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Cytoskeleton Reorganization and Cytokine Production of Macrophages by Bifidobacterial Cells and Cell-Free Extracts

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Abstract Bifidobacteria have been previously shown to stimulate the immune functions and cytokine production in macrophages and T-lymphocytes. Accordingly, the RAW 264.7 murine macrophage cell line was used to assess the effects of *Bifidobacterium* on the proliferation and cytoskeleton reorganization of the cells. Cytokine production after exposure to *Bifidobacterium* was also monitored in both whole cells and cell-free extracts. When RAW 264.7 cells were cultured for 24 h in the presence of heat-killed *Bifidobacterium bifidum* BGN4, the proliferation of macrophages was slowed down in a dose-dependent manner and cell differentiation was observed by staining with the actin-specific fluorescent dye, rhodamin-conjugated phalloidin. Although EL-4 cells, a T-cell line, stimulated RAW 264.7 cells to produce TNF-α and IL-6, the stimulatory activity of *B. bifidum* BGN4 decreased as the EL-4 cell mumber increased. When disrupted and fractionated BGN4 was used, the whole cell fraction was more effective than the other fractions for the TNF-α production. In contrast, the cell-free extract exhibited the highest IL-6 production level among the fractions, which was evident even at a 1 µg/ml concentration. The current results demonstrate that *Bifidobacterium* induced differentiation of the macrophages from the fast proliferative stage and that the cytokine production was differentially induced by the whole cells and cell-free extracts. The *in vitro* approaches employed herein are expected to be useful in further characterization of the effects of bifidobacteria with regards to gastrointestinal and systemic immunity.

Key words: *Bifidobacterium*, macrophage activation, cytoskeleton, actin

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An immunopotentiator has been defined as a substance that directly or indirectly enhances a particular immunological function. Immunopotentiators of bacterial origin occupy an important position in the field of immunology related to vaccination and immunotherapy. Many studies have already shown that pathogenic bacteria or their fractions stimulate cytokine production both *in vitro* and *in vivo* [9]. Although these bacteria can increase immune functions, they are undesirable in hosts. Strains of the genera *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* which are commonly referred to as lactic acid bacteria (LAB) are considered to be nonpathogenic and believed beneficial to human health [16]. In particular, bifidobacteria are excellent candidates for immunomodulation studies of the intestinal system, because they are widely used in commercial fermented dairy products and are one of the most abundant groups of microorganisms in the human gut, with 3–4 logs more cells per gram of feces than *Lactobacillus* [1]. It has recently been reported that bifidobacteria enhance several immune functions, namely macrophage and lymphocyte activation [8, 28], antibody production [12, 13, 36, 37], and a mitogenic response in spleen and Peyer's patches [9, 10, 12, 33]. Such stimulation of immune responses by bifidobacteria has been proposed to enhance resistance to infection by pathogenic organisms [4, 23, 35] and potentially prevent cancer [5, 21, 25, 26].

Macrophages play a major role in a host's defense against infection and tumor formation. It is believed that macrophages regulate immunity through the production of several mediators, such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) [11], NO, and H_2O_2, thereby inhibiting tumor cells, bacteria, fungi, and parasites [14, 29]. While these mediators play key homeostatic functional roles, they are also potentially capable of injuring host

tissues [6]. Thus, the regulation of these mediadiators is important for maintaining a normal physiological immune state. *Bifidobacterium* can differentially upregulate the production of macrophage cytokines in a dose-dependent fashion. The exact mechanism for the induction of an immune response by *Bifidobacterium* is unclear because of the complexity of the immune reactions involving different interacting cells and poor characterization of the *Bifidobacterium*-derived components in the immune system. The cell components of *Bifidobacterium*, which function as immunomodifiers of the host, have been reported to include peptidoglycans, intra and extracellular polysaccharide products, cell-free extracts, and cell wall preparation [7, 8, 9, 17, 25, 26]. Since most previous studies have focused on the cell wall component for the immunopotentiators from *Bifidobacterium* [25, 26], little attention has been paid to other fractions. Furthermore, there is not as yet a clear understanding of the molecular and cellular basis for bifidobacteria immunostimulation.

In previous studies, by the current authors, various strains of bifidobacteria were compared for the macrophage activation [18]. The data revealed that high-activating strains tended to induce all the macrophage activating markers: TNF-α, IL-6, H_2O_2, NO, and a phagocytic ability, at higher level than low stimulating strains. Accordingly, further characterization of the effect of *Bifidobacterium* strain specificity on macrophages may provide more insight into the role of specific *Bifidobacterium* strains in the structure-functional aspects of macrophage activation.

The current study observed that the activation of macrophages by bifidobacteria was accompanied by slow proliferation and greater differentiation, especially through the action of filament formation. Also, TNF-α and IL-6 production was differentially induced by whole cells and cell-free extracts, respectively.

MATERIALS AND METHODS

Bifidobacterium Cultures

The identification and experimental use of *B. bifidum* BGN4 and *Bifidobacterium* sp. CN2 were previously reported [18]. All strains were cultured and subcultured anaerobically in an MRS broth (Difco, Detroit, MI, U.S.A.) containing 5% lactose (wt/vol, MRSL) at 37°C until the late log phase. The cells were collected by centrifugation at 1,000 ×g for 15 min at 4°C and washed twice with PBS, followed by a final washing with distilled water. The cells were then dried using a Speed-Vac (Speed-Vac Instruments, Inc., NY, U.S.A.) and resuspended with Hanks' buffered salt solution (Sigma Chemical Co., St. Louis, MO, U.S.A.) to the desired bacterial concentration on a dry weight basis. For introduction into a tissue culture, the bifidobacteria were heat-killed by heating at 95°C for 30 min. The heat-killed cultures were aliquoted and stored at −80°C until use.

Chemicals and Reagents

The TNF-α, IL-6, purified antibodies to TNF-α or IL-6 (rat anti-mouse), and biotinylated rat anti-mouse TNF-α or IL-6 were obtained from PharMingen (San Diego, CA, U.S.A.). The Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Chagrin Falls, IL, U.S.A.). The tetramethylbenzidine (TMB) was from Fluka Chemical Corp. (Ronkonkoma, NY, U.S.A.), and the MTT [3-(4,5-di-methylthizol-2-yl)-2,5-diphenyltetrazolium bromide] and Phalloidinrhodamine were from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Cell Culture

The mouse macrophage cell line RAW264.7 (American type culture collection) and mouse thymoma cell line EL-4 were grown in DMEM supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, streptomycin (100 μg/ml), and penicillin (100 U/ml). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. The cell number and viability were assessed by the trypan blue dye exclusion method [32] on a Neubauer hemacytometer (American Optical, Buffalo, NY, U.S.A.). The cells were grown to confluence in sterile tissue culture dishes and gently detached by repeated pipetting. For the experiments, the cells were cultured in triplicate at various densities in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, U.S.A.). To investigate the effect of co-culturing, macrophages, prepared as described above and pretreated with the culture medium alone or with various bacterial concentrations, were incubated together with EL-4 cells at a ratio of 1:0.3 to 1:10 for 24 h. After incubation, the culture supernatants were used for the TNF-α and IL-6 analysis.

TNF-α and IL-6 Quantitation

The production of TNF-α and IL-6 was monitored by ELISA (Enzyme Linked ImmunoSorbent Assay) using a modification of the procedure of Dong *et al.* [3]. Briefly, microtiter strip wells (Immunolon IV Removawell; Dynatech Laboratories, Chantilly, VG, U.S.A.) were coated overnight at 4°C with 50 μl of 1 μg/ml purified antibodies to TNF-α or IL-6 antibodies (rat anti-mouse) in a 0.1 M sodium bicarbonate buffer (pH 8.2). The wells were then 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 any nonspecific protein binding. Next, standard recombinant murine TNF-α, IL-6, and the samples were diluted in 10% (v/v) FBS RPMI-1640, and 50 μl aliquots were added to appropriate wells, and incubated at 37°C for 1 h. After washing four times

with PBST, the biotinylated rat anti-mouse TNF-α or IL-6 antibodies were diluted in BSA-PBST to 1 μg/ml and 1.5 μg/ml, respectively, and a further 50 μl of these were added and the mixture was incubated at room temperature for 1 h. The plates were washed six times and incubated with 50 μl of a streptavidin-horseradish peroxidase conjugate (1.5 μg/ml in BSA-PBST) at room temperature for 1 h. After washing eight times, the bound peroxidase conjugate was detected by adding a 100 μl/well solution of a substrate consisting of 25 ml of a 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 then added to stop the reaction. The plates were read at 450 nm on a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA, U.S.A.). The TNF-α and IL-6 were quantitated using V_{max} Software (Molecular Devices).

MTT Assay

A colorimetric MTT cleavage test was performed as described by Visconti *et al.* [34], to assess the proliferation in the RAW 264.7 cultures. Briefly, 50 μl of a filter-sterilized stock MTT solution (5 mg/ml in phosphate buffered saline) was added to each well, then the cultures were incubated for 3 h at 37°C. The plates were centrifuged at 450 ×g and the supernatant removed. Isopropanol (200 μl) was added to the wells and mixed thoroughly to completely dissolve the crystalline material. The absorbance of each tissue culture well was read on a V_{max} Kinetic Microplate Reader at 570–690 nm.

Lysosome Staining with Acridine Orange

To locate the macrophage lysosomes, the RAW 264.7 cells exposed to *Bifidobacterium* were incubated with DMEM containing acridine orange (10 μg/ml) for 10 min, then the excess fluorescence dye was removed by washing twice with DMEM without the dye at 5-min intervals for 5 min [38]. The cells were observed to be alive with an Olympus epifluorescence microscope (DX-05F-3, Olympus Co., Japan) and photographed using TMAX 400 film (Kodak, Rochester, NY, U.S.A.)

Actin Filament Staining

An actin filament was visualized by staining with phalloidin-rhodamine. The cells treated with bifidobacteria were fixed with 3% paraformaldehyde for 10 min and washed 3 times with phosphate-buffered saline (PBS). For staining, the cells were incubated in PBS containing 0.1 μg/ml phalloidin-rhodamine for 10 min and washed extensively with PBS. The cells were mounted on a glass slide and observed under an Olympus epifluorescence microscope.

Preparation of *Bifidobacterium* BGN4 Cell Fractions

The cells were fractionated by a modification of the method of Okitsu-Negishi *et al.* [18]. The cells grown in the MRS medium were pelleted by centrifugation (1,000 ×g for 20 min). The pellets were then washed twice with PBS and centrifuged again. The packed cells were suspended homogeneously in 30 ml of distilled water, and then disintegrated using a French Press (Spectronic, Rochester, NY, U.S.A.). The whole cells and debris were removed by centrifugation at 3,000 ×g for 10 min at 4°C. The cell walls were sedimented by centrifugation at 10,000 ×g for 30 min at 4°C, and the supernatant used as the cell-free extract. The crude-wall fractions were checked microscopically. The cell walls were washed another two times in 15 mM NaCl, followed by a wash in 50 mM TrisHCl, and then diluted in twice the volume of 10 mM potassium phosphate buffer (pH 7.0). RNase and DNase were added to a final concentration of 50 μg/ml. The mixture was incubated at 37°C for 90 min and then centrifuged at 10,000 ×g for 40 min. The washed cell walls were treated with 2% sodium dodecyl sulfate (SDS) and heated at 70°C for 2 h to remove the membrane. The cell walls were then washed extensively with distilled water to remove the SDS, collected by centrifugation, and lyophilized for use as the purified cell-wall preparation.

Statistical Analysis

The data were analyzed by the Student-Newman-Keuls (SNK) test following a one-way analysis of variance (ANOVA) using the Sigmastat Statistical Analysis System (Jandel Scientific, San Rafael, CA, U.S.A.). A probability of $p<0.05$ was used in the two-tailed test as the criterion for statistical significance.

Results

Effect of *Bifidobacterium* on Macrophage Cytoskeleton Organization

The current authors previously demonstrated that both human and commercial *Bifidobacterium* strains can stimulate H_2O_2, NO, TNF-α, and IL-6 from the macrophage cell line, and this effect is strain-specific and dose-dependent [18]. Among the 33 *Bifidobacterium* strains tested, *B. bifidum* BGN4 showed the highest stimulation effect for cytokine production even at a low concentration of cells (10 μg/ml), whereas strain CN2 showed the lowest activity. To further examine the morphological change at a subcellular level during the activation of the macrophages by bifidobacteria, the staining of a lysosome-like vacuole and actin filament were performed. Lysosome-like vacuole staining is based on the use of lysosomotropic weak-base acridine orange, which in its stacked form as it occurs within lysosomes, emits red fluorescence when excited by blue light. After treatment with the *Bifidobacterium* CN2 strain which exhibited a lower potency for macrophage activation, the macrophages showed a slight change in the red fluorescence intensity of the small vacuoles (Fig. 1Ab). However, when

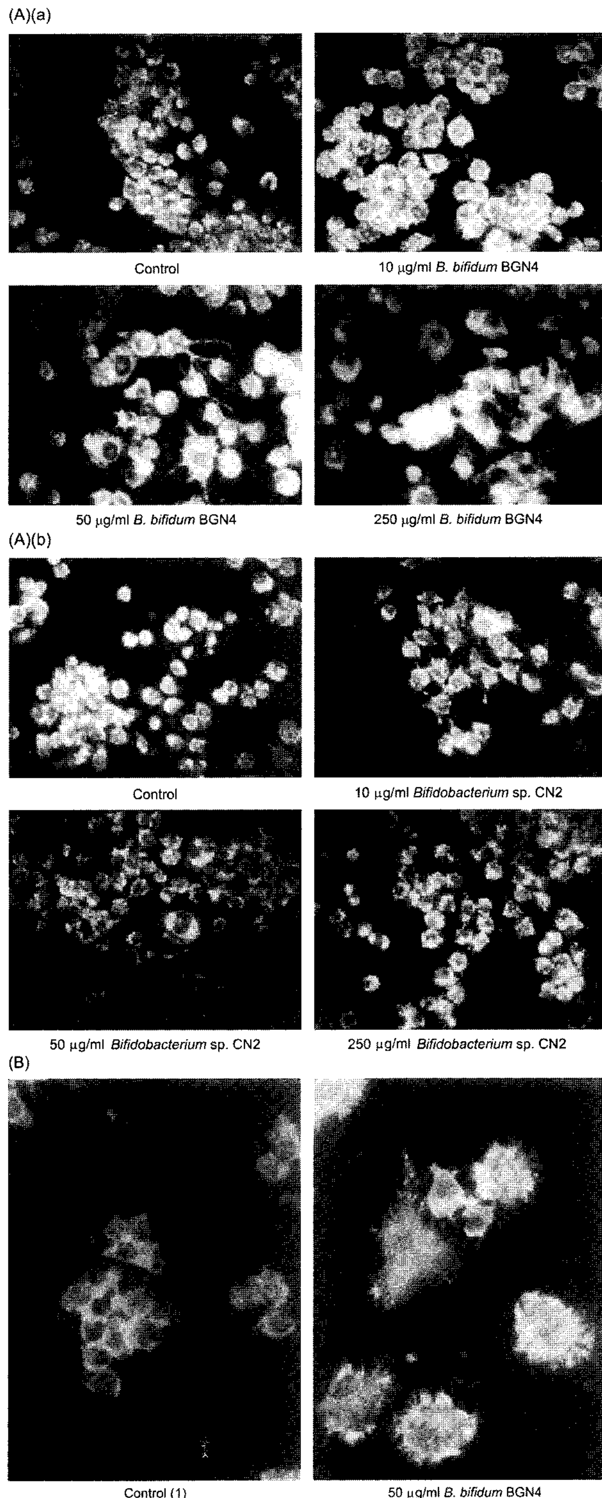

Fig. 1. Acridine orange staining (A) and actin filament staining (B) of murine macrophage RAW 264.7 cells treated with *Bifidobacterium* cells.

A: Macrophage cells treated with the high-macrophage activating strain BGN4 (a) showed an increased number of activated vacuoles compared to those treated with the low-macrophage activating strain CN2 (b). B: The *B. bifidum* BGN4-treated macrophage cells showed a much greater level of actin filament formation and filopodia development (magnification ×400).

treated with the *B. bifidum* BGN4 strain, the cells showed greater numbers and larger sizes of activated vacuoles with a significantly increased red fluorescence intensity. This increase was markedly potentiated when increasing the cell concentration of *B. bifidum* BGN4 (Fig. 1Aa). Besides the activation of the lysosome-like vacuoles, the exposure of the macrophages to *B. bifidum* BGN4 enlarged the size of the macrophage cells 3–4 times. Since the altered macrophage morphology could be contributed to a change in the microfilament arrangement, the organization of the actin filaments was determined. Phalloidin, a toxin from the plant *Amanita phalloides*, irreversibly polymerizes actin filaments, and thus can recognize filamentous actin. The changes in the cell shape and in the patterns of the microfilament distribution were analyzed using cell monolayers before and after exposure to the bacteria. A few minutes after the addition of the bifidobacteria, the staining of the F-actin with rhodamine phalloidin revealed the formation of large numbers of filopodia around the periphery of the cells (Fig. 1B). A simultaneous increase in the fluorescence intensity also occurred in response to the bifidobacteria. The MTT cleavage test has previously been used as an indirect measure of the proliferation of cultured cells [34]. As the concentration of *Bifidobacterium* BGN4 increased from 0 to 50 μg/ml the MTT value decreased in a dose-dependent manner (Fig. 2). This result suggests that, upon activation of the macrophages by the *Bifidobacterium*, the proliferation activity of the macrophages decreased.

Effect of *Bifidobacterium* on Cytokine Production in Co-Culture System of Macrophage and T-Cell Lines

To examine the effect of the *Bifidobacterium* on cytokine production in a system more resembling an *in vivo* situation, T-cells were added to the assay. The co-culture system of macrophages and T-cell lines was considered to reflect *in vivo* conditions better than a single cell line culture. For the experiment, RAW 264.7 cells (5×10^5 cells/ml) were co-incubated with an increasing ratio of EL.4

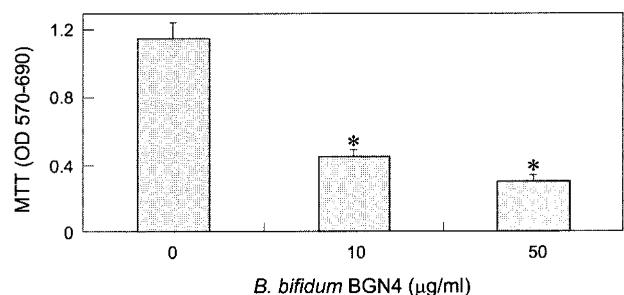

Fig. 2. Effect of *B. bifidum* BGN4 on MTT activity of RAW 264.7 cells (5×10^5 cells/ml).

The cells were incubated in the presence of different cell concentrations of *Bifidobacterium* for 24 h. Data are means±SD of triplicate cultures. Asterisk indicates significant difference from control (*B. bifidum* BGN4, 0 cells/ml) ($P<0.05$).

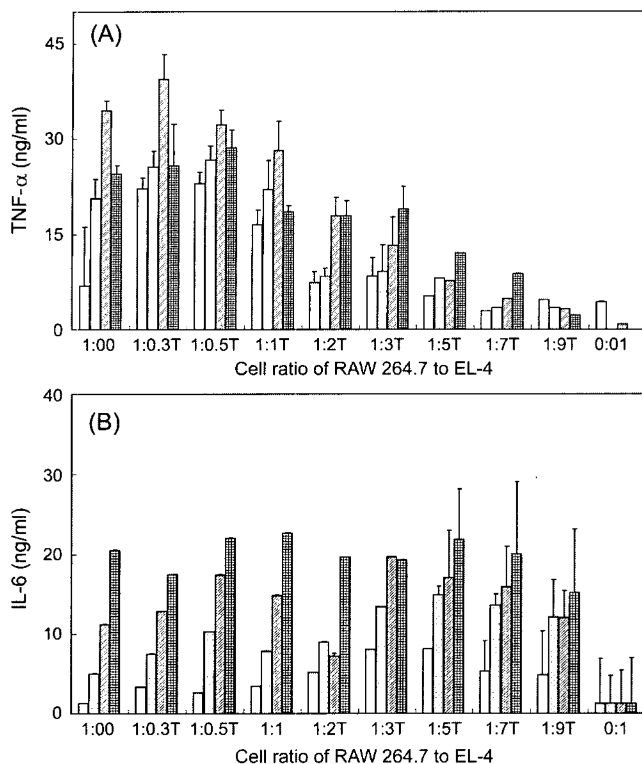

Fig. 3. Effect of *B. bifidum* BGN4 cells on the production of TNF-α (A) and IL-6 (B) by co-cultured RAW 264.7 and EL-4 cells.

Data are means±SD of triplicate cultures. Symbols: □ 0, ▒ 10, ▨ 50, ▦ 250 μg/ml *B. bifidum* BGN4.

IL-2 thymoma cells (T-helper cell model) (Fig. 3) from 1:0 to 1:9. In the absence of the *Bifidobacterium*, the production of cytokines gradually increased up to an 1:0.5 (RAW 264.7: EL-4) ratio for TNF and up to a 1:5 ratio for IL-6, followed by a gradual decrease as the EL-4 concentration increased further. These results show that the EL-4 cells were able to influence the production of cytokine from the RAW 264.7 cells. Although the EL-4 cells stimulated the RAW 264.7 cells to produce TNF-α and IL-6, the stimulatory activity of the *Bifidobacterium* decreased, especially with regard to the production of TNF-α, as the EL-4 concentration increased, although *B. bifidum* BGN4 alone increased the production of TNF-α and IL-6.

Effect of Cell Fractions of *B. bifidum* BGN4 on TNF-α and IL-6 Production

To examine the effect of cell fractions of *B. bifidum* BGN4 on TNF-α and IL-6 production from macrophages, RAW 264.7 cells were incubated with 0–250 μg/ml of bacterial fractions, and the cytokine secretion in the culture supernatant was monitored by ELISA. The results for TNF-α and IL-6 production are shown in Figs. 4A and 4B, respectively. While the whole cell fraction was more effective for TNF-α production than the other fractions, the cell-free extracts exhibited the strongest IL-6 production, followed by the purified cell wall and whole cells. The cell-free extract produced IL-6 even at 1 μg/ml concentration.

Discussion

Bifidobacteria and other lactic acid bacteria have been previously shown to stimulate immune functions [7, 10, 12, 19] and antitumor activity in a host [5, 21, 25, 27]. Thus, their ability to stimulate macrophages and T-cells [8, 26, 27] may play a crucial role in these activities. Macrophages are known to facilitate the presentation of various antigens to lymphocytes and provide signals in the form of cell-to-cell and humoral-to-cell interactions that result in an enhanced lymphocyte proliferation and elaboration of various cytokines [11]. Therefore, cytokine production is likely to be a good indicator of the degree of macrophage activation. Previously, we showed that both human and commercial *Bifidobacterium* strains could stimulate H_2O_2, NO, TNF-α, and IL-6 production and this effect was strain dependent. These results were consistent with previous reports that bifidobacteria enhance

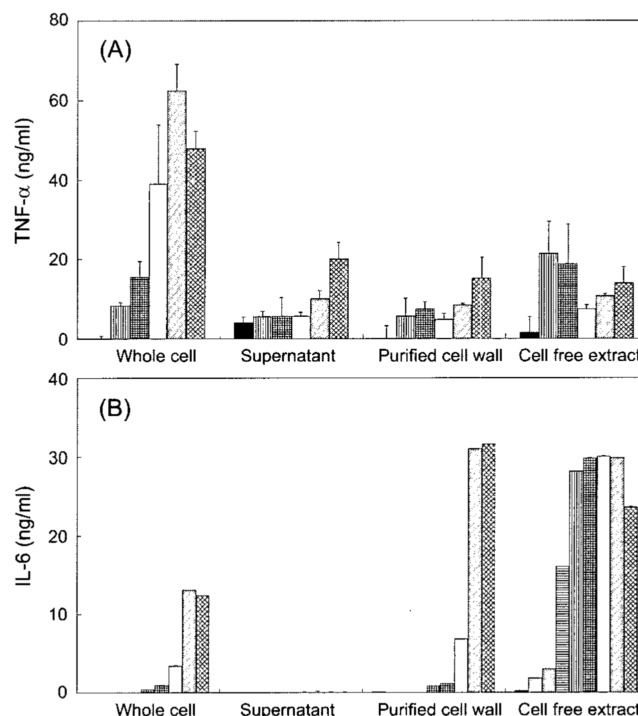

Fig. 4. Effect of various cell fractions of *B. bifidum* BGN4 on the production of TNF-α (A) and IL-6 (B) by murine macrophage cell lines.

RAW 264.7 cells (5×10^5 cells/ml) were cultured for 24 h in the presence of various bacterial components. Data are means±SD of triplicate cultures. Symbols: ■ 0, ▒ 0.1, ▧ 0.25, ▤ 0.5, ▥ 1, ▦ 5, □ 10, ▨ 50, ▩ 250 μg/ml *B. bifidum* BGN4.

the production of TNF-α and IL-6 by human peripheral blood mononuclear cells [16, 30] and mouse peritoneal cells [27]. Lee *et al.* [12] reported that bifidobacteria significantly increased IL-2 production in EL-4 cells with PMA stimulation. However, the results were in contrast with Solis Pereyra and Lemonnier [30] who were unable to detect any increase of IL-2 production during the incubation of blood mononuclear cells with the *Bifidobacterium* sp. used in dairy production. The co-culture system of macrophages and T-cell lines employed in the current study appeared to reflect *in vivo* conditions better than a single cell line culture. In the control groups, either RAW 264.7 cells or EL-4 cells were cultured with *B. bifidum* BGN4 to measure the production of BGN4-stimulated TNF-α and IL-6. No production of the macrophage cytokine TNF-α and IL-6 was detected or minimally detected in the EL-4 thymoma cell line, when incubated with *Bfidobacterium* (data not shown). Marin *et al.* [15] also reported that *Bifidobacterium* had negligible effects on interleukin production in a culture of EL-4 cells without PMA stimulation. The current results showed that the co-incubation of the RAW 264.7 cells with *B. bifidum* BGN4 or EL-4 cells enhanced the production of both TNF-α and IL-6 from the RAW 264.7 cells, yet there was no synergistic effect between the *Bifidobacterium* and the T-cells. Perhaps, the EL-4 cells compete with the *Bifidobacterium* in the activation of RAW 264.7, thereby reducing the stimulatory effect of the *Bifidobacterium* on the RAW 264.7 activation.

The development of stress fibers may be related to an altered cellular morphology in the macrophages. These changes probably result from the rearrangement of cellular cytoskeletons and extracellular matrix components. Actin is one such cellular component that has already been identified to be involved in the cellular process. The current study also provides evidence to relate stress fiber formation with the enlargement of the macrophage cell size and increased fluorescence intensity. This highly developed actin may aid the activated macrophage to migrate toward the target site with a higher chemotactic activity. In addition to actin, myosin also plays an important role in several cellular responses including contractility [31], thus the filamentous myosin structure may play a role in the *Bifidobacterium*-induced cell-shape change. It would be interesting to see if *Bifidobacterium* induces the rearrangement of myosin in the macrophages. Furthermore, with highly developed vacuoles, they may ingest pathogenic organisms more vigorously. In fact, the BGN-4 activated macrophages exhibited an enhanced phagocytic activity when challenged with fluorescein-labeled *E. coli* (unpublished results). Taken together, the present findings that a high-stimulating strain induced more marked development of actin filament and filopodia formation support the above-mentioned results related to the cytokine production.

The major site for immune responses in the intestinal tract is considered to be Peyer's patches, which are distributed differently depending on both age and species. Human Peyer's patches within the duodenum are small and consist of a few lymphocyte follicles, yet become larger in more distal areas in the ileum; as such, they are considered to be induced by the presence of endogenous microflora. The survival of a cell and processing of its cell component may also be related to the efficiency of the immunostimulatory effect. The death of *Bifidobacterium* may occur rapidly once they enter the immune system, due to their strict anaerobic characteristic. The ease of cell death and lysis is also related to structural integrity, composition of the cell material, and sensitivity to enzymatic or chemical attack. In this context, the binding of an intact cell or cell component may play a role in the immune activation with mechanisms different from each other. Indeed, it was noticed that whole cells stimulated TNF-α production, while cell-free extracts favored IL-6 production. In particular, the cell-free extract fraction exhibited IL-6 production even at a 1 μg/ml concentration. It is very interesting that the stimulative fractions for TNF-α or IL-6 were different in the *B. bifidum* BGN4 cell fractions. Lee *et al.* [12] reported that disrupted cells of *B. adolescentis* M101-4 enhanced the *in vitro* proliferation and secretion of antibodies specific for the nominal antigen, suggesting the polyclonal activation of B-cells and/or antibody-producing plasma cells. Yasui *et al.* [37] reported that *Bifidobacterium* stimulated IgA production in the gut. IL-6, secreted by macrophages, makes a critical contribution to the development of mucosal IgA responses. IL-6 drives IgA production when it is added to Peyer's patch B-cell cultures [2]. The increased production of macrophage IL-6 by the cell-free extract from *B. bifidum* BGN4 observed in the current study suggests that this may affect the IgA production in the B-cells. In contrast, Hatcher and Lambrecht [8] reported that extracts from *Bifidobacterium* produced activation in the absence of either viable organisms or cell wall fractions. An attempt was made to further purify the IL-6 stimulating components by Sephdex G-200 gel filtration chromatography, and DEAE- and Mono Q- ion-exchange chromatography (data not shown). Various fractions containing proteins or carbohydrates showed IL-6 stimulating activities. Some peaks containing carbohydrates without proteins also showed activity. The highly active fractions consisted of both carbohydrates and proteins. Hosono *et al.* [9] reported that three quarters of the dry weight of the isolated immunoactive fraction from *B. adolescentis* M101-4 consisted of carbohydrates, leading them to suggest that proteins did not seem to be directly related to the immunopotentiating activity, whereas polysaccharides were. Gomez *et al.* [7] reported that exocellular products from *Bifidobacterium*, which were carbohydrate-rich fractions, stimulated DNA synthesis in LPS-sensitive B-lymphocytes.

The current results suggest that both the protein components and the carbohydrates from the cell-free extract were important in enhancing IL-6 production. In conclusion, the results reported here suggest that the activation of macrophages was accompanied by actin filament formation and vacuole development. In addition, this stimulatory capacity was also affected by the dose, strain, and composition of the *Bifidobacterium*. The *in vitro* approaches employed here should be useful in future mechanistic characterization of the effects of bifidobacteria on gastrointestinal immunity and the possibility of enhancing gastrointestinal immune functions. Further elucidation of the differential effects of the cell-free extracts on cytokine production would contribute to a better understanding of the role of the *Bifidobacterium* in the intestinal immune system.

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