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Modulatory Activity of CpG Oligonucleotides from *Bifidobacterium longum* on Immune Cells

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Abstract The purpose of this study was to characterize and investigate the immune activity of CpG oligodeoxynucleotides (ODNs) from *Bifidobacterium longum*. Bacterial CpG motifs have attracted considerable interests because of their immunomodulatory activities. Genomic DNA from *B. longum* was prepared and amplified for 4 different 180-188-mer double-stranded ODNs (BLODN1-BLODN4). When immune cells (RAW 264.7 murine macrophages and JAWS II dendritic cells) with these ODNs were treated, BLODN4 induced the highest immune activity. To assess the effectiveness of the CpG sequences within BLODN4, single-stranded 40-mer ODNs containing CpG sequences (sBLODN4-1, sBLODN4-2) were synthesized. sBLODN4-1 induced higher level of cytokines such as interleukin (IL)-12p40 and tumor necrosis factor (TNF)-α by macrophage and IL-6 and TNF-α by dendritic cells than did sBLODN4-2. The results suggest that CpG ODNs-enriched components of *B. longum* might be useful as an immunomodulatory functional food ingredient.

Keywords: immune cell, probiotics, *Bifidobacterium longum*, CpG DNA

Introduction

Bacterial DNA is known to stimulate immune cells and initiate Th1 type immunity (1). These abilities are partially related to their unmethylated CpG oligodeoxynucleotides (ODNs) motif, which are involved in pathogen-associated molecular pattern (PAMP). Bacterial CpG DNA is more frequently unmethylated than vertebrate CpG DNA and it shows stronger immunoactivity than that of vertebrate CpG DNA (2). Therefore, bacterial CpG ODNs are considered an important immune adjuvant for the gene therapy in various murine models of disease. The probiotic microorganisms such as *Bifidobacterium* species are interesting because they belong to the high-guanine, cytosine (GC) probiotic microorganisms and exert beneficial functions in normalizing unbalanced intestinal microflora by suppressing the growth of harmful bacteria (3). *Bifidobacterium* species are also known to promote potential anti-allergenic processes through the induction of Th1 type immunity and enhancement of regulatory lymphocytes (4,5). Although several studies have investigated the application of genomic DNA of probiotic microorganisms for the regulation of various immune diseases (6-9), CpG ODNs have not been investigated from probiotic microorganisms yet. In this study, we attempted to identify immunomodulatory CpG DNA sequences from *Bifidobacterium* species and evaluated their effects on macrophages and dendritic cells.

Materials and Methods

Bacterial strains and growth conditions Various *Bifidobacterium* strains (*B. longum* KCCM 12099, *B. bifidum* ATCC 15521, *B. infantis* KCTC 3249, *B. adolescentis* ATCC 15703, and *B. breve* ATCC 15700) were anaerobically cultured at 37°C for 18 hr in brain-heart infusion (BHI) broth (Difco, Detroit, MI, USA) containing 0.05% L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) and 1% glucose. The bacteria were collected by centrifugation at 1,000×g for 15 min at 4°C and washed twice with phosphate buffered saline (PBS). After being washed, the collected bacteria were stored at −20°C for DNA extraction and polymerase chain reaction (PCR).

Preparation of JAWS and RAW 264.7 cells JAWS II immature mouse bone-marrow-derived dendritic cells were maintained in minimum essential medium (alpha-MEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Sigma-Aldrich), 20% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen), and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 5-6 days. RAW 264.7 immune mouse bone-marrow-derived macrophages were maintained in medium RPMI (Gibco-BRL) supplemented with 20% heat-inactivated FBS and 1% penicillin/streptomycin, and incubated at 37°C in a humidified atmosphere of 5% for 3-4 days.

Preparation of bacterial DNA Cultured *Bifidobacterium* cells were washed twice with TES buffer [50 mM NaCl, 30 mM Tris (pH 8.0), 5 mM ethylenediamide tetraacetic acid (EDTA)], resuspended in 12 mL of lysis buffer [25% sucrose, 50 mM Tris, 1 mM EDTA (pH 8.0)] with lysozyme (1%, w/v), and incubated at 37°C for 1 hr. Then, 12 mL of 0.25 M EDTA (pH 8.0) was added and the cells were incubated at room temperature for 5 min, after which 4.8 mL of 20% sodium dodecyl sulfate (SDS) was added and the cells incubated at 65°C for 30 min. Proteinase K (60 μL of 20 mg/mL) was added, and the samples were incubated

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Table 1. PCR primer sequences for specific CpG motif, their expected target sites in the double-stranded chromosomal DNA in *B. longum*, and synthesized complementary 2 single stranded ODNs (sBLODN4-1, sBLODN4-2)

	Primer sequence and target site	CpG motif	Accession No.
BLODN1	5'-TACTGGCGTCGACAAGAATGCGGCACC-3' 5'-CGGGCGCATGCTTTGGATCAATCAGAGC-3'	**TCGTCG**TTG	NC004307
BLODN2	5'-CGCTGATCGTGGAGCATCGCGACCGgcT-3' 5'-GGCCCCGCGGCGTCCGTACAGTCTCGC-3'	**TCGTCGTCG**TGG	NC004307
BLODN3	5'-GCGGTGGCCATGGAGGCACGGGCGCCG-5' 5'-GGACGGCACCGTGcGTCTTGAAGGCCTG-3'	ATCGACGTT	NC004307
BLODN4	5'-TCGTCGTCGTGGACGACACGGGACTCGA-3' 5'-CTGCCGTGGGGTCGGGTCGAGCCGGATTC-3'	**TCGTCGTCG**TGG	NC004307
	Synthesized single stranded ODNs		
sBLODN4-1	5-TCGTCGTCGTGGACGACACGGGACTCGACGACGATCTGGT-3'		
sBLODN4-2	5-ACCAGATCGTCGTCGAGTCCCGTGTCGTCCACGACGACGA-3'		

for another 15 min at the same temperature. To extract the DNA, the incubated mixture was gently mixed with an equal volume of phenol:chlorophorm:isoamyl alcohol (25:24:1). After centrifugation at 1,000×g for 30 min, the aqueous phase was transferred to a clean tube, to which an equal volume of benzyl chloride was added, and the sample was gently mixed. After centrifugation at 1,000×g for 10 min, the aqueous phase was transferred to a clean tube. To this solution, RNAase solution (10 mg/mL in autoclaved distilled water) was added to a final concentration of 20 μg/mL, and it was incubated at 37°C for 1 hr. Finally, 2 volumes of absolute ethanol were added to the mixture. After the sample had been mixed slowly, the DNA was spooled, rinsed with 70% ethanol, and resuspended in TE buffer.

Preparation of double-stranded ODNs by PCR To amplify 4 CpG-motif-specific ODNs, BLODN1- BLODN4, from *B. longum* with PCR, the corresponding primer sets were synthesized (Dyne Bio Inc., Seoul, Korea). Their sequences and expected target sites are listed in Table1.

Synthesis of single-stranded ODNs The positive control ODN containing CpG motif (5'-TCCATGACGTTCCTGA TG-3') and the negative control ODN containing no CpG motif (5'-TGCTGCTTTTGTGCTTTTGTGCTT-3') were purchased from Dyne Bio Inc. Two complementary 40-nt single-stranded ODNs (sBLODN4-1, sBLODN4-2) were also synthesized from Dyne Bio Inc. The 2 synthesized oligonucleotides had the following sequences:
sBLODN4-1: 5'-TCGTCGTCGTGGACGACACGGGAC TCGACGACGATCTGGT-3'
sBLODN4-2: 5'-ACCAGATCGTCGTCGAGTCCCGTGT CGTCCACGACGACGA-3'

Cytokine measurement The levels of interleukin (IL)-6, IL-12p40, and tumor necrosis factor (TNF)-α from immune cells were assayed by using enzyme-linked immunosorbent assay (ELISA) protocol (BD Bioscience, San Jose, CA, USA).

Statistical analysis Comparisons between groups were performed using SAS systems V8 and analysis of variance (ANOVA) was used for comparisons among all groups. One-way ANOVA followed by Duncan's multiple-comparison tests. All data are expressed as mean±standard deviation (SD). Values of p less than 0.05 are considered significant.

Results and Discussion

Identification and synthesis of CpG oligonucleotides from *B. longum* First, we applied the genomic DNA (10 μg/mL) from various strains of *Bifidobacterium* to murine macrophages and measured the levels of cytokines. The DNA of *Bifidobacterium* species induced similar cytokine levels except *B. longum* DNA which induced higher levels of cytokines than did the DNAs of the other *Bifidobacterium* species (data not shown). Previously, specific CpG ODN containing the 5'-TCGTCG-3' was reported to induce particularly strong immune activity compared with various other CpG motifs (10,11). Based on this information, specific ODNs containing the 'TCGTCG' sequences in the genome of *B. longum* were confirmed by applying the PSI-BLAST program. Consequently, 3 different 180-188-mer double stranded CpG ODNs (BLODN1, BLODN2, and BLODN4) containing the 'TCGTCG' motif and another 190-mer double-stranded CpG ODN (BLODN3) which did not contain 'TCGTCG' motif were amplified by PCR from the chromosomal DNA of *B. longum.*

Effect of CpG oligonucleotides on production of cytokines from immune cells When they were treated with JAWS II and RAW 264.7 cells at 10 μg/mL the BLODN4 showed the highest level of cytokines such as IL-12p40 and IL-6 from RAW 264.7 cells and IL-12p70 from JAWS II cells, respectively (Fig. 1-3). To further characterize the functional strand of BLODN4, we treated immune cells with 2 single stranded-complementary 40-mer ODNs (sBLODN4-1, sBLODN4-2) at 10 μg/mL. The sBLODN4-1 induced greater production of IL-6, IL-12p70, and TNF-α from JAWS II cells and IL-12p40 from RAW 264.7 cells, respectively, than did sBLODN4-2 and negative control ODN (Fig. 4 and 5).

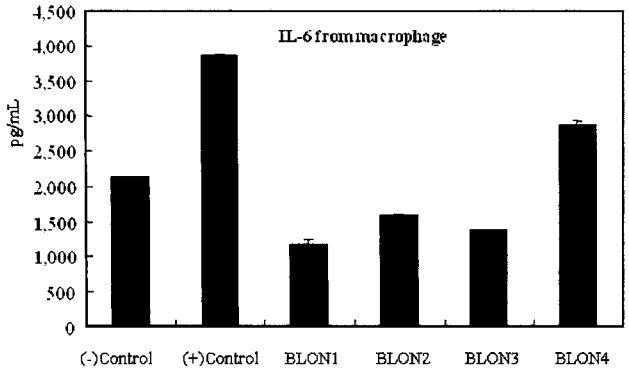

Fig 1. Production of IL-6 from macrophage cells treated with amplified 4 different ODNs (BLODN1-BLODN4) (10 μg/mL).

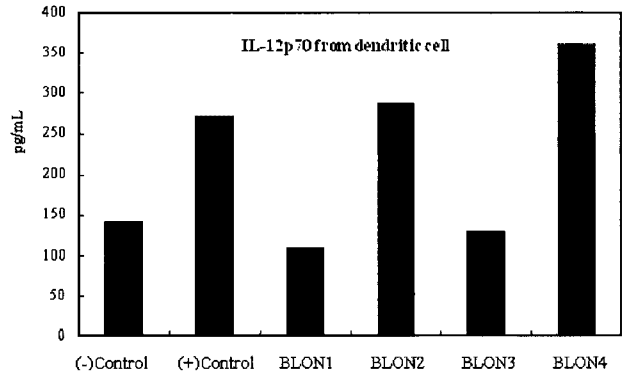

Fig 2. Production of IL-12p70 from dendritic cells treated with amplified 4 different ODNs (BLODN1-BLODN4) (10 μg/mL).

Significance of CpG oligonucleotides from *Bifidobacterium* in immune cells Bacterial DNA containing unmethylated CpG motifs is known to be PAMP, which interacts with host immune cells via a Toll-like receptor to induce various immune responses (12). The gut contains diverse bacterial species that together play important roles affecting the physiology of their host. Among them, the probiotic microorganisms have been reported to improve the balance of the intestinal microflora (13), contribute to the development of regulatory T cells in the gut, and suppress chronic inflammation (14,15). In particular, *Bifidobacterium* species with high-GC genomes are predominant in the gastrointestinal tracts of breast-fed infants and are believed to play an important role in gut homeostasis and the normal development of intestinal immunity (4). The intake of *Bifidobacterium* is potentially effective in relieving allergic symptoms and modulating the Th1/Th2 balance (16). Despite these reported immunoregulatory functions of *Bifidobacterium*, little is known about CpG DNA motifs on the immune cells. In this study, both of the synthetic single-stranded ODN (sBLODN4-1) and double-stranded ODN (BLODN4) containing the 'TCGTCG' motif were able to induce cytokines such as IL-12p40 and TNF-α from murine macrophages and IL-12p70, IL-6, and TNF-α from dendritic cells, respectively. Cytokines such as IL-6, IL-12p40, and TNF-α are among the first proinflammatory cytokines, produced by phagocytic cells in response to encounters with pathogenic agents. The production of these cytokines from the immune cells exposed to *Lactobacillus*

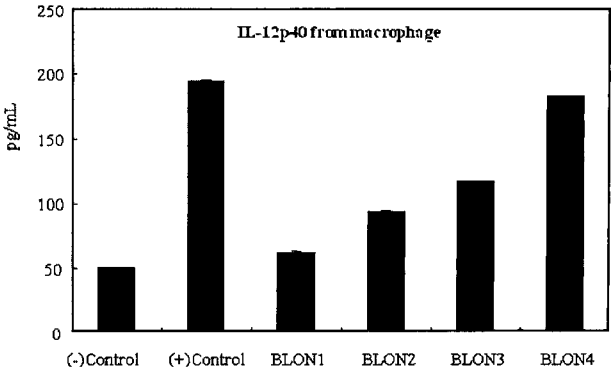

Fig 3. Production of IL-12p40 from macrophage cells treated with amplified 4 different ODNs (BLODN1-BLODN4) (10 μg/mL).

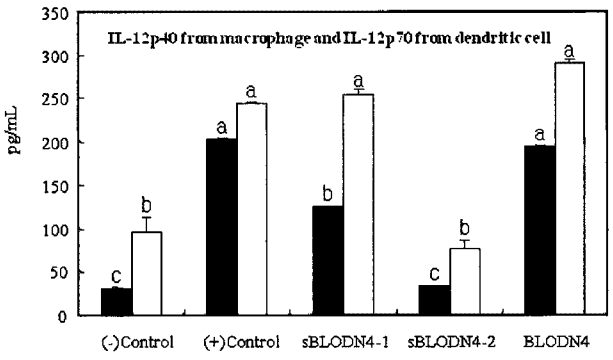

Fig 4. Production of Il-12p40 from macrophage cells and IL-12p70 from dendritic cells treated with synthetic CpG ODNs (sBLODN4-1, sBLODN4-2) (10 μg/mL).

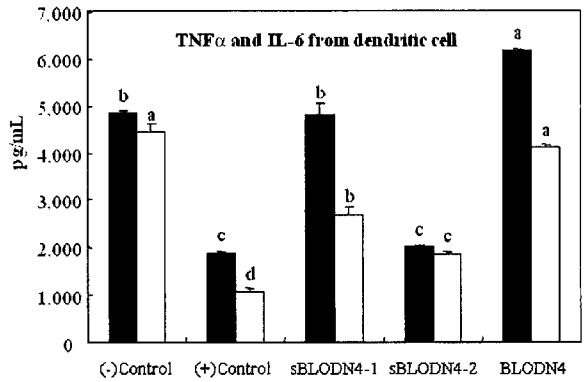

Fig 5. Production of TNFα and IL-6 from dendritic cells treated with synthetic CpG ODNs (sBLODN4-1, sBLODN4-2) (10 μg/mL).

cells was also related to the suppression of food allergy by *L. rhamnosus* GG (17). Several studies have shown that the *Bifidobacterium* is able to activate immune cells such as macrophage, natural killer, and B cells (18). However, the specific functional sequences with immune regulatory activity have not been characterized before. Thus, the successful identification and characterization of the immunoregulatoy CpG ODNs from *Bifidobacterium* genome in the present study would contribute to the development of probiotic-based immune therapeutic strategies including those related to allergies and cancer and development of DNA vaccines.

Acknowledgments

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