Effects of Orally Ingested *Bifidobacterium longum* on the Mucosal IgA Response of Mice to Dietary Antigens

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To study the effects of lactic acid bacteria on the mucosal defence against dietary protein antigens, we compared the mucosal IgA responses to \(\beta\)-lactoglobulin (\(\beta\)-LG) of two groups of mice fed a whey protein diet with and without a culture condensate of *Bifidobacterium longum*. Both total IgA and anti-\(\beta\)-LG IgA levels in tissue extracts of the small intestinal wall were significantly higher in mice fed the *B. longum* diet for 2 weeks than in control ones. Peyer's patch (PP) cells from *B. longum*-fed mice had a much larger increase in *in vitro* IgA production than those from control mice. Furthermore, the *in vitro* IgA response to \(\beta\)-LG was detected only when PP cells from *B. longum*-fed mice were assayed. These results suggest that orally ingested lactic acid bacteria may protect a host from invasion of the intestinal mucosa by dietary antigens that have escaped enzymatic digestion in the intestine.

**Key words:** *Bifidobacterium longum*; \(\beta\)-lactoglobulin; mucosal IgA response; Peyer's patch

It is well known that orally administered lactic acid bacteria increase the systemic immune response in a host. For example, when given orally, lactic acid bacteria increase phagocytic activity\(^1\) and cytokine production.\(^2\) Such immunomodulating activities of lactic acid bacteria have been demonstrated not only in animal models but also in man.\(^3,4\) It was recently reported that orally administered lactic acid bacteria increase the host mucosal immunity. It was previously shown that mice fed *Lactobacillus casei* for 2 d showed increased mucosal IgA responses to *Salmonella typhimurium* and *Escherichia coli*, being protected against infection by them.\(^5\) Furthermore, infants fed fermented milk containing *Lactobacillus* GG, a human *Lactobacillus* strain, showed an increase in the specific IgA response to rotavirus, compared to the control group.\(^6\) Since invasion of a host by pathogenic bacteria and viruses occurs on the mucosal surface, dietary lactic acid bacteria are thought to contribute to the host defence against such organisms.

It is currently recognized that some dietary protein antigens remain partly intact in the gastrointestinal tract.\(^7\) This suggests that mucosal defence against dietary protein antigens may also be important for prevention of systemic sensitization to them. However, there have been few reports on the effects of lactic acid bacteria on the host defence against luminal protein antigens. In this study, we investigated the effects of orally administered lactic acid bacteria on the mucosal IgA response of mice to \(\beta\)-lactoglobulin, a whey protein antigen often ingested with lactic acid bacteria in dairy foods such as yogurt.

**Materials and Methods**

*Mice.* Five-week-old female BALB/c mice were used in all experiments. These mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and bred in our laboratories.

Microorganism and its cell fraction. *Bifidobacterium longum* OLL6001, isolated from human feces, was used. The cell fraction of *B. longum* was prepared as described previously.\(^8\) In brief, *B. longum* was inoculated into an EG medium, and then incubated anaerobically at 37°C overnight. The bacteria were then harvested from the EG medium, and washed three times with distilled water. To obtain the bacterial cell fraction, the bacteria were sonicated with a Branson 200 cell disruptor (Danbury, CT, USA) for 30 min on ice after resuspension in distilled water. The supernatant obtained on centrifugation (the cytoplasmic fraction of *B. longum*) was lyophilized and used for the following experiments.

Antigens. Whey protein isolate was obtained from Le Sueur Isolates (MN, USA), and egg albumin, 5× crystallized, from Seikagaku Corp. (Tokyo, Japan). Bovine \(\beta\)-lactoglobulin (\(\beta\)-LG) was prepared from fresh raw milk as described elsewhere.\(^9\)

Feeding procedure. Mice were fed ad libitum a whey protein diet (WPI diet) with 5% (W/W) lyophilized culture condensate of *B. longum* for 2 wk, which was prepared as described previously.\(^9\) The lyophilized culture condensate of *B. longum* contained about 8×10\(^{11}\) nonviable cells/g. We estimated that approximately 8×10\(^{10}\) nonviable cells were given orally to each mouse daily, because the mice ingested about 2 g of the diet per day throughout the feeding period. The control group was fed the WPI diet without the culture condensate of *B. longum*. Each group consisted of five to ten mice. In the dose-response experiments, the WPI diet with 1 or 0.2% (W/W) lyophilized culture condensate of *B. longum* was also used.
Preparation of tissue extracts of the small intestinal wall. The procedure for the preparation of a tissue extract of the small intestinal wall was a modification of that described by Yamazaki et al.10 The small intestine was carefully removed from B. longum-fed mice or control ones. The intestinal contents were washed out with 1 ml of phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) three times. After the washed small intestine was weighed, a four-fold volume of PBS-T was added, followed by thorough disruption with a glass homogenizer. The suspension was centrifuged at 10,000 g for 15 min, and the supernatant obtained was used as the tissue extract of the small intestinal wall for the detection of a intestinal IgA response.

Cell culture. Peyer’s patches (PP) from B. longum-fed or control mice were used for a culture. A single-cell suspension of PP was prepared as described previously.11 In brief, PP dissected from the small intestine were placed in 10 ml of an RPMI 1640 medium in a glass dish, and then mechanically dissociated. The treated cells were washed twice with the RPMI 1640 medium. The PP cells (1 x 106 cells/0.2 ml/well) were cultured in a flat-bottom, 96-well microtest plate (Falcon Cat. No. 3072; Becton Dickinson, Franklin Lakes, NJ, USA) in RPMI 1640 medium containing 100 U/ml of penicillin (Life Technologies, Inc., Rockville, MD, USA), 100 µg/ml of streptomycin (Life Technologies), 5 x 10^{-5} M 2-ME and 1% fresh autologous normal mouse serum. Triplicate wells were challenged with various doses of the substance. After incubation for 5 d at 37°C under 5% CO2 in air, the culture supernatants were collected and stored at -80°C until the assay.

ELISA. Total IgA and the anti-β-LG IgA antibody response were measured by an enzyme-linked immunosorbent assay (ELISA), as described by Makinen-Kiljunen and Palosuo11 with some modifications. Microtiter plates with 96 wells (Nunc, Roskilde, Denmark) were coated at 4°C overnight with 100 µl of sheep anti-mouse IgA (0.1 mg/ml) (The Binding Site Ltd., Birmingham, UK), or β-LG (0.1 mg/ml) dissolved in a 0.1 M carbonate buffer (pH 9.6). The unbound materials were removed by four washings with 125 µl of PBS-T. The plates were incubated with 125 µl of 1.5% gelatin (Difco, Detroit, USA) for 30 min at room temperature, and then washed four times. Tissue extracts of the small intestinal wall, supernatants of cultures, and standard mouse IgA (mouse myeloma protein; ICN Biomedicals, Inc., Lisle, IL, USA) were diluted with 0.01 M PBS (pH 7.2) containing 0.5 M NaCl, 0.1% Tween 20, and 3% polyethylene glycol 6000 (Nacalai Tesque, Inc., Kyoto, Japan), and then 100 µl of diluted samples and standards were added to triplicate wells. For each sample, an uncoated well blocked with 1.5% gelatin was used as a control for non-specific binding. After incubation overnight at 4°C, the plates were washed, and then 100 µl of biotinylated rat anti-mouse IgA monoclonal antibodies (Kanto Chemical Co., Inc., Tokyo, Japan) were added. Following further incubation at room temperature for 2 h, the plates were washed, and 100 µl of alkaline phosphatase-conjugated avidin (Organon Teknika Corp., PA, USA) was added to each well. Finally, 100 µl of p-nitrophenyl phosphate (1 mg/ml) dissolved in a diethanolamine buffer (pH 9.8) was added. After incubation at room temperature for 30 min, color development was stopped by adding 20 µl of 5 M NaOH, and then the absorbance at 405 nm of each well was measured. The total IgA content of each sample was calculated from a calibration curve of A405 vs. the log concentration of standard mouse IgA. The values for the intestinal IgA were adjusted for the volume of suspension recovered and expressed as the total IgA per gut wet weight (µg/g). For measurement of the anti-β-LG IgA response, the incubation for color development was done at 37°C for 2 h, and the results were expressed as the A405.

Assessment of the Induction of oral tolerance by feeding the WPI diet with or without 5% (W/W) culture condensate of B. longum. To find whether oral tolerance was induced in the mice fed on the WPI diet with or without 5% (W/W) culture condensate of B. longum, mice were fed with the diets for 2 wk, subsequently immunized intraperitoneally with 100 µg of β-LG in complete Freund’s adjuvant (CFA; Difco, Detroit, USA) and then boosted with 100 µg of the antigen in incomplete Freund’s adjuvant (IFA; Difco) 2 weeks later. Mice were bled 1 week after the second immunization and serum was tested for its binding to β-LG by ELISA as previously described10 with minor modifications. Briefly, microtiter plates were coated with 100 µl of the antigen (0.1 mg/ml). After incubating 125 µl of 1% egg albumin, 100 µl of the samples were added to the wells. Following further incubation, 100 µl of alkaline phosphatase-conjugated anti-mouse Ig (Zymed, San Francisco, USA) was added to each well. Finally, 100 µl of an enzyme substrate buffer solution (0.1% p-nitrophenylphosphate in a diethanolamine buffer at pH 9.8) was added. The reaction was stopped by adding 20 µl of 5 M NaOH, and the absorbance at 405 nm was measured. The antibody titers from the mice fed on the WPI diet with or without 5% (W/W) culture condensate of B. longum were compared with the antibody titers of the group fed on a commercial diet (MFS; Oriental Yeast, Tokyo, Japan) which did not contain milk proteins.

Statistics. The results, expressed as means ± SD, were compared by Student’s t-test.

Results
Mucosal IgA response in vivo
To discover the effects of orally administered lactic acid bacteria on the mucosal immune response, we compared the mucosal IgA responses between B. longum-fed mice and control ones. A tissue extract of the small intestinal wall, which contains mucosal IgA antibodies produced in mucosal effector tissues such as the lamina propria, was collected from each mouse in both groups, and then the total IgA and anti-β-LG IgA levels in the extract were measured by ELISA. Figure 1 shows the IgA levels in tissue extracts of the small intestinal wall. Feed-
Fig. 1. Total IgA (A) and Anti-β-LG IgA (B) Levels in Tissue Extracts of the Small Intestinal Wall of Mice Measured by an Enzyme-linked Immunosorbent Assay (ELISA).

Mice were fed the WPI diet with or without 5% (W/W) culture condensate of *B. longum* for 2 wk. The results are the means ± SD for 5 individual mice. Significant difference from the control: *P* < 0.05, **P** < 0.01.

ing with *B. longum* for 2 wk increased the total IgA level in these extracts (p < 0.05). Furthermore, the anti-β-LG IgA level was also significantly higher in *B. longum*-fed mice (p < 0.01) than in control ones. The whole body weight and gut wet weight were not significantly different between the two dietary groups at the time of death (Table I).

In vitro IgA response of PP cells.

These experiments showed that orally administered *B. longum OLL6001* increased the mucosal IgA response of the mice. Since PP cells include immunocompetent cells necessary for induction of the mucosal IgA response,1) we next examined the in vitro IgA response of PP cells derived from *B. longum*-fed and control mice.

We assayed PP cells with the cytoplasm of *B. longum* instead of intact cells, because PP cells from *B. longum*-fed mice responded much better to the cytoplasm of *B. longum* than its cell wall in our previous study.5) When stimulated with the cytoplasm of *B. longum in vitro*, PP cells from *B. longum*-fed mice had a marked increase in IgA production (Table II). Such an increase in IgA production was not observed with PP cells from control mice. On the other hand, when stimulated with β-LG, a coadministered dietary antigen, the IgA response of PP cells from *B. longum*-fed mice was almost as low as that on stimulation with ovalbumin, an irrelevant antigen, or the background response. The IgA response to β-LG was detected only when PP cells from *B. longum*-fed mice were stimulated with the cytoplasm of *B. longum*. Such an adjuvant effect of *B. longum OLL6001* on the in vitro IgA response was also observed when mice were fed with the WPI diet with 1 or 0.2% (W/W) lyophilized culture condensate of *B. longum* (Table III).

Induction of oral tolerance by feeding the WPI diet with 5% (W/W) culture condensate of *B. longum*.

It has been shown that feeding mice a WPI diet induced oral tolerance to β-LG in a previous study.7) We found whether a WPI diet with 5% (W/W) culture condensate of *B. longum* could also induce oral tolerance to β-LG. As shown in Fig. 2, feeding with *B. longum* for 2 wk did not affect the induction of oral tolerance to β-LG.

**Discussion**

Recently, it was recognized that the mucosal immune system plays a crucial role in the protection of a host against invasion by microbes and dietary antigens.13,14) Thus, various maneuvers have been used to improve the mucosal immune system. For instance, nonreplicating particles such as liposomes, microcapsules, and immunostimulating complexes have been shown to induce secretory immune responses.15) Replicating vectors in-

**Table I** Whole body weight and gut wet weight of control and *B. longum*-fed mice.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+5% <em>B. longum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole body weight (g)</td>
<td>17.54±0.82</td>
<td>18.21±0.60</td>
</tr>
<tr>
<td>Gut wet weight (g)</td>
<td>0.52±0.02</td>
<td>0.54±0.05</td>
</tr>
</tbody>
</table>

Mean ± SD (n = 5)

**Table II** Dose-response Profiles of Total IgA and the Anti-β-LG IgA Responses of PP Cells in the Presence of Various Substances.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Dose (µg/ml)</th>
<th>Total IgA (µg/ml)</th>
<th>Anti-β-LG IgA (A405)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+5% <em>B. longum</em></td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.58±0.06</td>
<td>6.60±0.76</td>
</tr>
<tr>
<td>The cytoplasm of</td>
<td>0.1</td>
<td>1.3±0.2</td>
<td>1350±628</td>
</tr>
<tr>
<td><em>B. longum</em></td>
<td>1</td>
<td>1.8±0.2</td>
<td>2160±628</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.6±4.4</td>
<td>166±25</td>
</tr>
<tr>
<td>β-LG</td>
<td>0.1</td>
<td>0.98±0.12</td>
<td>15.1±2.0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.90±0.04</td>
<td>6.5±0.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.84±0.09</td>
<td>18.5±0.3</td>
</tr>
<tr>
<td>OVA</td>
<td>0.1</td>
<td>0.60±0.05</td>
<td>9.3±5.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.64±0.10</td>
<td>9.1±5.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.80±0.05</td>
<td>8.4±1.7</td>
</tr>
</tbody>
</table>

Mice were fed the WPI diet with or without 5% (W/W) culture condensate of *B. longum* for 2 wk. PP cells removed from five to ten mice of each group were cultured for 5 d. The culture supernatants were examined by ELISA. The results are the means ± SD for triplicate wells. n.d. = not detected.
Table III  Dose Dependence of Total IgA and the Anti β-LG IgA Responses of PP Cells.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Total IgA (µg/ml)</th>
<th>anti β-LG IgA (A405)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 0.2 (%)</td>
<td>B. longom-fed 1 (%)</td>
</tr>
<tr>
<td>None</td>
<td>0.58 ± 0.06</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>The cytoplasm of B. longum</td>
<td>1.8 ± 0.2</td>
<td>88 ± 29</td>
</tr>
<tr>
<td>β-LG</td>
<td>0.90 ± 0.04</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>OVA</td>
<td>0.64 ± 0.10</td>
<td>2.1 ± 0.3</td>
</tr>
</tbody>
</table>

Mice were fed the WPI diet with various B. longum contents (0, 0.2, 1, 5% (W/W)) for 2 wk. PP cells removed from each group of mice were cultured in the presence of various substances (1 µg/ml). The culture supernatants were examined by ELISA. The results are the means ± SD for triplicate wells. n.d. = not detected.

Fig. 2. Induction of Oral Tolerance by Feeding the WPI Diet with 5% (W/W) Culture Condensate of B. longum.

Mice were fed the WPI diet with or without 5% (W/W) culture condensate of B. longum, or commercial diet (MF), and subsequently immunized with β-LG. Each serum was diluted and tested for anti-β-LG antibodies by ELISA.

including relatively safe bacteria and viruses have also been used to mediate mucosal immunity to infection.14) Although such agents differ in composition and characteristics, all of them are constructed for targeting on the PP, an inductive site of local secretory immunity.12) Therefore, it seems to be necessary for induction of the mucosal immune response that the adjuvant can stimulate PP cells. Indeed, it was reported that cholera toxin, the most potent oral adjuvant, increases the PP B cell response induced by orally administered influenza virus.17) As we previously found for the proliferative response,8) B. longum OLL6001 stimulated PP cells when given orally. This implies that the microorganism may improve mucosal antibody responses. Thus, we examined the adjuvant effect of the microorganism on the IgA antibody responses at mucosal sites.

IgA is the predominant antibody in the local immune system. It is generally accepted that IgA in mucosal secretions is protective in the mucosal immune system.18) There are two approaches for increasing the levels of IgA antibodies at mucosal effector sites in a host. One approach is the provision of exogenous IgA. It is well known that human milk, especially colostrum, contains high levels of IgA antibodies, which contribute to the improved immunity of the breast-fed infant.19) Furthermore, IgA antibodies purified from human milk or saliva were shown to be effective for mucosal protection against invasion by poliovirus20) and M6 streptococci.21) However, except for the breast-fed infant, the application of exogenous IgA to host defense at the mucosal surface is still very limited, because the large-scale separation of IgA from mucosal secretions is difficult, and the industrial production of IgA, especially secretory IgA, by biotechnology has not been done yet. On the other hand, another approach is the stimulation of endogenous IgA production. This approach is likely to be rather favorable in that the host immune system develops and matures with immunostimulatory agents, and the effect seems to be long-lasting. As mentioned above, there are several immunomodulatory agents that augment the endogenous synthesis of IgA. However, most of them were developed for pharmaceutical or experimental use, not for dietary use. Therefore, it is noteworthy that orally ingested B. longum OLL6001 can increase endogenous IgA production (Fig. 1), as reported for several strains of lactic acid bacteria.22) Since the IgA response of PP cells was much higher in B. longum-fed mice than that in control ones (Table II), the adjuvant effect of B. longum OLL6001 on the mucosal IgA response was thought to be due to the stimulation of PP cells by the microorganism.

It was recently demonstrated that lactic acid bacteria such as bifidobacteria and lactobacilli have many health and nutritional benefits.23) Furthermore, on the basis of the results of a study on these microorganisms, the term probiotic was proposed for "a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance."24) Since several benefits, such as improvement of lactose intolerance symptoms by a microbial enzyme, would depend on viable bacteria, the importance of live bacteria has been stressed. Also, in the case of adjuvant activity, it was reported that the effect of yogurt consumption on increasing human lymphocyte interferon-γ (IFN-γ) production was much stronger with live active culture yogurt than heat-treated yogurt.25) In a recent study, it was shown that viable bacteria, but not heat-killed ones, induce interleukin 12 expression by macrophages, which is important for the induction of IFN-γ-producing T cells.26) However, in this study, non-viable bifidobacteria given orally were found to improve the mucosal immune system. Therefore, it seems that the active component responsible for the adjuvant activity remains intact, at least partially, in non-viable bifidobacteria. This is also implied by the
fact that the adjuvant effect of orally ingested \textit{B. longum} OLL6001 on the IgA response of PP cells increased in a dose-dependent manner (Table III). We consider that the cellular components increasing mucosal IgA responses may exist in the cytoplasm of \textit{B. longum} OLL6001. Interestingly, polysaccharides with immunopotentiating activity for PP cells have been more recently found in the soluble fractions from bifidobacteria.\textsuperscript{27} Similar substances may be involved in the adjuvant effect of \textit{B. longum} OLL6001. Their characterization is now under way.

It has been indicated that the mucosal immune responses to orally administered antigens differ with immunoglobulin classes. For example, while IgG and IgE responses may be suppressed, IgA responses may be unaffected.\textsuperscript{28} It is generally more difficult to produce mucosal IgA responses to dietary protein antigens than to bacterial antigens.\textsuperscript{29} However, it has been reported that dietary antigen-specific IgA antibodies have been detected in milk.\textsuperscript{30,31} This implies, according to the concept of a common mucosal immune system, that IgA antibody responses to dietary antigens, which would be considerably weaker, are elicited at different mucosal sites, such as the gastrointestinal tract. Our results suggest that orally ingested \textit{B. longum} OLL6001 can increase such mucosal IgA responses to dietary antigens, which may lead to the mucosal protection against dietary proteins remaining antigenically intact in the gut. With regard to the host defence at mucosal barriers, this adjuvant effect of \textit{B. longum} OLL6001 may be consistent with the previous finding that \textit{Lactobacillus} GG, a strain studied as a human probiotic,\textsuperscript{32} evoked down-regulation of the increased intestinal permeability induced by cow milk in suckling rats.\textsuperscript{33} Further study is needed to clarify the details of the mechanism of the interaction of dietary lactic acid bacteria and the mucosal barrier system.

In our previous study,\textsuperscript{30} a systemic immune response to \textit{B. longum} cells was not produced in mice fed with \textit{B. longum} for 2 wk. Therefore, it is possible to increase a host’s mucosal immunity without sensitizing the host to the orally ingested microorganism. Furthermore, oral tolerance to β-LG was induced in mice fed with \textit{B. longum} for 2 wk as well as in control mice (Fig. 2). This is in contrast to the abrogation of the oral tolerance to a coadministered protein antigen by cholera toxin, a strong mucosal adjuvant.\textsuperscript{34} These findings imply that orally ingested \textit{B. longum} OLL6001 may not have negative effects on the host.

In conclusion, we now present evidence that \textit{B. longum} OLL6001 stimulates PP cells, increasing the IgA antibody responses to dietary antigens in the small intestinal mucosa without other harmful side effects. These results suggest that orally administered lactic acid bacteria may protect a host from invasion of the intestinal mucosa not only by pathogenic microorganisms but also by dietary antigens that have escaped enzymatic digestion in the gastrointestinal tract. Thus, this study may form the basis for possible application of lactic acid bacteria as immunomodulatory agents for preventing the systemic sensitization to dietary protein antigens.

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