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Effect of *Bifidobacterium* Cell Fractions on IL-6 Production in RAW 264.7 Macrophage Cells

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Abstract *Bifidobacterium* has been previously shown to potentiate immune function, which was mediated through the stimulation of cytokine production by macrophage. This study was performed to further characterize the effective component of *Bifidobacterium* by measuring the level of interleukin (IL)-6 cytokine using the RAW 264.7 murine cell line as a macrophage model. RAW 264.7 cells were cultured for 24 h in the presence of whole cells (WCs), cell walls (CWs), and cell-free extracts (CFEs) from various strains of *Bifidobacterium* and other lactic acid bacteria at various concentrations. The most effective component was different depending on the strains and the concentrations used. When tested with each cell fraction from *Bifidobacterium* sp. BGN4, heat treatment of the cell fractions lowered the production of IL-6. Synergistic effect was obtained, especially when CWs and CFEs were combined. Sonicated WCs stimulated IL-6 production more than intact WCs. The *in vitro* approaches employed here should be useful in further characterization of the effects of *Bifidobacterium* on gastrointestinal and systemic immunity.

Key words: *Bifidobacterium*, IL-6, macrophage, cell fractions

Bifidobacterium is predominant in the lumen of the large intestine. In breast-fed infants, *Bifidobacterium* comprises more than 90% of the gut bacterial population [19], but, their numbers gradually decrease over the life time of the host. *Bifidobacterium* spp. is used in commercial fermented dairy products and has been suggested to exert health promoting effects on the host by maintaining intestinal microflora balances, improving lactose tolerance, preventing inflammatory bowel disease, and aiding anticarcinogenic activity [10, 11, 13]. In addition, Hattori *et al.* [8] reported that the administration of *Bifidobacterium* for 1 month to children with atopic dermatitis showed significant improvement of allergic symptoms.

Other beneficial effects of the intake of *Bifidobacterium* are reported to include reinforcement of immune functions [28]. It has been shown that *Bifidobacterium* enhances several immune functions, namely, macrophage and lymphocyte activation [7, 25], cytokine secretion [16], and mitogenic response in spleen and Peyer's patches [9, 12, 28]. Such stimulation of the immune response by *Bifidobacterium* has been proposed to enhance resistance to infection by pathogenic organisms [24] and potentially prevent cancer [25, 26]. Cell components of *Bifidobacterium* that function as immunomodifiers of the host reportedly include peptidoglycan, intra- and extracellular polysaccharide products, cell-free extracts (CFEs), and cell walls (CWs) [6, 7, 9, 20, 25, 26]. However, at the present time, there is not yet a clear understanding of the molecular and cellular basis for immunomodulation by *Bifidobacterium.* To utilize a potential of the *Bifidobacterium* for immunomodulation of the host, a better understanding of the quantitative and qualitative assessment of the immunomodulatory effect of the *Bifidobacterium* is needed.

Miettinen *et al.* [18] measured production of tumor necrosis factor (TNF)-alpha, interleukin (IL)-6, and IL-10 from human peripheral blood mononuclear cells after stimulation with live or glutaraldehyde-fixed bacteria and suggested that lactic acid bacteria can stimulate nonspecific immunity. Using macrophage as a model system, we earlier showed that the modulatory effect of the *Bifidobacterium* on IL-6 production was strain- and dose-dependent. In the present study, we further characterized the different cell components and their processing on the immunostimulation in the RAW 264.7 murine macrophage model by measuring IL-6 production as a representative macrophage cytokine.

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The results showed that the stimulatory activity of the *Bifidobacterium* cell fractions differed, depending on the strains and the concentrations used as well as the processing conditions of the cell components. Our study suggests that better understanding of the effective component and the immunological state of the host is necessary to achieve a desirable immunological balance for the host.

MATERIALS AND METHODS

Bifidobacterium Cultures

B. adolescentis 15703, *B. longum* 15707, *B. infantis* 15697, and *B. breve* 15700 were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The identification and experimental use of *Bifidobacterium* sp. BGN4, *Bifidobacterium* sp. JH-2, *Bifidobacterium* sp. MS-1, *Bifidobacterium* sp. SJ342, *Bifidobacterium* sp. UN-4, and other lactic bacteria were reported before [3, 22]. All strains were cultured and subcultured anaerobically in MRS broth (Difco, Detroit, MI, U.S.A.) containing 5% (wt/vol) lactose (MRSL) at 37°C until the late-log phase [15, 21]. Cells were collected by centrifugation at 1,000 ×*g* and 4°C for 15 min and washed twice with PBS, followed by final washing with distilled water [14]. They were dried by Speed-Vac (Instruments, Inc., N.Y., U.S.A.) and resuspended in Hank's buffered salt solution (Sigma Chemical Co., St. Louis, MO, U.S.A.) to desired bacterial concentration on a dry weight basis. For introduction into tissue culture, *Bifidobacterium* was ordinarily killed by heating at 100°C for 20 min. Heat-killed cultures were aliquoted and stored at −80°C until used.

Preparation of Cell Fractions

For the preparation of the cell fractions, cultured bacterial cells were collected by centrifugation at 2,000 ×*g* for 20 min (Hanil MF-80, Inchun, Korea) and washed twice with autoclaved PBS, followed by final washing with autoclaved distilled water. For the preparation of CWs and CFEs, washed WCs were disrupted by French Pressure Cell Press (Spectronic, Rochester, N.Y., U.S.A.). The WCs were removed from the suspension by centrifugation at 2,000 ×*g* for 20 min. CWs were sedimented by centrifugation at 15,000 ×*g* and 4°C for 45 min (Hanil Micro 17R+, Inchon, Korea) and the supernatant was used as CFEs. Each fraction was freeze dried and resuspended with DMEM to the desired concentration on a dry weight basis. Suspended bacterial fractions were stored at −20°C until used.

Chemicals and Reagents

IL-6, purified antibodies to IL-6 antibodies (rat anti-mouse), and biotinylated rat anti- or IL-6 were obtained from PharMingen (San Diego, CA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Chagrin Falls, IL, U.S.A.).

Cell Culture

The mouse macrophage cell line RAW 264.7 (American Type Tissue Collection) was grown in DMEM supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 1% (v/v) NCTC-135, streptomycin (100 μg/ml), and penicillin (100 U/ml). All cultures were carried out at 37°C in a humidified atmosphere with 5% CO_2 [29]. Cell number and viability were assessed by trypan blue dye exclusion [27] on a Neubauer hemacytometer (American Optical, Buffalo, N.Y., U.S.A.). Cells were grown to confluency in sterile tissue culture dishes and gently detached by repeated pipetting. For experiments, cells were cultured in triplicate at a density of 5×10^5 cells/ml in 96-well flat-bottomed tissue culture plates (Costar, Cambridge MA, U.S.A.). Cultures containing bacterial cell fractions were incubated for various time intervals and analyzed for IL-6. Heat treatment of the fractions were ordinarily done at 100°C for 20 min to sterilize bacterial contaminants. When unheated fractions were assessed, they were treated at 10 cm below ultraviolet light (Sankyo Germicidal UV Lamp G40TO, Japan) for 30 min.

IL-6 Quantitation

Production of IL-6 was quantitated by ELISA using modification of the procedure of Dong *et al.* [4]. Briefly, microtiter strip wells (Immunolon IV Removawell; Dynatech Laboratories Inc., Chantilly, VA, U.S.A.) were coated overnight at 4°C with 50 μl of 1 μg/ml purified antibodies to IL-6 antibodies (rat anti-mouse) in 0.1 M sodium bicarbonate buffer (pH 8.2). Wells were incubated with 300 μl of 3% (v/v) bovine serum albumin (BSA) in 0.01 M PBS (pH 7.2), containing 0.2% (v/v) Tween 20 (PBST), at 37°C for 30 min to block nonspecific protein binding. Standard recombinant murine IL-6 and samples, diluted in 10% (v/v) FBS RPMI-1640, were added in 50 μl aliquots to appropriate wells and incubated at 37°C for 1 h. After washing four times with PBST, biotinylated rat anti-mouse IL-6 antibodies were diluted in BSA-PBST to 1 μg/ml and 1.5 μg/ml, respectively, and 50 μl each were added and incubated at room temperature for 1 h. Plates were washed six times and incubated with 50 μl of streptavidin-horseradish peroxidase conjugate (1.5 μg/ml in BSA-PBST) at room temperature for 1 h. After washing eight times, bound peroxidase conjugate was detected by adding 100 μl/well solution of substrate consisting of 25 ml of 0.1 M citric-phosphate buffer (pH 5.5), 0.1 mg/ml TMB, and 100 μl of 1% H_2O_2. An equal volume of 6 N H_2SO_4 was added to stop the reaction. The plates were read at 450 nm on a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA, U.S.A.). The IL-6 was quantitated using

Table 1. Effect of *Bifidobacterium* and *Lactobacillus* cell fractions on IL-6 production by RAW 264.7 cells.

Strain	Treatment	IL-6 concentration (ng/ml)								
		WC (μg/ml)			CW (μg/ml)			CFE (μg/ml)		
		0.5	2.5	12.5	0.5	2.5	12.5	0.5	2.5	12.5
B. adolescentis ATCC 15703		ND	ND	1.5±0.3	ND	10.4±0.3	23.3±1.1	ND	1.7±0.2	4.2±0.3
B. breve ATCC 15700		ND	1.7±0.3	5.0±0.4	ND	2.1±0.2	11.3±0.2	ND	2.5±0.1	5.8±0.1
B. infantis ATCC 15697		ND	ND	4.5±0.7	ND	10.8±0.5	21.7±1.0	ND	ND	10.0±0.5
B. longum ATCC 15707		ND	ND	14.1±0.3	ND	1.8±0.2	22.5±0.9	ND	10.8±0.4	18.8±0.7
Bifidobacterium sp. BGN4		ND	1.5±0.3	9.3±0.5	ND	2.7±0.3	10.8±1.0	ND	5.2±0.1	15.8±0.4
Bifidobacterium sp. JH2		ND	ND	3.3±0.2	ND	ND	11.7±0.5	ND	8.3±0.9	12.1±0.3
Bifidobacterium sp. MS1		ND	ND	1.7±0.3	ND	3.0±0.2	8.2±0.3	ND	2.9±0.2	4.8±0.2
Bifidobacterium sp. SJ32		ND	ND	1.5±0.1	ND	8.3±0.4	14.8±0.2	ND	4.2±0.4	9.0±0.2
Bifidobacterium sp. UN4		ND	12.8±0.9	10.8±0.4	ND	9.2±0.2	12.7±0.1	ND	2.5±0.2	12.3±1.0
Lactobacillus sp. L1		ND	ND	ND	ND	ND	1.7±0.2	ND	ND	ND
Lactobacillus sp. L2		ND	ND	ND	ND	ND	2.5±0.3	ND	1.6±0.2	5.8±0.5
Lactobacillus sp. L4		ND	ND	ND	ND	ND	2.6±0.4	ND	ND	1.8±0.3
Lactobacillus sp. L7		ND	ND	ND	ND	ND	2.8±0.4	ND	ND	ND

Vmax Software (Molecular Devices). Triplicate samples were used throughout the experiments.

RESULTS

Effect of Cell Fractions of Various *Bifidobacterium* on IL-6 Production by RAW 264.7 Cells

To assess the effects of the cell fractions from various *Bifidobacterium* strains on the production of IL-6, RAW 264.7 cells (5×10^5 cells/ml) were incubated in the presence of 0.5, 2.5, and 12.5 μg/ml bacterial cell fractions. To prevent contamination, all bacterial fractions were routinely preheated at 100°C for 20 min. The results for IL-6 production are shown in Table 1. The degree of IL-6 production differed depending on the strains and the concentrations of the cell fractions used. All three fractions from the MS-1 strain showed low level of IL-6 production, compared with the other strains. CWs from strains *B. adolescentis* 15703, *B. longum* 15707, *B. infantis* 15697, *B. breve* 15700, *Bifidobacterium* sp. MS-1, and *Bifidobacterium* sp. SJ-32 showed stronger activity than WCs or CFEs, whereas CFEs from strain BGN4 showed higher IL-6 production than WCs or CWs. The production of IL-6 tended to increase in a dose-dependent manner up to 12.5 μg/ml of all three fractions from most of the strains. For comparison, cell fractions from four strains of lactic acid bacteria were assessed. Fractions from *Lactobacillus* strains showed much lower activity than those from *Bifidobacterium*.

Effect of Heat Treatment and Combination of the Cell Fractions on IL-6 Production

To assess the effects of combination of the cell fractions, the mixed fractions from *Bifidobacterium* sp. BGN4 were added to RAW 264.7 cells. The combinations were as follows; WCs plus CWs, CWs plus CFEs, and WCs plus CFEs. Their final concentrations were 0, 0.5, 12.5, and 25 μg/ml, and each combination contained equal amount

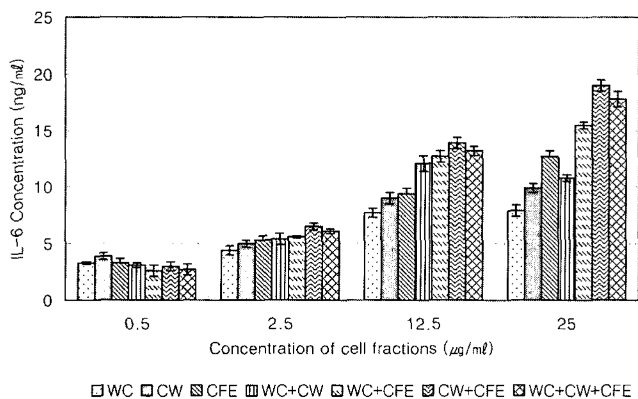

Fig. 1. Effect of combination of BGN cell fractions on IL-6 by RAW 264.7 cells.

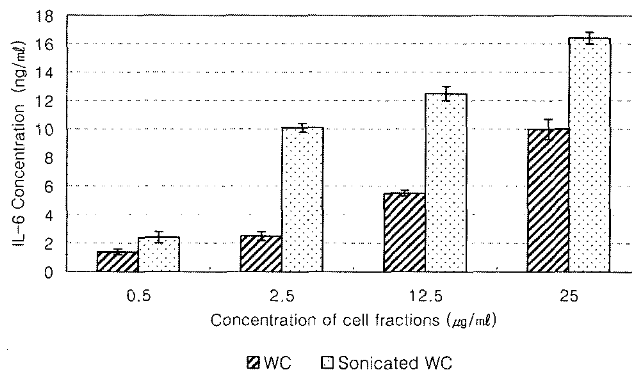

Fig. 2. Effect of sonication of BGN4 WC on IL-6 production by RAW 264.7 cells.

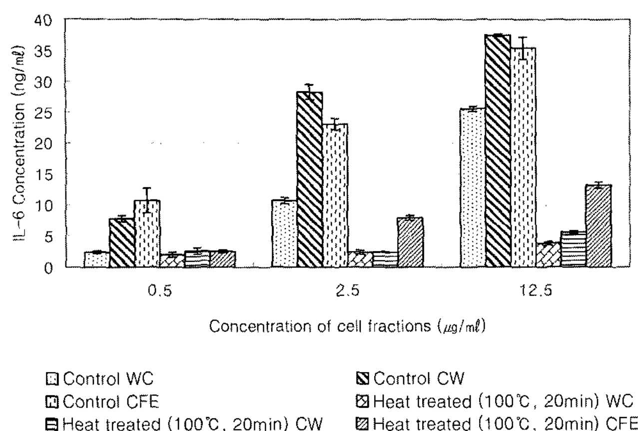

Fig. 3. Effect of heat treatment of BGN4 cell fractions on IL-6 production by RAW 264.7 cells.

of respective fractions. The results are shown in Fig. 1. A synergistic effect was observed when mixed cell fractions were used on the macrophage stimulation. The combination of CWs and CFEs showed the highest stimulation. In addition, sonicated WCs, which was a mixture of different cell fractions, showed higher stimulation than WCs (Fig. 2). When heated and unheated fractions were compared, unheated fractions had much stronger stimulatory activity than heated fraction (Fig. 3). The experimental values of IL-6 production under the same treatment conditions or the control values at the base line slightly differed from experiment to experiment, but showed similar tendency.

DISCUSSION

Bifidobacterium and other lactic acid bacteria have been previously shown to stimulate immune function [6, 12, 17]. Furthermore, *Bifidobacterium* and other lactic acid bacteria can improve antitumor activity of the host [5, 23, 26]. It has been suggested that this activity may be due to their ability to stimulate macrophages and T cells [7, 25]. Sekine *et al.* [26] have proposed critical roles of cytokines played in the antitumor-promoting properties of *B. infantis*; however, the mechanisms by which such bacteria modulate the immune response remains unclear. There is extensive evidence that cytokines play pivotal roles in host defense, inflammatory responses, and autoimmune disease [1, 2].

Park *et al.* [22] showed that both human and commercial *Bifidobacterium* strains can stimulate hydrogen peroxide, nitric oxide, TNF-alpha, and IL-6 productions, and this effect was dependent on dose and strain. In the present study, this phenomenon was further confirmed by the fact that the stimulating activity of each fraction was different, depending on the strain and the dose. This may explain the reason of why different authors reported different cell components of *Bifidobacterium* as immunomodifiers of the host, including peptidoglycan, intra- and extra-cellular polysaccharide products, CFEs, and CWs [6, 7, 9, 20, 22, 25, 26]. Park *et al.* [22] suggested that even morphology and composition of the *Bifidobacterium* cell might play an important role in macrophage activation, and showed that the morphology of the *Bifidobacterium* varied depending on the growth temperature, thus suggesting that even the same strain may show varying stimulatory effect on macrophage depending on the growth conditions or environmental conditions to which *Bifidobacterium* was exposed. Furthermore, processing conditions of the active materials affect the IL-6 production by different degrees. In the present study, heat treatment decreased the production of IL-6, and combination of cell fractions had a synergistic effect on the production of IL-6. From the point of view of health, a balanced immune state is desired, whereas too much enhancement or too much suppression leads to the state of various immune diseases. Therefore, the control of activity of the immune cells is very important. Although these organisms are incorporated into dairy foods or pharmaceuticals, the benefits of these products have not typically been backed by clinical and/or microbiological studies. The present study provides some insight into how and what to consider when designing the application of the probiotic *Bifidobacterium* in order to provide health benefits from the immunological standpoint. However, further studies are necessary.

In conclusion, the results reported here showed that various sources of *Bifidobacterium* increased the secretion of several mediators by macrophage, thereby potentially modulating the host immune response. The results also showed that this stimulatory capacity was affected by dose and strain of the *Bifidobacterium* strain. The *in vitro* approaches employed here should be useful in future characterization to understanding the mechanism of the effects of *Bifidobacterium* on gastrointestinal immunity, and in exploitation of possible enhancement of health benefits using *Bifidobacterium* products.

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