleted diet (MF, Oriental Yeast, Tokyo, Japan).

2.3 Cells and cell culture

PP and SPL cells were excised and isolated from BALB/cA mice. These immune tissue cells were prepared with Collagenase D (Roche, Mannheim, Germany) and Dnase I (Roche). Both cell types were cultured in RPMI 1640 medium (NISSUI Pharmaceutical CO., LTD., Tokyo, Japan) containing 5% heat-inactivated FBS (MP Biomedicals,

Inc, Irvine, CA), 100U/ml penicillin (BANYU Pharmaceutical CO., LTD., Tokyo, Japan), 100μg/ml streptomycin (MEIJI SEIKA KAISHA, LTD., Tokyo, Japan), 3mM L-glutamine, and 50μM 2-ME for the indicated times at 37°C in a humidified atmosphere containing 5% CO_2.

2.4 Measurement of cytokine and IgA production *in vitro*

PP cells and splenocytes were prepared from 6-8 weeks old BALB/c mice. Mouse immune tissue cells (2.5×10^6cells/well in a total volume of 1ml) were cultured with 50-200μg/ml DS-βG, 20μg/ml Lipopolysaccharide (LPS; from Esherichia coli O55:B5) or 100μg/ml zymosan (Sigma, St. Louis, MO) in 48-well flat-bottomed plates. Control cultures were incubated in base medium alone. Culture supernatants were collected after 72 h and assayed for IL-5, IL-6, IL-12(p40/p70) and IFN-γ levels. At the same time, mouse immune tissue cells (2.0×10^5cells/well in a total volume of 200μl) were cultured with mitogens in 96-well flat-bottomed plates and supernatants collected after 1 week and assayed for IgA production. Cytokine and IgA levels were measured by sandwich ELISA.

2.5 Measurement of cell proliferation *in vitro*

PP cells and splenocytes (2.0×10^5cells/well in a total volume of 200μl) were cultured with 50-200μg/ml DS-βG, 20μg/ml LPS or 100μg/ml zymosan in 96-well flat-bottomed plates. Control cultures were incubated in base medium alone. After 40 h incubation, 20μl of CellTiter 96® AQueous One Solution Reagent (Promega Corporation, Madison, WI) was added to each well and the plate incubated for 3 h. Total cell count was measured by the absorbance of light at 490 nm via a microplate reader. Relative proliferation activity was quantified by comparison with control cultures.

2.6 Determination of cytokine and IgA levels by ELISA

Ninety-six-well plate (Immuno Plate; Nunc, Inter-Med, Denmark) were coated with relevant purified anti-mouse cytokine anti-bodies (BD Bioscienses Pharmingen, San Jose, CA) or goat anti-mouse IgA (MP Biomedicals Inc., Irvine, CA) in 0.1M $NaHCO_3$ (pH 8.4) or 0.1M Na_2HPO_4 (pH 9.0) and incubated overnight at 4°C. Wells were washed with phosphate buffered saline (PBS) buffer containing 0.05% Tween-20 (PBS-T). Uncoated binding sites in the wells were blocked with PBS containing 1% bovine serum albumin (Sigma) at room temperature for 2 h.Wells were washed with PBS-T and then either standard recombinant cytokines or IgA dilutions or culture supernatant samples in PBS-T were added to the wells and incubated overnight at 4°C. Wells were then washed and relevant biotinylated anti-mouse cytokine antibodies (Pharmingen) or IgA antibody (Sigma) in PBS-T added and incubated at

room temperature for 2 h. Wells were washed and streptavidin-alkaline phosphatase (Invitrogen Corp., Carlsbad, CA) in PBS-T added and incubated at room temperature for 1 h. Plates were washed and the substrate solutions added (i.e. *p*-nitrophenyl phosphate) and incubated at 37°C for about 30 minutes. The absorbance of each well at 405 nm was measured using an automated spectrophotometer (microplate reader, BIO-RAD, Hercules, CA). Cytokines and IgA concentrations in culture supernatants were calculated from the standard curves produced by dilutions of the recombinant standard.

2.7 Statistical analyses

Results are expressed as means + S.E.M. and were compared using the Tukey test. Differences between control and stimulation groups were considered statistically significant at $p < 0.05$*, $p < 0.01$** or $p < 0.001$***.

3. RESULTS AND DISCUSSION

Beta-(1,3-1,6)-linked glucan (DS-βG) was prepared via specific treatments to reduce its viscosity, improve filtration and recovery. The molecular mass of DS-βG was determined to be between 5.0×10^4 and 3.0×10^5 by gel filtration chromatography, and the average mass approximately 1.0×10^5. The structure consisted of a main chain of β-(1,3)-linked β-D-glucose with β-(1,6)-linked side-chains. The integral ratio of branches of β-1,6 for β-1,3 was estimated to be 50-80% from NMR and emzymatic analyses. Finally, DS-βG was prepared to 85 to 95% purity (Suzuki, T. 2005, Iizuka, M. 2002).

To investigate the effects of DS-βG on mouse immune tissue, cell proliferation, cytokine and IgA production were assessed *in vitro* following stimulation with 0-200μg/ml of DS-βG. As a positive control, LPS or zymosan were simultaneously added to the cultured immune cells. DS-βG stimulated cell proliferation of both PP cells and splenocytes in a dose-dependent manner (Table 1). DS-βG also stimulated IL-5 or IL-6 production by cultured PP cells in a dose-dependent manner (Table 2). A similar result was obtained for IgA production (Table 2). The culture supernatant from DS-βG-treated PP cells showed a significantly higher amount of IL-5, IL-6 and IgA comparable to those from zymosan-treated cultures at the same concentration (100μg/ml) (Table 2). As such, it appears that highly purified DS-βG causes a more effective immune response than zymosan. IL-12(p40/p70) and IFN-γ production were also stimulated by DS-βG (data not shown). These results clearly show that DS-βG stimulates PP

cells to produce cytokines or IgA *in vitro*. In addition, DS-βG stimulated IL-6 production by cultured splenocytes in a dose-dependent manner (data not shown).

We then postulated whether oral administration of DS-βG could stimulate IgA production in PP cells and splenocytes. IgA production was increased in PP cells from mice given DS-βG in comparison to the control group with results for both groups being almost the same (data not shown). IgA production by splenocytes from both groups was not significantly different (data not shown).

Table 1. Effects of DS-βG on cell proliferation by Peyer's patches cells and splenocytes of BALB/cA mice *in vitro*.

		Relative activities	
	(μg/ml)	PP	SPL
Control		1.000	1.000
DS-βG	50	1.523 ± 0.035***	1.243 ± 0.033**
DS-βG	100	1.636 ± 0.086***	1.373 ± 0.026***
DS-βG	200	1.784± 0.018***	1.652 ± 0.048***
LPS	20	2.396 ± 0.072***	1.795 ± 0.023***
Zymosan	100	0.889 ± 0.004	0.811 ± 0.027

PP cells and splenocytes were stimulated with DS-βG (50-200μg/ml), LPS (20μg/ml) or zymosan (100μg/ml). Control cultures were incubated in culture medium with sterile saline/diluent added. Cell proliferation levels show relative activity compared with control cultures as baseline. The results represent the mean ± S.E.M. of triplicate independent assays. Statistically significant differences are shown as $p < 0.01$**, $p < 0.001$***.

Table 2. Effects of DS-βG on IL-5, IL-6 and IgA productions by Peyer's patches cells from BALB/cA mice *in vitro*.

		Cytokine and IgA production (ng/ml)		
	(μg/ml)	IL-5	IL-6	IgA
Control		n.d. (+/−)	+/−	+/−
DS-βG	50	n.d.	+	+
DS-βG	100	+	+	++
DS-βG	200	+	++	+++
LPS	20	n.d.	++	+
Zymosan	100	n.d.	−	−

PP cells were stimulated with DS-βG (50-200μg/ml), LPS (20μg/ml) or zymosan (100μg/ml). Control cultures were incubated in culture medium with sterile saline/diluent added. Supernatant cytokine and IgA levels were measured by ELISA. The results represent + / − (control), − (decreased), + (increased), ++ (increased, $p < 0.05$) and +++ (increased, $p < 0.01$). Statistically significant differences are shown as $p < 0.05$*, $p < 0.01$**.

In this study, we propose that DS-βG stimulates mucosal immune tissues, such as PP. IL-5 and IL-6 production was enhanced in PP cells

stimulated by DS-βG *in vitro*. IL-5 and IL-6 are known to further enhance IgA production. IgA production was enhanced in PP cells by DS-βG *in vitro*. Therefore, we suggest that DS-βG may prevent infection by pathogens through interactions with the intestinal immune system. In addition, IFN-γ production was enhanced in cultured PP cells by DS-βG. This result suggests that elimination of intracellular pathogens may also be effectively induced. We here suggest that DS-βG up-regulates intestinal immune responses and is available as a health food material.

4. REFERENCES

Adachi, Y., Okazaki, M., Ohno, N., and Yadomae, T. (1994) Enhancement of cytokine production by macrophages stimulated with (1→3)-β-D-glucan, grifolan (GRN), isolated from *Grifola frondosa*. Biol. Pharm. Bull. 12, 1554-1560.

Brown, G.D. and Gordon, S. (2001) Immune recognition. A new receptor for beta-glucans. Nature 413, 36-37.

Hamada, N., and Tsujisaka, Y. (1983) The structure of the carbohydrate moiety of an acidic polysaccharide produced by Aureobasidium sp. K-1. Agric. Biol. Chem. 47, 1167-1172.

Hobbs, C. (1995) Medicinal mushrooms: an exploration of tradition, healing and culture. Botanica Press, Santa Cruz, Calif.

Hodge, J. E. and Hofreiter, B. T. (1962) Method in Carbohydrate Chemistry 1, 338.

Iizuka, M. (2002) Great Development of Microorganisms (Imanaka, T. ed.) pp. 1012-1020, NTS Inc., Tokyo.

Janelle, A. C., Graham, E. K., and Alan, J. H. (1999) The effect of molecular weight and β-1,6-linkages on priming of macrophage function in mice by (1,3)-β-D-glucan. Immunol. Cell Biol. 77, 395-403.

Konopski, Z., Rasmussen, L.T., Seljelid, R., and Eskeland, T. (1991) Phagocytosis of beta-1,3-D-glucan-derivatized microbeads by mouse peritoneal macrophages involves three different receptors. Scand. J. Immunol. 33, 297-306.

Ladányi, A., T'már, J., Lapis, K. (1993) Effect of lentinan on macrophage cytotoxicity against metastatic tumor cells. Cancer Immunol. Immunother 36, 123-126.

Onderdonk, A.B., Cisneros, R.L., Hinkson, P., and Ostroff, G. (1992) Anti-infective effect of poly-beta 1-6-glucotriosyl-beta 1-3-glucopyranose glucan in vivo. Infect. Immun. 60, 1642-1647.

Suzuki, T. (2005) Food Style 21, vol.9, pp. 61-64, Food Chemicals Newspaper, Inc., Tokyo.

Xia, Y., Vetvicka, V., Yan, J., Hanikyrova, M., Mayadas, T., and Ross, G.D. (1999) The beta-glucan-binding lectin site of mouse CR3 (CD11b/CD18) and its function in generating a primed state of the receptor that mediates cytotoxic activation in response to iC3b-opsonized target cells. J. Immunol. 162, 2281-2290.

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In Vivo Immunopotentiating Effects of Cellular Components from *Lactococcus lactis* ssp. *lactis*

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Abstract Cellular components of *Lactococcus lactis* ssp. *lactis* (heat-killed whole cells, cytoplasm, and cell walls) were tested for their *in vivo* immunopotentiating activity. Peritoneal macrophages from mice orally administered with heat-killed whole cells exhibited significantly greater phagocytic activity than the groups administered with cell-wall fraction or cytoplasm fraction. The cytotoxicity of natural-killer cells was the highest in the group administered with whole cells, and the production of cytokines (IFN-γ, IL-2, and IL-12) in spleen cells was significantly higher, when cellular components were injected, and it tended to be higher in the cell-wall and cytoplasm groups than in the whole-cell group. Interestingly, the cytokine production of Peyer's patch cells was high, when cytoplasm fractions were administered. These results demonstrate that whole cells and cytoplasm and cell-wall fractions of *L. lactis* ssp. *lactis* have immunopotentiating activities, which are related to the stimulation of Peyer's patches.

Key words: Cytokine, immunopotentiating activity, *Lactococcus lactis* ssp. *lactis*, NK cell activity, phagocytic activity

The capacity of certain lactic acid bacteria (LAB) to function as probiotics, when ingested as a part of fermented dairy products or dietary adjuncts, is receiving increasing attention, and an extensive volume of literature on the possible health benefits associated with the consumption of LAB is now available [1, 5, 11, 13, 15]. The mechanisms underlying these favorable effects include changes in viable populations of microorganisms in the intestinal flora, competition for adhesion sites and nutrients between ingested bacteria and potential pathogens, production of antibacterial substances, and stimulation of the immune system. With regards immunity, Perdigon *et al.* [17] observed enhanced macrophage and lymphocyte activities in mice after administration of a mixed culture of *Lactobacillus acidophilus* and *Lb. casei*, and also reported that peritoneal macrophages in mice were activated by the oral administration of *Lb. casei* and *Lb. bulgaricus* [16, 18]. Similar results have been found for *Streptococcus thermophilus* and *Lb. acidophilus* orally administered [19], and heat-killed *Lb. casei* injected into mice [20]. The oral administration of LAB and fermented milk has been demonstrated to increase mitogenic responses [3]. These reports indicate that orally administered LAB and fermented milk stimulate the host's immune system. However, most reports on the immunopotentiating activity of LAB have focused on whole LAB cells and their peptidoglycans, with little attention being paid to the soluble fraction, although the potential in food applications is different between soluble and insoluble materials. We have recently described the *in vitro* immunopotentiating activity of the cellular component, containing the soluble fraction of *Lactococcus lactis* [12]. Those results indicated that *L. lactis* cytoplasm and cell-wall fractions as well as whole cells are capable of stimulating lymphocytes and macrophages to produce several cytokines. Based on these observations, we demonstrated the systemic and mucosal immune responses of mice administered orally with whole cells and cell-wall and cytoplasm fractions of *L. lactis* ssp. *lactis*.

L. lactis was cultured in M17 media (Difco, Detroit, MI, U.S.A.) for 18 h at 30°C. After cultivation, the cells were harvested in a refrigerated centrifuge (Vision, Seoul, South Korea), washed three times with distilled water, and lyophilized for storage. The lyophilized cells were resuspended

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