

Modulatory Activity of *Bifidobacterium* sp. BGN4 Cell Fractions on Immune Cells

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Abstract Bifidobacteria has been suggested to exert health promoting effects on the host by maintaining microbial flora and modulating immune functions in the human intestine. We assessed modulatory effects of the different cell fractions of *Bifidobacterium* sp. BGN4 on macrophage cells and other immune cells from the spleen and Peyer's patches (PP) of mouse. Cell free extracts (CFE) of the BGN4 fractions induced well-developed morphological changes in the macrophages and increased the phagocytic activity more effectively than other fractions in the mouse peritoneal cells. Nitric oxide (NO) production was significantly reduced by both the cell walls (CW) and CFE in the cultured cells from the spleen and PP. The production of interleukin-6 (IL-6) and interleukin-10 (IL-10) was eminent in the spleen cells treated with experimental BGN4 cell fractions. However, in the PP cells, IL-6 was slightly decreased by the treatment with the whole cell (WC) and CW, whereas IL-10 was significantly increased by the treatment with the CW and CFE. These results suggest that different types of bifidobacterial cell fractions may have differential immunomodulatory activities depending on their location within the host immune system.

Key words: *Bifidobacterium* sp. BGN4, immune cell, phagocytosis, NO, IL-6, IL-10

Mucosal epithelial cells and microflora cover the large intestines and prevent colonization of the gut by pathogens. Probiotics are strains of microorganisms that confer health benefits to the host. Their beneficial effects include the production of various antimicrobial products, competitive exclusion of enteric pathogens, and modulation of mucosal immune responses [24]. As a probiotic microorganism, *Bifidobacterium* is a nonpathogenic, Gram-positive, and

anaerobic bacteria that inhabits the intestinal tracts of humans and animals. *Bifidobacterium* is the most abundant flora in breast-fed babies [1]; however, the proportion of *Bifidobacterium* continuously decreases after the weaning period. The reduction of bifidobacterial flora was also observed in Crohn's disease patients through an epidemiological study [30]. *Bifidobacterium* showed an immunopotentiating activity in the culture of macrophages and lymphocytes [5, 13, 21], in addition to a mitogenic response in the spleen and PP [6, 9, 10, 12].

Macrophages play a critical role in a host's defense system through physical uptake activity and secretion of immune mediators such as NO and various cytokines [16, 28], which inhibits tumor cells, bacteria, fungi, and parasites. Moreover, the macrophages execute a conservative process through engulfment of extracellular materials through phagocytosis [31]. The PP are collections of subepithelial lymphoid follicles burgeoning amongst villi and distributed throughout the intestine, mainly on the antimesenteric side. A mixture of lymphocytes, macrophages, and dendritic cells found on top of the follicle towards the gut lumen [8] are involved in the induction of a mucosal immune response in the intestine [17, 18]. The secretion of the various immune mediators by the intestinal immune cells needs to be controlled at an appropriate level since they can potentially injure host cells and tissues [3, 4]. Thus, investigation of the secretion and regulation of immune mediators is warranted, when probiotic strains are considered for the modulation of immune balance.

In our previous study, *Bifidobacterium* sp. BGN4 was found to exhibit a prominent adhesive capacity for intestinal epithelial cells [11] as well as modulatory activity on the production of IL-6 and tumor necrosis factor- α (TNF- α) [15]. To further elucidate the immunomodulatory mechanisms of *Bifidobacterium* sp. BGN4, we examined macrophage activation by morphological differentiation and antigen uptake using a macrophage cell line, RAW 264.7, and observed

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secretions of NO, IL-6, and IL-10 in the spleen and PP cells.

MATERIALS AND METHODS

Experimental Animals

BALB/c and C3H/He mice were purchased from the laboratory animal center of Seoul National University. Eight- to twelve-week-old mice were used in this study.

Bifidobacterium Cultures

The identification and experimental use of *B. bifidum* BGN4 was previously reported [21]. All strains were cultured and subcultured anaerobically in MRS broth (Difco, MI, U.S.A.) containing 5% lactose (w/v, MRSL) at 37°C until the late-log phase. Cells were collected by centrifugation at 1,000 ×g for 15 min at 4°C and washed twice with PBS (phosphate-buffered saline) followed by a final washing with distilled water. Freeze-lyophilized BGN4 was resuspended with the cell culture medium to obtain a desired bacterial concentration on a dry weight basis. BGN4 was heat-killed before introduction into the cell culture plate by heating at 95°C for 30 min. Heat-killed cultures were aliquoted and stored at -80°C until use.

Preparation of *Bifidobacterium* sp. BGN4 Cell Fractions

Harvested BGN4 cells were fractionated by a modification of the method according to Okitsu-Negishi *et al.* [20]. Cells grown in the MRS medium were pelleted by centrifugation (1,000 ×g for 20 min). The pellets were washed twice with PBS and were centrifuged again. The packed cells were homogeneously suspended in 30 ml of distilled water followed by disintegration by French Press (Spectronic, NY, U.S.A.). The whole cells were removed from the suspension by centrifugation at 2,000 ×g for 20 min. The cell walls were sedimented by centrifugation at 15,000 ×g for 45 min at 4°C, and the supernatant was used as the CFE. Each fraction was freeze dried (Ilshin, Korea) and resuspended with Dulbecco's modified Eagle's medium (DMEM) (Sigma, MO, U.S.A.) to the desired bacterial concentration on a dry weight basis. Suspended bacterial cells were stored at -20°C until use.

Cell Culture

RAW 264.7 cells were grown to confluence in sterile tissue culture dishes (Costar, MA, U.S.A.) and gently detached by repeated pipetting. Spleens were aseptically extirpated, and single-cell suspensions were prepared by a standard mechanical disruption procedure. The PP was also aseptically detached and washed by stirring at 37°C in RPMI 1640 (containing 1 mg/ml of collagenase IV, Sigma, MO, U.S.A.). After additional washes with RPMI1640 [containing 2% fetal bovine serum (FBS)], the cell suspensions

were passed through a 200-mesh nylon membrane to remove incompletely dissociated epithelial tissue sheet, and then washed twice with RPMI 1640 (containing 2% FBS). All experimental cells were cultured in DMEM or RPMI 1640 supplemented with 10% (v/v) FBS (BioWhittaker, MD, U.S.A.) and 1% (v/v) penicillin-streptomycin (Gibco Brl, NY, U.S.A.). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂ (Sanyo, Japan) for 48 h. Cell number and viability were assessed by the trypan blue dye exclusion method [27].

Chemicals and Reagents

IL-6, IL-10, purified antibodies to IL-6 or IL-10 (rat anti-mouse), and biotinylated rat anti-mouse IL-6 or IL-10 were obtained from PharMingen (San Diego, CA, U.S.A.). DMEM and FBS were obtained from Gibco Laboratories (Chagrin Falls, IL, U.S.A.). Tetramethylbenzidine (TMB) was purchased from Fluka Chemical Corp (Ronkonkoma, NY, U.S.A.).

Scanning Electron Microscopy

A 2×10⁵ cells/ml sample of RAW 264.7 cells was incubated on a coverslip to use bottom-attached cells. Overnight-grown RAW 264.7 cells were co-cultured with BGN4 cell fractions for 24 h. After 24 h of incubation, the cell media and residual particles were decanted, and the cell-attached coverslips were prefixed with glutaraldehyde, washed, and rinsed in a cacodylate buffer. After fixation on osmium tetroxide, the cells were stained with uranyl acetate and dehydrated in a series of graded ethanols. The cell coverslips were critical-point dried using carbon dioxide, and then coated with gold and observed under the scanning electron microscope (SEM). SEM analysis was accomplished with the help of the National Institute of Crop Science (Suwon, Korea).

Phagocytosis Assay

The cells in peritoneal exudates of the sacrificed BALB/c mice were collected by washing the peritoneal cavity with autoclaved PBS [29]. The cells were diluted to 2×10⁵ cells/ml and incubated on coverslips for 2 h with the BGN4 cell fractions. After washing with PBS, 4×10⁵ *Candida albicans* cells were co-incubated with peritoneal cells on the coverslip for 45 min. After washing again with PBS, the coverslips were stained with Wright stain for 10 min. Phagocytic activity was expressed as the percentage of phagocytic cells that had phagocytosed *C. albicans*.

Measurement of NO

The concentration of nitrite in the cell culture supernatant was measured using the Griess Reagent System (Promega, Madison, U.S.A.), which is based on the chemical reaction between sulfanilamide and *N*-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid)

conditions. At first, 50 μ l of each experimental sample was mixed with 50 μ l of the sulfanilamide solution and incubated for 5 min at room temperature. After incubation, 50 μ l of the NED solution was added and incubated for 5 min at room temperature in the dark. Then, the optical density was measured within 30 min in a plate reader with a filter between 520–550 nm.

Quantification of IL-6 and IL-10

The production of IL-6 and IL-10 was detected by ELISA (Enzyme Linked ImmunoSorbent Assay) according to Dong *et al.* [2]. The ELISA plates were read at 450 nm on a V_{\max} Kinetic Microplate Reader (Molecular Devices, CA, U.S.A.). IL-6 and IL-10 were quantitated using V_{\max} Software (Molecular Devices).

Statistical Analysis

Each set of experiments was performed at least three times. All data were presented as mean \pm SEM. The data were analyzed by one-way analysis of variance (ANOVA) using the SAS system (SAS Institute Inc., NC, U.S.A.) and t-test. A probability of $p < 0.05$ was used as the criterion for statistical significance.

RESULTS AND DISCUSSION

Effect of BGN4 Cell Fractions on Macrophage Activation

Previously, our data showed that various fractions of *Bifidobacterium* sp. BGN4 exerted differential effects on the production of cytokines by the macrophage cell line, RAW 264.7 [15]. In this study, we assessed the macrophage activation capacity of BGN4 cell fractions through the SEM and phagocytosis analysis. Macrophage cells stimulated with WC or CFE were larger in size, as depicted by the enlarged surface area [Fig. 1A(b) and (c)] than the control [Fig. 1A(a)]. Furthermore, macrophage cells treated with CFE expressed more differentiated pseudopodia than those treated with WC.

C. albicans is a ubiquitous pathogenic fungus associated with infections of the immunocompromised host [29]. Thus, an efficient and prompt clearance of these organisms is critical in innate immune systems. In this context, we performed a phagocytosis test with the murine peritoneal cells, which consist largely of phagocytic immune cells. Peritoneal cells treated with WC, CW, and CFE showed significantly increased levels of phagocytosis, with CFE showing higher stimulatory activity than WC or CW (Fig. 1B).

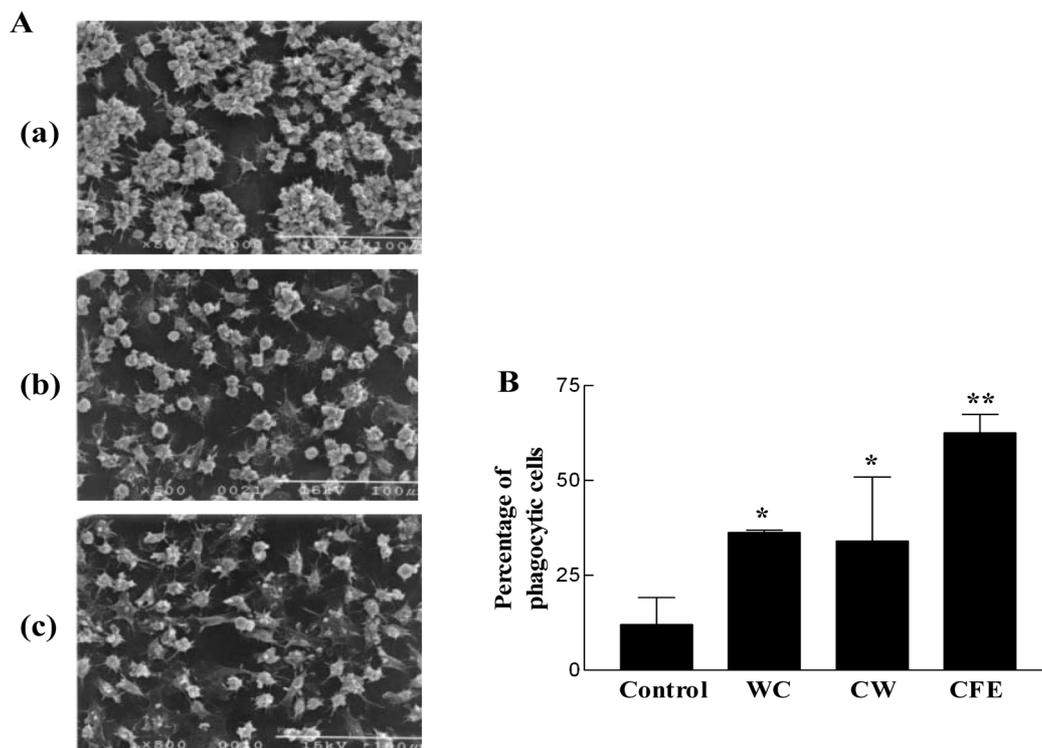


Fig. 1. Effect of BGN4 cell fractions on the activation of macrophage. RAW 264.7 cells and BGN4 cell fractions were co-cultured for 24 h and observed using a scanning electron microscope.

A. (a) Control (RAW 264.7 cells), (b) BGN4 WC (50 μ g/ml) for 24 h, (c) BGN4 CFE (50 μ g/ml) for 24 h. *C. albicans* (4×10^5 cells) was co-incubated with mouse (BALB/c) peritoneal cells and pretreated with BGN4 cell fractions on the coverslip for 45 min. Phagocytic activity was expressed by the percentage of phagocytic cells B. The results are expressed as mean \pm SEM of triplicates (* is defined different from the control, ** is defined by increased activity than the same concentration of WC or CW, $p < 0.05$).

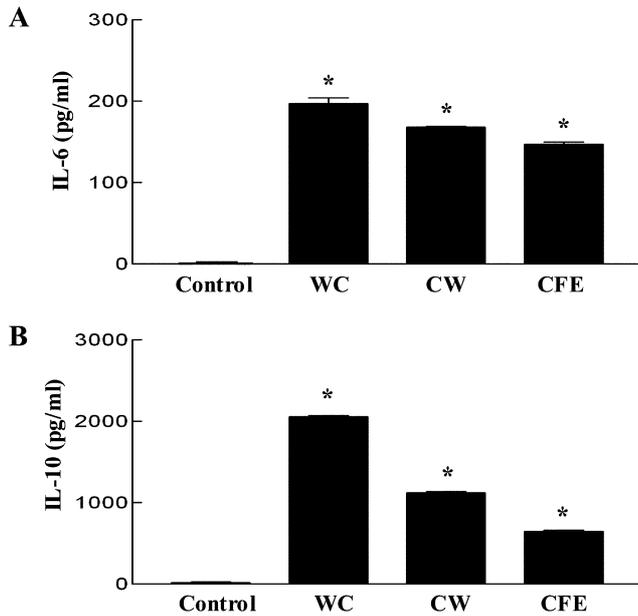


Fig. 2. Effect of BGN4 cell fractions on the production of NO by splenocytes and PP.

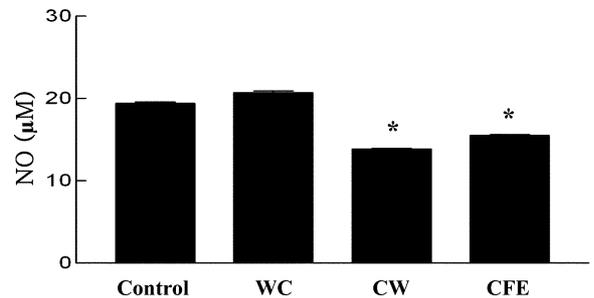
Splenocytes (A) and PP cells (B) (5×10^5 cell/ml) were cultured for 48 h in the presence of BGN4 cell fractions for the detection of NO. The BGN4 cell fractions were preheated at 95°C for 30 min. Data are presented as mean \pm SEM of triplicates (* is defined different from control, $p < 0.05$).

Inhibitory and Regulatory Effect of BGN4 Cell Fractions on NO, IL-6, and IL-10

Figures 2A and 2B indicate that WC did not show any significant difference in the production of NO, when compared with the control. Interestingly, CW and CFE showed significantly reduced levels of NO production. NO is mainly produced from macrophage and monocytes, and is an important mediator of macrophage phagocytosis [12]. However, NO-mediated cell damage enhances the release of a proinflammatory mediator from the macrophages. Enhancement of IL-8 and TNF- α release is also partially NO-dependent in the activated peritoneal neutrophils [24]. Our results suggest that the immunomodulatory effects of *Bifidobacterium* are exerted in a rather sophisticated manner in the spleen and PP than mere reflection of the stimulatory effects observed in the macrophage cells, possibly causing a noted differential effect of WC compared with CW and CFE.

Probiotics are suggested to have direct effects on the intestinal lumen or intestinal immune cells via cytokine induction [19]. Macrophage and lymphocytes play a central role in cell-mediated and humoral immunity through the release of different cytokines such as TNF- α , IL-6, and IL-10 [32]. In the spleen cells, strong IL-6 and IL-10 productions by each fraction were observed, with WC showing the highest secretion level of cytokines (Fig. 3). In PP, the patterns of the production of IL-6 and IL-10 were considerably different from those in the spleen cells.

A. Spleen



B. PP

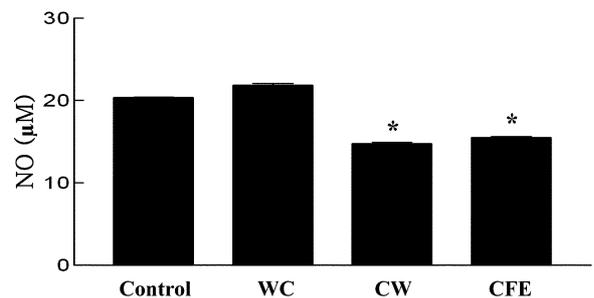


Fig. 3. Effect of BGN4 cell fractions on the production of IL-6 and IL-10 in the splenocytes culture.

Splenocytes (5×10^5 cells/ml) were cultured for 48 h in the presence of various bacterial fractions for the detection of IL-6 (A) and IL-10 (B) production. The bacterial fractions were preheated at 95°C for 30 min. Data are presented as mean \pm SEM of triplicates (* is defined as significantly different from the control, $p < 0.05$).

Interestingly, WC and CW significantly decreased IL-6 production, although by a slight margin. In the case of IL-10 production, CW and CFE showed significantly increased levels, compared with the control (Fig. 4). The results from the PP might reflect better the *in vivo* situation than those from the spleen cells, since the PP continuously contacts with the luminal intestinal bacteria (Figs. 3 and 4) [25]. Furthermore, the differences in the cell composition between spleen and PP could have been due to the differential effects of the different BGN4 cell fractions. Additionally, it was of interest to note that CFE had stronger stimulatory activity for IL-6 and IL-10 production in the PP cells, whereas WC showed greater stimulation in the spleen cells (Figs. 3 and 4).

Although BGN4 cell fractions apparently activated the immune cells, the stimulatory effects of BGN4 varied, depending on the types of the BGN4 cell fractions or the host immune cells. Taken together, BGN4 induced cell differentiation and phagocytosis, and promoted secretion of the anti-inflammatory cytokine, IL-10. Commensal microorganisms continuously interact with the epithelial cell layers and present a number of innate immunity associated antigens via receptors for the pathogen-associated molecular patterns (PAMS). A recent report showed that nonpathogenic

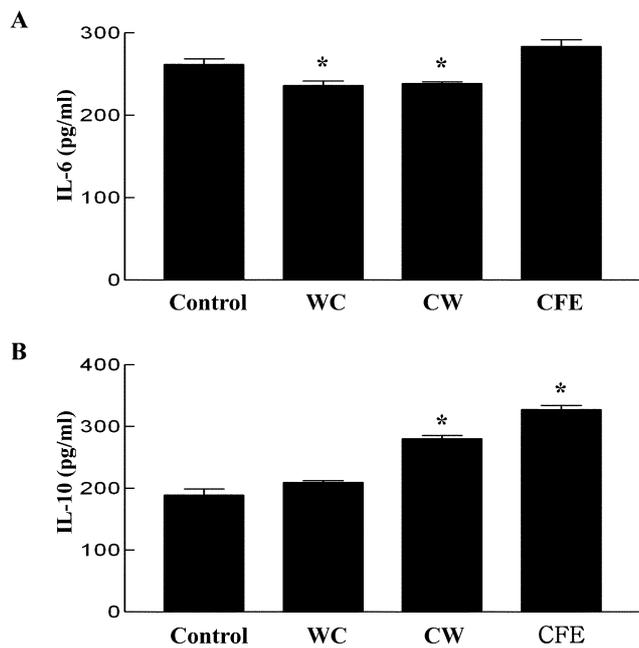


Fig. 4. Effect of BGN4 cell fractions on the production of IL-6 and IL-10 in the PP.

PP cells (5×10^5 cells/ml) were cultured for 48 h in the presence of various bacterial fractions for the detection of IL-6 (A) and IL-10 (B) production. The bacterial fractions were preheated at 95°C for 30 min. The results are expressed as mean \pm SEM of triplicates (* is defined as significantly different from the control, $p < 0.05$).

intestinal bacteria could induce dendritic cells (DC) to migrate into the epithelial layer for antigen sampling from the gut lumen [22]. Particularly in the gut, recruited DC by luminal antigen, including intestinal bacteria, is important for the induction and maintenance of peripheral self-tolerance [7]. Further study on the strain-dependent or bacterial fraction-dependent effects on cytokine production and immune cell activation may deepen the understanding of the role of *Bifidobacterium* in the intestinal immune system.

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