

Effect of Orally Administered Non-Viable *Lactobacillus* Cells on Murine Humoral Immune Responses

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Abstract: BALB/c mice were immunized intraperitoneally with the food antigen ovalbumin (OVA) while they were fed with *Lactobacillus* GG heated killed cell preparation. The oral administration of *Lactobacillus* GG did not appear to modify the antigen-augmented serum IgE in the tested mice but significantly augmented serum OVA specific IgG in the tested mice fed with a diet containing 0.1% *Lactobacillus* GG as the non-viable cell preparation ($P<0.05$). The fecal OVA specific IgA of the tested mice fed with non-viable *Lactobacillus* GG cells was also significantly elevated ($P<0.05$) compared to those from OVA immunized mice. The spleen cells of mice fed with non-viable *Lactobacillus* GG cells secreted more IL-6 ($P<0.01$). These results suggest that the non-viable *Lactobacillus* GG can augment the systemic and mucosal immune responses in a host animal favoring secretory IgA but not IgE in an adjuvant-like manner.

Key words: *Lactobacillus* GG, Probiotic, Humoral immunity, Immunoglobulin

The prevalence of allergic disease characterized by enhanced immunoglobulin E (IgE) responses to common environmental antigens is increasing worldwide especially in Western industrialized countries (29). Although the etiology of such allergic disease is still not completely understood, a disturbance of the intestinal microflora, especially of endogenous lactic acid bacteria and bifidobacteria, has been reported as one of the important events associated with allergic diseases (2, 3, 6, 13, 22). Probiotics can offer a safe and practical means of modulating composition and activity of the human intestinal microbiota (26, 27).

Lactobacillus GG is widely used in the dairy industry as a probiotic bacterium with well documented health promoting benefits (28). Majamaa and Isolauri (17) demonstrated that the clinical symptoms of atopic eczema could be improved in nursing infants whose mothers were given *Lactobacillus* GG and in milk-hypersensitive infants who consumed a hydrolyzed whey formula supplemented with *Lactobacillus* GG. In a 4 year follow up clinical study, this probiotic bacteri-

um effectively protected infants with predisposition for allergic diseases (14). However, the underlying mechanisms behind these documented clinical effects remain unclear. In the present study, BALB/c mice were immunized with a common food protein, ovalbumin (OVA) while they were orally fed with heat-killed *Lactobacillus* GG. The immune responses in the tested mice were investigated by determining the concentration of antibodies in serum and feces, and the cytokine secretion by the spleen cell *in vitro* after re-stimulation with OVA.

Inbred BALB/c mice were purchased from Charles River, Japan, Inc. (Yokohama, Japan). Mice were kept in plastic cages, were given a standard diet (MF-1, Oriental Yeast Co., Tokyo), and were allowed free access to water. The temperature and humidity were controlled at 24 ± 1 C and $55\pm10\%$, respectively. The animal study was conducted based on the Guidelines for Use and Care of Laboratory Animals (Prime Minister's Office announcement No. 6, Japan, 1980). Lyophilized *Lactobacillus* GG (GG powder) was kindly supplied by

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Abbreviations: cfu, colony forming units; IFN, interferon; Ig, Immunoglobulin; IL, interleukin; OVA, ovalbumin; PBS, phosphate-buffered saline; sASC, specific antibody secreting cells.

Valio, Ltd. (Helsinki, Finland). For heat-inactivation, GG powder was suspended in phosphate-buffered saline (PBS, pH 7.4) at a concentration of 1 g/ml, subsequently heated in a water bath at 65 C for 30 min, and stored at -70 C until use. The *Lactobacillus* GG containing diet was processed by Oriental Yeast Co. by adding 0.1% GG powder to the standard diet.

In administration of Exp. 1, mice were divided to three groups ($n=6$ per group, 7 weeks old). Two groups were i.p. injected on day 0 and day 14 with 20 μ g of OVA (Sigma Grade V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) and 2 mg of $\text{Al}(\text{OH})_3$ (Wako, Tokyo) in PBS with a total volume of 0.2 ml. Of these two groups of mice, one group was fed a diet with 0.1% heat-inactivated *Lactobacillus* GG throughout the experiment, while the other group was fed a standard diet. The third group was fed a standard diet without the immunization. Sera and feces were collected from the mice on day 0 and day 21. In administration of Exp. 2, mice were divided into three groups ($n=6$ per group, 7 weeks old). Two groups were immunized with OVA as described above. *Lactobacillus* GG (1.0×10^9 cfu in 200 μ l PBS) was administered intragastrically to the test mice every 2 days. Feeding started 3 days before immunization and continued throughout the experiment. The groups of OVA-immunized mice and the non-immunized mice as the control received intragastrically 200 μ l PBS instead of *Lactobacillus* GG-containing preparations. Sera and feces were collected from the mice on day 0 and day 21.

Fresh feces were collected from each mouse, weighed and diluted 1:10 with PBS. The mixture was subsequently incubated for 30 min at room temperature, mixed to suspension, and then centrifuged at $2,000 \times g$ for 10 min. The supernatant was removed and stored at -80 C for antibody measurement. The mice were bled from the retroorbital plexus. Serum was obtained after incubation at room temperature and centrifugation at $10,000 \times g$ for 15 min. Serum samples were stored in -80 C for the analysis of IgG, IgE and IgA.

Mice were sacrificed 1 week after the second immunization with OVA, and their spleens were aseptically removed. Single cell suspensions were then prepared by opening each spleen gently in a 5 cm petri dish with 5 ml of RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Rockville, N.Y., U.S.A.), 100 μ g/ml streptomycin, 100 μ g/ml penicillin, 10 mM Hepes and 50 μ M 2-mercaptoethanol. The cells were washed three times with RPMI 1640 medium, then resuspended in RPMI 1640 medium to 1.0×10^7 cells/ml. Spleen cells were seeded to 24-well multi-plate (Nunc, Roskilde, Denmark) at 5.0×10^6 cells/well, and cultured in the presence of OVA

at a final concentration of 200 μ g/ml/well at 37 C for 72 hr under 5% CO_2 . The supernatants of the cell cultures were collected by centrifugation at $2,000 \times g$ for 10 min, and stored at -70 C until use.

Total and anti-ovalbumin immunoglobulin (OVA sIg) titers were measured by sandwich-type ELISA as described by Fukushima et al. (5) with some modification. Briefly, 96-well immunoplates were coated with anti-mouse IgA (α chain specific, Zymed, San Francisco, Calif., U.S.A.), or 0.01% of OVA for the measurement of total IgA and anti-ovalbumin immunoglobulin. After overnight incubation at 4 C and blocking with 1% bovine serum albumin solution at 37 C for 1 hr, the appropriately diluted sample solutions were added and incubated at 37 C for 2 hr. After washing three times, biotin-conjugated anti-mouse antibodies were added, which were followed by adding alkaline-phosphates labeled avidin. The visualization was performed by incubating with *p*-nitrophenylphosphate at room temperature for 30 min. After stopping the reaction with 5 N NaOH, absorbance was measured at 450 nm/595 nm using a microplate reader (BioRad Japan, Tokyo). The concentration of total IgA was determined based on the calibration curves with myeloma IgA as standard. Biotinylated-rabbit anti-mouse IgG, IgG₁, IgG_{2a} (γ chain specific) (Zymed); goat anti-mouse IgA (Southern Biotechnology Associates, Inc., Birmingham, Ala., U.S.A.); and rat anti-mouse IgE (ϵ chain specific) (Southern Biotechnology Associates, Inc.) were used as captive antibodies in the measurements of anti-OVA specific IgG, IgG₁, IgG_{2a}, IgA and IgE titers, respectively. The concentration of total IgE in the serum samples was analyzed with a commercial ELSIA kit (Mouse IgE Eia Kit Yamasa, Yamasa Co., Chiba, Japan). IL-2, IL-4, IL-5, and IFN- γ in the supernatant of spleen cell culture were tested with the commercial ELISA kits (Amersham Pharmacia Biotech., Buckinghamshire, U.K.), and IL-6, IL-10, IL-12 (p40 and p70) were examined with the commercial ELISA kits (Endogen, Inc., Woburn, Mass., U.S.A.).

The results were expressed as mean values with standard error from three independent experiments in triplicate. JMP[®] 4.0 (SAS Institute, Inc., Cary, N.C., U.S.A.) was used for analysis. Statistical differences between the test group and control group were determined by unpaired student *t* test. The differences were considered significant if *P* value for the effect was less than 0.05.

Serum antibodies of the tested mice in Exp. 1 and the cytokine production of their spleen cells *in vitro* after re-stimulation with OVA were analyzed (Table 1). The immunization with OVA elevated the production of total IgE and OVA-specific IgG in the serum. No sig-

nificant difference of total IgE was found between sera from the mice orally administered with the diet containing 0.1% heat-inactivated *Lactobacillus* GG and control mice. However, OVA-specific IgG in the serum from mice fed with heat-inactivated *Lactobacillus* GG diet was significantly increased compared to that of the control mice ($P<0.05$) (Table 1). The spleen cells from the mice fed with *Lactobacillus* GG diet secreted signif-

icantly more IL-2 and IL-6 ($P<0.05$ and $P<0.01$) *in vitro* in the presence of OVA than those from control mice (Table 1).

In Exp. 2, heat-killed *Lactobacillus* GG was administered intragastrically. The serum OVA-specific IgG titer and the fecal OVA specific IgA titer of the mice fed with heat-killed *Lactobacillus* GG were significantly higher ($P<0.05$) than those of control mice (Table 2).

Table 1. Serum antibodies and spleen cytokines in the mice of Experiment 1^{a)}

Antibodies & cytokines	Test group ^{b)} (n=6)	Control group ^{c)} (n=6)	Normal group ^{d)} (n=6)
Total IgE (nm/ml)	158.95±39.61	145.76±45.21	28.48±5.56
OVA specific IgG (A ₄₅₀ nm)	0.73±0.11*	0.52±0.16	—
IFN-γ (pg/ml)	2,796.29±603.85	3,646.29±2,095.7	2,034.54±1,229.11
IL-2 (pg/ml)	1,814.41±564.78*	1,237.61±262.15	1,168.53±409.39
IL-4 (pg/ml)	543.43±196.01	482.03±303.41	—
IL-5 (pg/ml)	416.61±109.01	380.12±73.31	14.30±3.71
IL-6 (pg/ml)	789.08±354.50**	295.36±15.45	6.58±3.16

^{a)} Data are presented as mean and standard error of mean; —: not detected.

^{b)} Mice were fed with 0.1% *Lactobacillus* GG containing diet and immunized with OVA.

^{c)} Mice were fed with standard diet and immunized with OVA.

^{d)} Mice were fed with standard diet without immunization.

* $P<0.05$ significantly different from control.

** $P<0.01$ significantly different from control.

Table 2. Serum antibodies in mice in Experiment 2^{a)}

Antibodies	Test group ^{b)}	Control group ^{c)}	Normal ^{d)}
Total IgE (nm/ml)	270±12.10	231.38±13.83	—
Serum OVA sIgG (A ₄₅₀ nm)	0.83±0.14*	0.09±0.04	—
Serum OVA sIgG ₁ (A ₄₅₀ nm)	0.67±0.07	0.58±0.22	—
Serum OVA sIgG _{2a} (A ₄₅₀ nm)	0.25±0.16	0.27±0.04	—
Fecal OVA sIgA (A ₄₅₀ nm)	0.31±0.09*	0.06±0.03	—

^{a)} Data are presented as mean and standard error of mean; —: not detected.

^{b)} Mice were fed with heat-killed *Lactobacillus* GG (1.0×10^9 cfu/2 days) and immunized with OVA.

^{c)} Mice were fed with standard diet and immunized with OVA.

^{d)} Mice were fed with standard diet without immunization.

* $P<0.05$ significantly different from control.

Table 3. Cytokines production by spleen cells of the mice in Experiment 2 immunized by OVA re-stimulated *in vitro*^{a)}

Cytokines	Test group ^{b)}	Control group ^{c)}	Normal ^{d)}
IL-2 (pg/ml)	346.53±61.62	275.49±94.31	495.08±93.81
IL-4 (pg/ml)	156.66±7.48	213.94±15.59	13.95±7.92
IL-5 (ng/ml)	1,137.28±41.61	1,311.68±69.96	—
IL-6 (pg/ml)	617.79±12.23*	506.57±30.62	312.49±19.13
IL-10 (pg/ml)	171.64±0	283.75±0.00	—
IL-12 (pg/ml)	268.73±1.40	385.95±0.09	1,026.62±0.28
IFN-γ (pg/ml)	8,193.39±1,225.35	7,010.4±604.13	5,745.67±1088.50

^{a)} Data are presented as mean and standard error of mean; —: not detected.

^{b)} Mice were fed with heat-killed *Lactobacillus* GG (1.0×10^9 cfu/2 days) and immunized with OVA.

^{c)} Mice were fed with standard diet and immunized with OVA.

^{d)} Mice were fed with standard diet without immunization.

* $P<0.05$ significantly different from control.

Furthermore, the spleen cells from the mouse fed with heat-killed *Lactobacillus* GG secreted more IL-6 than those from control mice ($P < 0.05$). These cells also produced more IL-2 than spleen cells of the control mice, but the difference did not reach statistical significance (Table 3).

T cells are key immunocomponent cells which can be divided into two functional types, Th1 and Th2, based on their cytokine profile. Th1 cells produce IL-2, IFN- γ and tumor necrosis factor that are vital for cell-mediated immunity. On the other hand, Th2 cells predominantly produce IL-4, IL-5 and IL-10 and are associated with humoral immunity and allergic responses. The enhanced IgE expression is likely to be a consequence of the Th2 type skewing immune responses (4, 9). Recent studies have indicated that some specific probiotic lactic acid bacteria, like *L. casei* Shirota and *L. plantarum* L-137 activated macrophages to secrete more pro-inflammatory cytokine IL-12 which can favor Th1 type immunity (15, 20, 30). Consequently, these probiotic strains apparently down-regulated the antigen-induced serum IgE production in mice (18, 20). Based on these results, these lactic acid bacteria are believed to possess positive effects to allergy, although no clinical studies have been conducted to demonstrate this hypothesis.

In our previous study, *Lactobacillus* GG stimulated the secretion of macrophage derived pro-inflammatory cytokine IL-12 *in vitro* as did *L. casei* Shirota strain (19). In light of this finding, we hypothesized that *Lactobacillus* GG may suppress the IgE production induced by food antigen (OVA) in an animal model as has been observed for other probiotic lactic acid bacteria. However, the results from the present study indicated that orally fed *Lactobacillus* GG did not influence the mouse serum IgE production that was triggered by the food antigen. This is in good agreement with clinical observations that clinical improvement was not accompanied with an alteration in serum IgE level during *Lactobacillus* GG administration (14, 24). These results indicated that the underlying mechanisms of intervention on the antigen triggered IgE production in some probiotics strains does not seem to contribute to the ability of *Lactobacillus* GG to alleviate atopic diseases.

An elevated secretory IgA level in the intestinal lumen is the consequence of stimulation of the mucosal immunity of the host (1). The secretory IgA in the intestine can prevent infection by pathogenic microorganisms and aberrant absorption of allergenic food proteins and carcinogens (16). In clinical and animal studies, *Lactobacillus* GG increased the number of specific antibody secreting cells (sASC) against β -lactalbumin

and casein in patients with Crohn's disease and milk protein in suckling rats, and IgA sASC and serum IgA titers to rotavirus in rotavirus patients (10, 12). There was a propensity toward a greater increase in antigen specific sASC IgA among the subjects receiving the vaccine in combination with *Lactobacillus* GG (7). In a study to compare the effect of viable and heat-inactivated *Lactobacillus* GG on rotavirus immune responses in the patients with rotavirus diarrhea, only viable *Lactobacillus* GG promoted the serum rotavirus specific IgA secreting cells (11). The health benefits reported for the fermented milk have been documented using viable bacteria and data on non-viable preparation is often limited (23). In the present study, we found that the heat-killed *Lactobacillus* GG promoted more antigen specific IgA secretion. Recently, the chromosomal DNA from *Lactobacillus* GG was found to be a potent inducer of splenic B cell proliferation, CD86/CD69 expression and cytokine production in mice (8). Taken together with these results, the cell components rather than metabolites of *Lactobacillus* GG appear to be the key factors to alter the human immune responses.

Oral administration of a diet containing *Lactobacillus* GG significantly enhanced the secretion of IL-2 and IL-6 by the spleen cells. IL-2 is a cytokine produced by antigen-activated T cells that act in an autocrine manner to stimulate T cell proliferation and also potentiate apoptosis of antigen-activated T cells. Thus, IL-2 is required for both the induction and regulation of T cell mediated immune responses. IL-2 also stimulates proliferation and differentiation of natural killer cells and B cells. IL-6 stimulates the synthesis of acute phase proteins by hepatocytes and stimulates the growth of antibody-producing B-lymphocytes. Many studies both *in vitro* and *in vivo* have suggested a potential application of IL-6 in augmenting IgA antibody responses in mucosal B cells or at mucosal surfaces (21, 25). The results in the present study suggest that the enhancement of IL-2 and IL-6 production may be one possible underlying mechanisms of *Lactobacillus* GG to trigger the immune responses in an adjuvant manner.

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