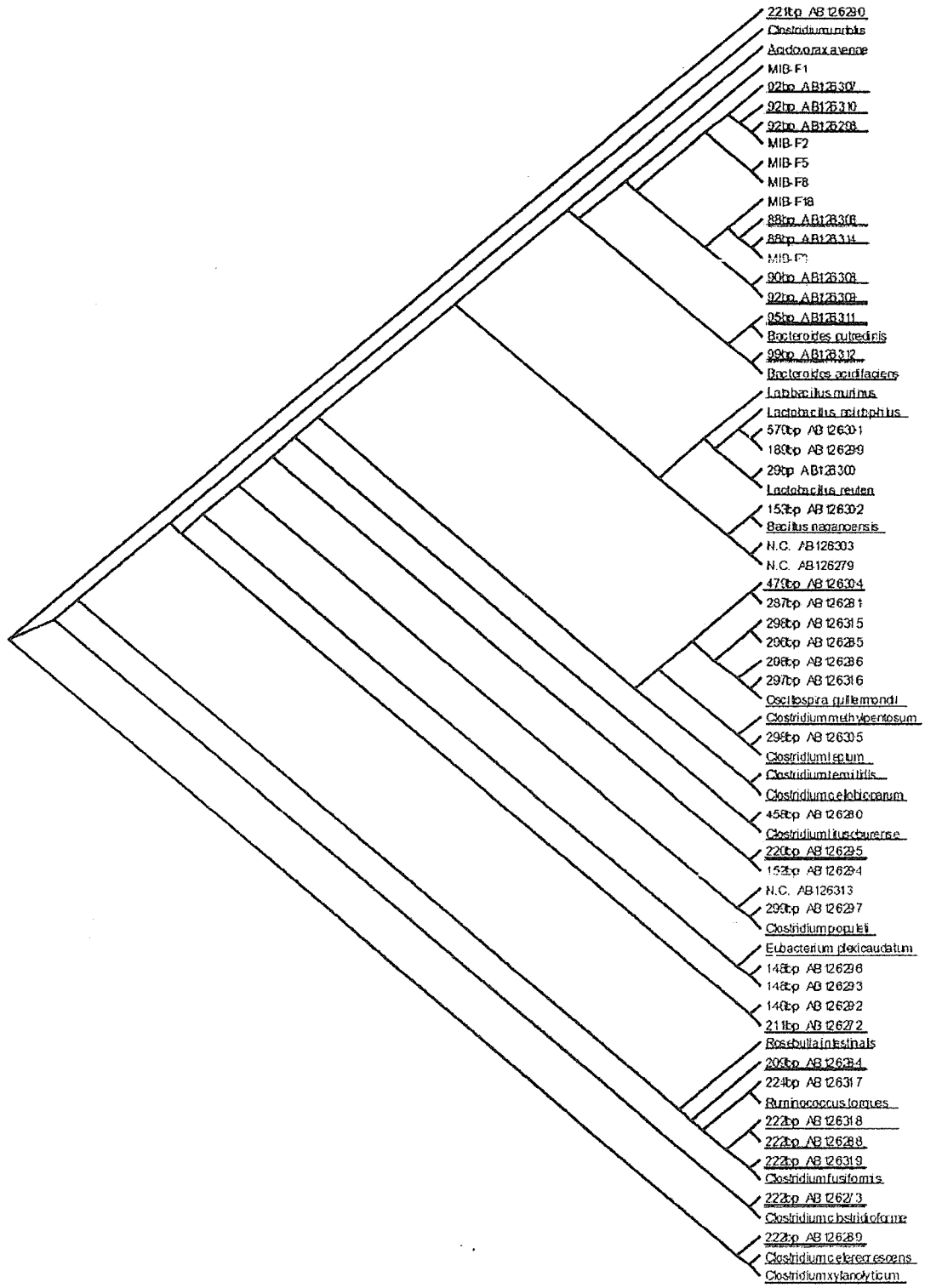

FIG. 1. Phylogenetic tree showing the relationships of obtained 16S rRNA partial genes in murine fecal microbiota with published sequences. The obtained 16S rRNA partial genes are displayed as accession numbers. The theoretical lengths of the T-RFs are also shown. The 16S rRNA partial genes with red underlining indicate candidate genes assigned to marker T-RFs at 83, 88, 93, and 95 bp. The genes with blue underlining and the gene with green underlining indicate candidate genes for marker T-RF at 215 bp and 475 bp, respectively.

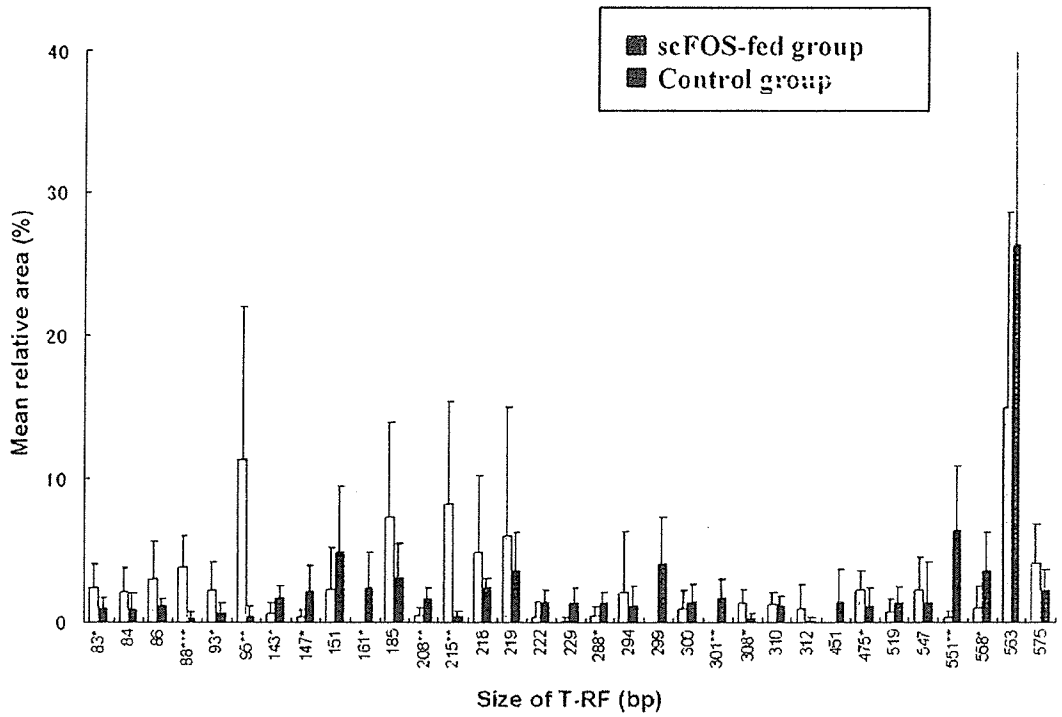

FIG. 2. Average T-RFLP profiles of the scFOS-fed group and control group at 5 weeks. Error bars indicate standard deviations of the mean relative areas. In the x axis of the figure, T-RF lengths are described. Asterisks next to the T-RF lengths indicate significance of difference of relative T-RF areas. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

rRNA partial genes are shown in Fig. 1. Also shown in Fig. 1 are the phylogenetic positions of these obtained 16S rRNA partial genes and those of known bacteria. Khan et al. indicated that the lengths of observed T-RFs were sometimes different from their theoretical lengths by 1 to 8 bp (9). In consideration of Khan et al.'s observation, the 16S rRNA partial genes, whose theoretical lengths were within ±8 bp of the lengths of the marker T-RFs, were selected as candidate 16S rRNA partial genes assigned to the marker T-RFs. We then attempted to assign the marker T-RFs to phylogenetic groups based on the phylogenetic position of the candidate 16S rRNA partial genes.

For marker T-RFs at 83, 88, 93, and 95 bp, we sought obtained 16S rRNA partial genes whose theoretical lengths were between 75 and 103 bp. Figure 1 shows that all 16S rRNA partial genes whose theoretical lengths were between 75 and 103 bp were similar to *Bacteroides* spp. or to the MIB group. The MIB group was found recently by Salzman et al. as a separate and still unrecognized branch of the *Bacteroides* group in the murine intestine (18). Therefore, we concluded that these marker T-RFs were derived from *Bacteroidetes*. For the marker T-RF at 215 bp, we sought the obtained 16S rRNA partial genes whose theoretical lengths were between 207 and 222 bp. Figure 1 shows that such 16S rRNA partial genes were scattered on the phylogenetic tree. For the marker T-RF at 475 bp, we sought the obtained 16S rRNA partial genes whose theoretical lengths were between 487 and 483 bp. Figure 1 shows that such 16S rRNA was only plasmid AB126304, which was also similar to some *Clostridium* spp.

Increase in T-RFs derived from *Bacteroidetes* after scFOS administration. As described above, four of six marker T-RFs were derived from *Bacteroidetes*. Another two T-RFs (at 84 and 86 bp), also derived from *Bacteroidetes*, were larger in the scFOS-fed group, although the differences were not significant. The total average relative areas of these six T-RFs derived from *Bacteroidetes* of the scFOS-fed group and the control group were 25.0% ± 12.5% and 4.1% ± 3.2% (Fig. 3), respectively. The difference between the total relative areas of the scFOS-fed group and the control group was significant ($P < 0.01$). These results indicated that the T-RFs derived from *Bacteroidetes* were consistently larger in the scFOS-fed group than in the control group; therefore, these T-RFs were considered good markers for murine intestinal microbiota change after scFOS administration. Figure 3 also shows that the total relative areas from *Bacteroidetes* of the scFOS-fed group were significantly larger than those at 0 weeks. On the other hand, the difference in total relative areas from *Bacteroidetes* between the control group and mouse at 0 weeks was not significant. These results suggest that scFOS administration significantly increased the total area of T-RFs from *Bacteroidetes*.

The 16S rRNA gene-based methods, including T-RFLP analysis, have inherent problems related to the quantification of bacterial populations (21). The amplification speeds of 16S rRNA genes in different bacteria genomes vary because of different operon number and PCR bias (21). Therefore, the quantity of PCR amplicons does not necessarily reflect the actual numbers of bacteria. Thus, the quantitative differences

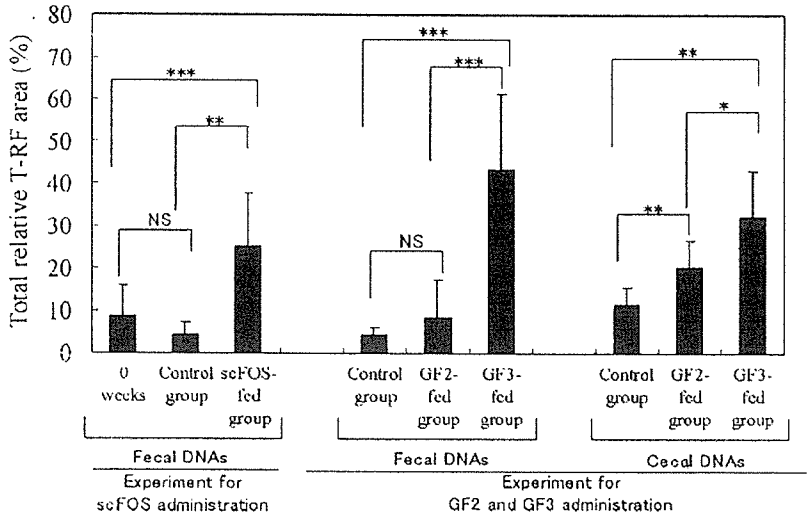

FIG. 3. Average total relative areas of T-RFs derived from *Bacteroidetes*. Average total relative areas of T-RFs derived from 16S rRNA genes of *Bacteroidetes* are shown. Error bars indicate standard deviations of the mean relative areas. Asterisks indicate significances of difference of relative T-RF areas. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. "NS" indicates that the difference was not significant.

in the relative areas of T-RFs between the scFOS-fed group and the control group do not necessarily reflect quantitative differences in their actual bacterial populations.

In this context, we preliminarily determined the relative amount of 16S rRNA genes of *Bacteroidetes* using real-time PCR with *Bacteroidetes*-specific primers to confirm that fecal samples of the scFOS-fed group had more *Bacteroidetes* bacteria than those from the control group. To confirm that the primers were specific for *Bacteroidetes*, we amplified the obtained 16S rRNA partial genes in plasmids with this primer set. All 16S rRNA partial genes which were similar to those of *Bacteroidetes*, with the exception of AB126311, were amplified. On the other hand, the other plasmids (AB126313, AB126301, AB126299, AB126300, AB126303, AB126302, AB126318, AB126316, and AB126302) were not amplified. Based on these results, the primers were judged to be specific to the *Bacteroidetes* bacteria found in mouse intestine.

Bacteroidetes bacteria in murine intestine have not been cultured so far (18). Therefore, DNA extracted from a known number of *Bacteroidetes* was not obtained. Thus, we quantified only a relative number of *Bacteroidetes* in mouse intestine. Real time PCR of fecal samples showed that the average amount of 16S rRNA genes derived from *Bacteroidetes* in the scFOS-fed group was 123 times more than that of the control group ($P < 0.01$). Therefore, as suggested by T-RFLP analysis, the fecal samples from the scFOS-fed group likely possessed more *Bacteroidetes* bacteria than the samples from the control group.

Effects of kestose and nystose administration on T-RFs derived from *Bacteroidetes*. The scFOS used in this study contained two major components, kestose (GF2) and nystose (GF3) (6). To compare the efficiency of the increase in T-RFs derived from *Bacteroidetes* between mice fed GF2 and GF3, BALB/c mice were fed a GF2 diet, a GF3 diet, or a control diet for 5 weeks, and then we determined the T-RFLP profile of the fecal DNA of each mouse. The total relative areas of the T-RFs derived from the 16S rRNA genes of *Bacteroidetes* are shown in Fig. 3. The results show that the total relative areas of T-RFs derived from *Bacteroidetes* were significantly larger in the GF3-fed group than in the GF2-fed group and the control group. These results suggest that GF3 is a more efficient component than GF2 to increase T-RFs derived from *Bacteroidetes* in fecal microbiota.

We also determined the T-RFLP profiles of cecal microbiota of mice fed a GF2 or a GF3 diet for 5 weeks. The total relative areas of the T-RFs derived from the 16S rRNA genes of *Bacteroidetes* are shown in Fig. 3. The results show that the total relative areas of T-RFs derived from *Bacteroidetes* were significantly larger in the GF3-fed group than in the GF2-fed group and the control group. These results suggest that GF3 is a more efficient component than GF2 to increase the T-RFs derived from *Bacteroidetes* in cecal microbiota as well as in fecal microbiota.

To our knowledge, this is the first report to describe markers of change in murine intestinal microbiota after scFOS administration. As described above, the T-RFLP is less suitable for quantification of an absolute number of intestinal bacteria than other methods, such as culturing, fluorescent in situ hybridization, and real-time PCR with internal standards. Therefore, the use of T-RFs as markers for diet evaluation could be limited. However, T-RF analysis could be a practical approach, as most mouse intestinal bacteria, including *Bacteroidetes*, are still uncultured. Also, T-RFLP is a high-throughput method. This feature should be preferable for determining changes in intestinal microbiota due to scFOS administration, since approximately 50 fecal samples need to be analyzed in even a small animal study. Therefore, we believe the results obtained here should contribute to our understanding of the mechanisms of physiological functions induced by scFOS administration in a murine model.

ACKNOWLEDGMENT

We thank Roy H. Doi (UC—Davis) for comments on the manuscript.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
2. **Clement, B. G., and C. L. Kitts.** 2000. Isolating PCR-quality DNA from human feces with a soil DNA kit. BioTechniques **28**:640–646.
3. **Felsenstein, J.** 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. **17**:368–376.
4. **Gibson, G. R., and M. B. Roberfroid.** 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. **125**:1401–1412.
5. **Hidaka, H., T. Eida, T. Takizawa, T. Tokunaga, and Y. Tashiro.** 1986. Effects of fructooligosaccharides on intestinal flora and human health. Bifidobacteria Microflora **5**:37–50.
6. **Hidaka, H., M. Hirayama, and N. Sumi.** 1988. A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC20611. Agric. Biol. Chem. **52**:1181–1187.
7. **Holton, T. A., and M. W. Graham.** 1991. A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. Nucleic Acids Res. **19**:1156.
8. **Hosono, A., A. Ozawa, R. Kato, Y. Ohnishi, Y. Nakanishi, T. Kimura, and R. Nakamura.** 2003. Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells. Biosci. Biotechnol. Biochem. **67**:758–764.
9. **Khan, A. A., M. S. Nawaz, L. Robertson, S. A. Khan, and C. E. Cerniglia.** 2001. Identification of predominant human and animal anaerobic intestinal bacterial species by terminal restriction fragment patterns (TRFPs): a rapid, PCR-based method. Mol. Cell. Probes **15**:349–355.
10. **Kibe, R., M. Sakamoto, H. Yokota, H. Ishikawa, Y. Aiba, Y. Koga, and Y. Benno.** 2005. Movement of fixation of intestinal microbiota after administration of human feces to germfree mice. Appl. Environ. Microbiol. **71**:3171–3178.
11. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrant and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York, N.Y.
12. **Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr., P. R. Saxman, R. J. Farris, G. M. Garrity, G. J. Olsen, T. M. Schmidt, and J. M. Tiedje.** 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. **29**:173–174.
13. **Manhart, N., A. Spittler, H. Bergmeister, M. Mittlbock, and E. Roth.** 2003. Influence of fructooligosaccharides on Peyer's patch lymphocyte numbers in healthy and endotoxemic mice. Nutrition **19**:657–660.
14. **Oku, T., T. Tokunaga, and N. Hosoya.** 1984. Nondigestibility of a new sweetener, "Neosugar," in the rat. J. Nutr. **114**:1574–1581.
15. **Rambaut, A.** 1996. Se-Al: Sequence Alignment Editor. [Online.] http://evolve.zoo.ox.ac.uk/.
16. **Reeves, P. G., F. H. Nielsen, and G. C. J. Fahey.** 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. **123**:1939–1951.
17. **Roberfroid, M. B., J. A. Van Loo, and G. R. Gibson.** 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. J. Nutr. **128**:11–19.
18. **Salzman, N. H., H. de Jong, Y. Paterson, H. J. Harmsen, G. W. Welling, and N. A. Bos.** 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiology **148**:3651–3660.
19. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673–4680.
20. **Tokunaga, T., Y. Nakada, Y. Tashiro, M. Hirayama, and H. Hidaka.** 1993. Effects of fructooligosaccharides intake on the intestinal flora and defecation in healthy volunteers. Bifidus **6**:143–150.
21. **Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie, and H. R. Gaskins.** 2004. Molecular ecology analysis of the gastrointestinal microbiota. J. Nutr. **134**:465–472.

THE ROLE OF *BIFIDOBACTERIUM* IN THE DEVELOPMENT OF GUT IMMUNE SYSTEMS: ANALYSIS USING GNOTOBIOTIC TCR-TRANSGENIC MICE

Masato Tsuda[1], Akira Hosono[1], Miran Fujioka[1], Satoshi Hachimura[2], Ryo Nakamura[1], Kazuhiro Hirayama[3], Kikuji Itoh[3], and Shuichi Kaminogawa[1]

[1]Department of Food Science and Technology, Nihon University,Fujisawa-shi, Kanagawa, 252-8510, Japan, and [2]Department of Applied Biological Chemistry, and [3]*Department of Veterinary Public Health, The University of Tokyo*

Abstract: We have previously established germfree ovalbumin (OVA)-specific T cell receptor transgenic (TCR-Tg) mice. Recent studies have shown that intestinal bacteria affect intestinal immunoresponses and that probiotic bacteria regulate allergic reactions in the host. However, the mechanisms of the development of the gut immune system by intestinal bacteria, especially antigen-specific immune responses, are not well understood. In this study we examined the antigen-specific immunoresponses, in particular of the intestinal immune systems derived from gnotobiotic TCR-Tg mice, associated with *Bifidobacterium* (BIF). The control group was associated with segmented filamentous bacteria (SFB) and 46 strains of clostridia, known to induce the development of the intestinal tissues. The BIF group was associated with *Bifidobacterium pseudocatenulatum* 7041, SFB and clostridia. Lymphocytes were isolated from spleen, Peyer's patch (PP) and lamina propria (LP) of the gnotobiotic TCR-Tg mice, and were co-cultured with OVA. We found that PP and LP cells from BIF mice secreted lower levels of interferon-γ (IFN-γ) and interleukin-6 (IL-6) than those from control mice in response to OVA stimulation. The pattern of regulated cytokine secretion of PP and LP cells from BIF mice was similar to that of CV mice. These results indicate that the colonization by *Bifidobacterium* regulates OVA-specific responses in the intestinal immune system. Our findings suggest that *Bifidobacterium* may contribute to the functional development of the intestinal immune system by down-regulating hyperesponsiveness to specific antigens.

Key words: *Bifidobacterium*, T cell receptor transgenic (TCR-Tg) mice, intestinal immune systems.

S. Iijima and K.-I. Nishijima (eds.), Animal Cell Technology: Basic & Applied Aspects, 93–99.

1. INTRODUCTION

The gut is consistently exposed to various dietary antigens, pathogenic bacteria, and intestinal commensal bacteria. There are intestinal immune systems specific to the intestinal mucosa whose role is to recognize and remove foreign antigens. It has recently been shown that intestinal bacteria play a role both in the development of intestinal tissues and of these intestinal immune systems.

In particular, members of the genus *Bifidobacterium* are one of the most prominent commensal bacteria in the human intestinal microflora and some species of *Bifidobacterium* have been expected to be probiotic bacteria with their -immunomodulatory activity moderating the response to infection(1-3). It is also possible that the presence of intestinal *Bifidobacterium* may moderate the allergic response. For example, one study examined the fecal flora of 2-year-old children with or without allergy. The allergic children had fewer *Bifidobacterium* in their fecal flora than the nonallergic (4). In addition, in a study using germ-free mice, it was shown that the presence of intestinal bacteria, especially members of the *Bifidobacterium*, was very important for the induction of oral tolerance (5). Thus, it is suggested that *Bifidobacterium* have immunomodulatory effects on intestinal immune systems, although the details of the mechanisms are unclear.

To examine the responsiveness to a specific antigen under conditions of regulated intestinal flora, we previously established the gnotobiotic model of ovalbumin (OVA) specific T cell receptor transgenic (TCR-Tg) mice in which mice were transfected with the sequence encoding the TCR recognizing OVA specifically. Using this experimental system, we have found some differing immunological characteristics when comparing conventional (CV) and germ-free (GF) breeding conditions. GF mice had less-developed intestinal immune tissues as evidenced by the size of Peyer's Patches (PP) from GF mice which were smaller than those from CV mice. Moreover, while immunocellular responsiveness to OVA was different between GF mice and CV mice, it was observed that PP cells from GF mice responded with higher cytokine production than those from CV mice(6). On this basis, we examined the effects of *Bifidobacterium* on the development of intestinal immune systems, focusing on the immune responses to the specifc antigen OVA.

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.

4. ACKNOWLEDGEMENT

We thank Dr. Sonoko Habu and Dr. Takehito Sato (Tokai University, School of Medicine) for generously providing the TCR-transgenic mice.

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

Yusuke Nakanishi, Akira Hosono, Teiji Kimura, and Shuichi Kaminogawa
Department of Food science and Technology, Nihon University, Fujisawa-shi, Kanagawa 252-8510, Japan

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.

S. Iijima and K.-I. Nishijma (eds.), Animal Cell Technology: Basic & Applied Aspects, 101–106.

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	Cont.	BIM-fed.
(μg/ml)	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

Takahiro Suzuki[1], Akira Hosono[2], Satoshi Hachimura[3], Toshio Suzuki[1], and Shuichi Kaminogawa [2]

[1]Research Laboratories, DAISO CO., LTD., Otakasu-cho, Amagasaki-shi, Hyogo, 660-0842, Japan. [2]Department of Food Science and Technology, Nihon University. [3]Department of Applied Biological Chemistry, The University of Tokyo

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

S. Iijima and K.-I. Nishijima (eds.), Animal Cell Technology: Basic & Applied Aspects, 369–375.

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,