

Communication

Oral Administration of an Immunostimulatory DNA Sequence from *Bifidobacterium longum* Improves Th1/Th2 Balance in a Murine Model

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We have reported the antiallergic activities of the immunostimulatory oligodeoxynucleotide (ODN) BL07S, identified from genomic DNA of *Bifidobacterium longum* BB536 from *in vitro* and *in vivo* studies. The present study evaluated the efficiency of ODN BL07S in preventing allergic responses by oral administration. Oral administration of BL07S suppressed serum ovalbumin (OVA)-specific immunoglobulin (Ig) E levels and improved the OVA-specific IgG2a/IgG1 ratio. ODN BL07S increased Th1 cytokine and decreased Th2 cytokine production in splenocytes. These results suggest that immunostimulatory ODNs are potentially associated with the antiallergic effects of probiotics.

Key words: probiotics; immunostimulatory DNA; immunoglobulin (Ig) E; allergy; *Bifidobacterium longum* BB536

Numerous studies have reported that intake of probiotics such as lactobacilli and bifidobacteria is associated with the prevention of various allergic diseases.^{1–5)} These reports have strongly indicated that probiotics have the potential to regulate allergic responses. We have reported that *Bifidobacterium longum* BB536 alleviates the subjective symptoms of Japanese cedar pollinosis, possibly by modulating effects on the helper T cell 1 (Th1)/helper T cell 2 (Th2) balance,⁶⁾ but the exact components in probiotics related to antiallergic activity remain unclear. Cell components in probiotics such as peptidoglycan and lipoteichoic acid reportedly show immunostimulatory activities.^{7,8)} Immunostimulatory sequence (ISS) oligodeoxynucleotides (ODNs), which mainly contain cytosine linked to guanine by a phosphate bond (CpG),⁹⁾ have recently been identified

from probiotics.^{10–15)} In general, ISS-ODNs have been found to suppress Th2 immune responses by stimulating innate immune cells *via* Toll-like receptor (TLR) 9 expressed on dendritic cells, macrophages, and the B-cell surface, affecting adaptive immune systems by releasing abundant Th1 cytokines.^{16,17)} ISS-ODNs are thus considered therapeutically significant reagents for allergic disease, but the effects of ISS-ODNs derived from probiotics on allergic diseases have not yet been well characterized. Assays *in vitro* and *vivo* have shown that ISS-ODN BL07S isolated from the genomic DNA of *B. longum* BB536 decreases allergen-induced immunoglobulin (Ig) E production.^{15,18)} Probiotics are usually delivered as foods such as yoghurt or lyophilized powder, and whether ISS-ODNs derived from probiotics show antiallergic activity upon oral administration represents important information. The objective of this study was to evaluate the effects of ODN BL07S on suppression of allergic reaction following oral administration.

The ODN used in this study was ODN BL07S (5'-GCGTCGGTTTCGGTGCTCAC-3') derived from the genomic DNA of *B. longum* BB536, as described in our previous study.^{15,18)} This ODN was chemically synthesized by SigmaGenosys (Ishikari, Japan), and was modified with phosphorothioate to increase resistance to nuclease degradation. ODN and ovalbumin (OVA) (grade V; Sigma, St. Louis, MO) were confirmed to be free of detectable endotoxin using a Limulus amoebocyte lysate kit (Seikagaku Kogyo, Tokyo). Specific pathogen-free 7-week-old female BALB/c mice were purchased from Charles River Japan (Atsugi, Japan) and housed in plastic cages. The room temperature and humidity were controlled at 23.5 ± 1 °C and 50 ± 5%.

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Abbreviations: IFN, interferon; Ig, immunoglobulin; IL, interleukin; ISS, immunostimulatory sequence; ODN, oligodeoxynucleotide; OVA, ovalbumin; TGF- β , transforming growth factor- β

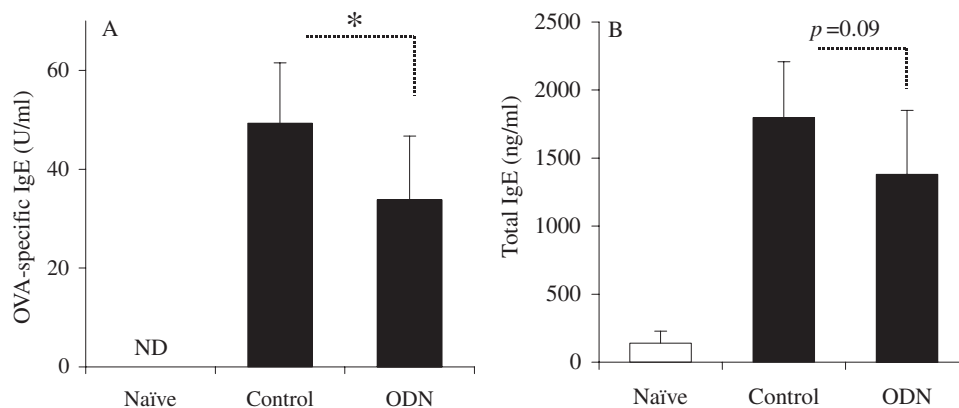


Fig. 1. ODN BL07S Inhibited IgE Production in OVA-Primed BALB/c Mice.

Mice were immunized using OVA (10 µg) in alum on days 2 and 12. ODN BL07S (1 mg) was administrated by gastric gavage on days 0–2 and 7–9 in the ODN group. PBS was given to naïve and control groups. Blood was collected on day 20 and IgE levels were determined using ELISA. Data represent mean $\pm$ SD from seven mice per group. A, OVA-specific IgE; B, Total IgE. * $p < 0.05$ versus controls. ND, not detected.

Mice were fed a standard CRF-1 diet (Oriental Yeast, Tokyo) and allowed *ad libitum* access to water throughout the experimental period. All experiments involving mice were performed according to the guidelines of the Prime Minister's Office of Japan (no. 6, March 27, 1980).

The antiallergic ability of ODN BL07S following oral administration was assessed using an allergen-induced Th2 murine model. Mice (8 weeks old on day 0) were intraperitoneally injected with 10 µg of OVA adsorbed into 1 mg of alum (Wako Pure Chemical, Osaka, Japan) on days 2 and 12. Mice in the naïve group were administered alum solution only. In a preliminary experiment, we checked the dose response at 0.1 and 1 mg of ODN BL07S, and found a trend toward reduction in serum OVA-specific and total IgE, especially at 1 mg. In the present study, ODN BL07S was administrated at 1 mg/200 µl PBS by gastric gavage on days 0–2 and 7–9. An equal volume of PBS was given to mice in naïve and control groups. Sera and spleens were collected on day 20 to estimate Ig levels in sera and cytokine responses in cultured splenocytes. Total IgE, IgG1, and IgG2a levels were assessed using an ELISA kit (Bethyl Laboratory, Montgomery, TX) according to the manufacture's instructions. OVA-specific IgE, IgG1, and IgG2a were evaluated by our previous methods.¹⁸⁾ Standard immune serum was obtained by intraperitoneal immunization of mice with 10 µg of OVA adsorbed to alum, repeated 3 times at 10-d intervals. OVA-specific IgE, IgG1, and IgG2a titers were calculated by comparison to standard immune serum. Concentrations of OVA-specific IgE, IgG1, and IgG2a in the standard immune serum were arbitrarily considered to be 100 U/ml. Single splenocyte suspensions were prepared in cold RPMI 1640 medium (Invitrogen, Carlsbad, CA) and cultured for each mouse at 4.0×10^6 cells/ml in medium containing 10% fetal calf serum (FCS; Invitrogen), 100 µg/ml penicillin, and 100 U/ml streptomycin

(Invitrogen). Cells were incubated in 24-well flat-bottomed culture plates with 100 µg/ml OVA at 37 °C in a 5% CO₂ atmosphere. Following incubation for 72 h, culture supernatants were harvested and kept at –20 °C until cytokine analysis. Induction of transforming growth factor (TGF)- β was performed as described by Nakao *et al.*, with some modifications.¹⁹⁾ Splenocytes were suspended at 4.0×10^6 cells/ml in AIM-V medium (Invitrogen) supplemented with 100 µg/ml penicillin and 100 U/ml streptomycin (Invitrogen) without FCS, and cultured for 96 h with 100 µg/ml OVA. Culture supernatants were activated by 1 N HCl for 10 min at room temperature and neutralized to pH 7.2 with 1.2 N NaOH/0.5 M HEPES buffer (Invitrogen) to measure TGF- β . The following cytokines were examined in this study: interferon (IFN)- γ , TGF- β , IL-4, IL-5, IL-10, and IL-12p40, using ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the manufacture's instructions. Experimental data were expressed as mean $\pm$ SD. All statistical analyses for obtained results were performed using SPSS version 11.0 statistical software (SPSS, Tokyo). The significance of differences in mean values was determined using unpaired Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

OVA-specific IgE levels were significantly lower and total IgE levels tended to be lower ($p = 0.09$) in the ODN group than in the control group (Fig. 1). Levels of OVA-specific and total IgG2a (Th1-associated) in the ODN group were significantly higher than in the control group (Table 1). No significant differences were found in levels of OVA-specific or total IgG1 (Th2-associated) between the two groups on day 20 (Table 1), but a significantly lower level of OVA-specific IgG1 was observed on day 17 in the ODN group than in the control group (control group: 22.2 ± 5.7 U/ml, ODN group: 11.7 ± 7.9 U/ml, $p < 0.05$). As an index of systemic Th1/Th2 balance, ratios of OVA-specific IgG2a levels/

Table 1. Effects of ODN BL07S on IgG2a and IgG1 Production in OVA-Primed BALB/c Mice

		Naïve	Control	ODN
Total	IgG2a (μg/ml)	43.6 ± 38.3	95.9 ± 39.0	150.2 ± 36.8*
	IgG1 (μg/ml)	145.2 ± 66.7	2617.7 ± 599.2	2119.4 ± 1003.3
OVA-specific	IgG2a (U/ml)	ND	68.5 ± 39.1	140.4 ± 60.9*
	IgG1 (U/ml)	ND	51.3 ± 8.3	44.1 ± 15.5
Th1/Th2 balance ratio (OVA-specific IgG2a/IgG1)		—	1.30 ± 0.64	3.53 ± 2.29*

Immunization and administration of ODN were performed as described in Fig. 1. Blood was collected on day 20 and immunoglobulin levels were determined using ELISA. Data are shown as mean ± SD from seven mice per group. * $p < 0.05$ versus controls. ND, not detected.

Table 2. Cytokine Levels in Supernatants of Cultured Splenocytes from Mice Sensitized with OVA

Cytokines (pg/ml)	Naïve	Control	ODN	<i>p</i> value
IL-12p40	40.1 ± 8.1	38.9 ± 7.7	44.4 ± 9.4	0.09
IFN-γ	ND	90.5 ± 37.6	233.7 ± 157.1	<0.01
IL-4	ND	102.4 ± 71.3	66.7 ± 32.0	0.10
IL-5	ND	1034.7 ± 450.9	786.5 ± 163.1	0.06
IL-10	ND	396.0 ± 105.1	351.6 ± 52.8	0.17
TGF-β	139.68 ± 20.1	141.3 ± 31.2	145.2 ± 33.0	0.64

Immunization and administration of ODN were performed as described in Fig. 1. Spleens were removed on day 20 and single splenocytes were incubated with 100 μg/ml OVA for 72 h. Cytokine levels were determined using ELISA. Data represent mean ± SD from seven mice per group. ND, not detected.

OVA-specific IgG1 in the ODN group were significantly elevated as compared to the control group (Table 1).

To clarify the mechanisms involved in the inhibition of IgE production by ODN BL07S, we investigated cytokine inductions from splenocytes (Table 2). Levels of IFN-γ were significantly higher in the ODN group than in the control group. IL-12p40 tended to be produced at higher levels in the ODN group than in the control group, but no significant difference was found ($p = 0.09$). Levels of IL-4 and -5 in the ODN group tended to be lower compared to the control group, but again, no significant differences were seen ($p = 0.10$ and 0.06 respectively). No differences in levels of regulatory cytokines IL-10 and TGF-β were found between the two groups.

From the above results, we found that oral administration of ODN BL07S stimulated Th1 immune responses and suppressed Th2 immune response, resulting in inhibition of antigen-related IgE production and improved Th1/Th2 balance. These results suggest a potential use of ODN BL07S in the treatment of type I allergic diseases. Recently, oral administration of a CpG-ODN has been reported to suppress OVA-induced allergic reaction,²⁰⁾ but most CpG-ODNs were designed based on genomic DNA of *Escherichia coli* and were partially modified to enhance immunostimulatory effects.^{21,22)} To the best of our knowledge, no previous reports have demonstrated the effects of a natural DNA sequence derived from probiotics in suppressing antigen-related IgE production on oral administration. Our previous *in vitro* and *in vivo* results suggest that ODN BL07S suppresses Th2 responses, but the regulatory mechanisms remain unclear. We speculate as to the involvement of regulatory T cells, secreting regulatory

cytokines IL-10 and TGF-β, but supporting data remains elusive. Similarly, no differences were found in the levels of these regulatory cytokines in the present study, suggesting that these cytokines might not be associated with the immunoregulatory effects of ODN BL07S. We observed induction of serum IL-12p40 (377.4 ± 73.0 pg/ml, average of three mice) following single oral administration of 1 mg ODN BL07S to non-sensitized BALB/c mice, although the levels of serum IL-12p40 in PBS-administrated mice used as a negative control were below the limits of detection in this experimental model. Induction of serum Th1 cytokines has rarely been reported on oral administration of food agents. These results suggest that induction of systemic Th1 responses might be associated with the suppression of Th2 responses by ODN BL07S, although further studies are needed.

Expression of TLR9, receptors of ISS-ODNs, was recently observed in gut-associated lymphoid tissue such as mesenteric lymph nodes and Peyer's patches in mammals.^{23–25)} Genomic DNA from probiotics also has been shown to exert anti-inflammatory effects in mice *via* TLR9 signaling.^{26,27)} Iliev *et al.* identified an ODN sequence with strong immunostimulatory activity from the genomic DNA of *Lactobacillus* GG, which has been investigated intensely for potential immunomodulatory and antiallergic effects *in vitro*, *in vivo*, and in clinical studies.^{13,28,29)} Taken together with our present results, these studies indicate that TLR9-mediated immunomodulatory effects by genomic or digested DNA containing ISS-ODNs from probiotics might affect intestinal immune responses and contribute to the antiallergic effects of probiotics.

In conclusion, we found that oral administration of an

ISS-ODN from *B. longum* BB536 inhibits IgE production, suggesting the possible involvement of ISS-ODNs in the antiallergic efficacy of this probiotic. Further studies are now underway to determine how ISS-ODNs from BB536 stimulate systemic Th1 immune responses and inhibit Th2 responses *via* the gut immune system.

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