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Enhancement of Antigen Presentation Capability of Dendritic Cells and Activation of Macrophages by the Components of *Bifidobacterium pseudocatenulatum* SPM 1204

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Abstract – Antigen presenting cells (APCs), dendritic cells (DCs) and macrophages, play a critical role not only in the initiation of immune responses, but also in the induction of immune tolerance. In an effort to regulate immune responses through the modulation of APC function, we searched for and characterized APC function modulators from natural products. *Bifidobacterium pseudocatenulatum* SPM1204 (SPM1204) isolated from feces of healthy Korean in the age of 20s was used in this experiment. DCs and macrophages were cultured in the presence of supernatants of SPM 1204 and then examined for their activities for the presentation exogenous antigen in association with major histocompatibility complexes (MHC) and macrophage activation. SPM1204 increased class I MHC-restricted presentation of exogenous antigen (cross-presentation) in a DC cell line, DC2.4 cells. The RAW 264.7 cell line was used to test the nonspecific effect of immune reinforcement of SPM1204 as a source of biological regulating modulator for the macrophage activation, include nitric oxide (NO) production and cytokine production. Results showed that the production of NO, tumor necrosis factor (TNF)-α, interleukin 1 (IL-1)-β and morphological changes in macrophages were largely affected by SPM1204 in a dose-dependent manner. Our results demonstrated that SPM1204 promote cross-presentation of dendritic cells as well as the induction of NO, TNF-α production, and activation of macrophage.

Keywords □ *Bifidobacterium* spp., exogenous OVA, MHC class I, antigen presentation, macrophage activation, IL-1β, TNF-α

INTRODUCTION

Antigen presenting cells (APCs), dendritic cells (DCs) and macrophages, play a critical role not only in the initiation of immune responses, but also in the induction of immune tolerance. Recent advances in cancer treatment, efforts to regulate immune responses through the modulation of APC function have been searched for and characterized APC function modulators from the naturally occurring biological active compounds. *Bifidobacterium* spp which inhabit in the intestinal tracts of humans and animals are used in commercially fermented dairy products and have been suggested to exert health promoting effects on the host by maintaining intestinal microflora balances, improving lactose tolerance, reducing serum cholesterol levels, increasing synthesis of vitamins, and aiding anti-carcinogenic activity (Mitsuoka, 1982; Gopal *et al.*, 1996; Homma, 1998; Hughes and Hoover 1991; Kumann and Raric 1998). Other beneficial effects of the intake of *bifidobacterium* are reported to include the reinforcement of immune function (Yasui and Ohwaki 1991; Yamazaki *et al.*, 1991).

Bifidobacteria apparently enhance several immune functions, including macrophage and lymphocyte activation (Hatcher and Lambrecht 1993; Sekine *et al.*, 1994), antibody production (Yasui and Ohwaki 1991, Lee *et al.*, 1993; Link-Amster *et al.*, 1994; You *et al.*, 1992), and the proliferative responses in spleen and Peyer's patches (Yasui and Ohwaki 1991; Lee *et al.*, 1993; Hosono *et al.*, 1997; Kado *et al.*, 1991; Takahashi *et al.*, 1993). Bifidobacteria ingestion has been proposed to enhance resistance to infection by pathogenic organisms (Duffy *et al.*, 1994; Sasaki *et al.*, 1994) and potentially to

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prevent cancer (Sekine *et al.*, 1994; Rafter, 1999).

However, the influence of exogenous factors on species composition of fecal bifidobacteria is still unclear. Protein must be processed into small peptides and presented either to major histocompatibility complex (MHC) class II molecules for activation of CD4 T cells or MHC class I molecules for activation of CD8 T cells. Exogenous antigens are normally processed and presented to class II MHC molecules. Recent studies documented that exogenous antigens can enter the class I MHC presentation pathway, a process known as cross presentation. Subsequently, it has shown that the cross presentation pathway is an obligatory mechanism in several situations (Shen *et al.*, 1997; Rafter, 1999). A strict requirement of cross presentation has been demonstrated in the induction of cytotoxic T lymphocyte (CTL) responses to graft tissues, tumor cells and viruses that infect only non-hematopoietic cells. Cross presentation may be a mechanism by which naive T cells can be primed to antigens that are present in nonprofessional APCs.

Macrophages play a major role in the host defense against infection and tumor formation. The production of nitric oxide (NO) and tumor necrosis factors (TNF)-α by macrophages mediate killing or growth inhibition of tumor cells, bacteria, fungi and parasites (Lorsbach *et al.*, 1993). Characterization of the effects on *Bifidobacterium* on the production of macrophage mediators may contribute to a better understanding of how this genus affects immune function at the cellular level (Rafter, 1999).

These abilities of APC to present exogenous antigens can be readily observed *in vitro* by DC 2.4 cell line and RAW 264.7 cell line, murine macrophage cell line. The culture supernatant of *Bifidobacterium pseudocatenulatum* SPM1204 was found to stimulate macrophages to release TNF-α and NO production. These results demonstrate that *Bifidobacterium pseudocatenulatum* SPM1204 increased Class I MHC-restricted presentation of exogenous antigen and activated macrophage function.

MATERIALS AND METHODS

Isolation and Identification of *Bifidobacterium pseudocatanulatum* SPM1204 from human feces

Fecal samples of healthy Koreans (20-30 years old) were collected by BBL's anaerobic sample collection & transport system in order to maintain anaerobic condition and were used within 24 hrs. Fecal samples were serially diluted 10 fold from 10^{-1} to 10^{-8} and 100 μl aliquot of appropriate dilutions was spread onto the selective BL agar containing 5% of sheep blood. Brown or reddish brown colonies having 2-3 mm in a diameter were selected for further identification after 48 hrs of incubation in anaerobic condition (Bactron Anaerobic Chamber, Sheldon Manufacturing Inc, U. S. A) (Han *et al.*, 1999; Bea *et al.*, 1998) onto GAM (General anaerobic medium, Nissue) at 37°C.

Bifidobacterium longum ATCC 15707 and *Bifidobacterium breve* ATCC 15700 were used as positive control strains. These colonies were examined by both Gram staining and microscopy for cell morphology. In addition, a fructose-6-phosphate phosphoketolase (F6PPK) test was performed (Mitsuoka, 1994; Roy and Ward 1990) to ensure that the colonies selected were bifidobacteria.

Cell culture

The murine macrophages cell line, RAW 264.7, was obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle Medium (DMEM), which was supplemented with high glucose, L-glutamine, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), and penicillin (100U/ml)/ streptomycin (100U/ml). LPS (Escherichia coli, 0127: B8 Westphal type) was purchased from Sigma Chemical Co. (St. Louis, MO. USA). B3Z86/90.14 (B3Z), a T cell hybridoma that recognizes K^b with peptide 257-264 (SIINFEKL) of OVA, was kindly provided by Dr. Nilabh Shastri (University of California, Berkeley, CA. USA). B3Z contains a DNA construct coding for the *lacZ* gene, under the control of the IL-2 regulatory elements. Upon activation of B3Z through the TCR, the *lacZ* gene is expressed, allowing determination of the activation of the T cell hybrid through colorimetric assays (Yeh *et al.*, 1998). DC2.4 cells were obtained from bone marrow cells infected with a retrovirus encoding *myc* and *raf* by using supernatant from NIH J2 Leuk cells, as previously described (Shen *et al.*, 1997).

B3Z T hybridoma activation assays

Activation of B3Z cells was measured by *lacZ* activity. Briefly, 4×10^4 cells/96 well DC2.4, the cells were treated with different supernatant of bifidobacteria strains (1 μl/96well) in 5.5% CO_2 humidified air for 2 hrs at 37°C, and then particulate OVA was added for another 2 hrs. The cells were washed with phosphate buffered saline and then incubated with 1×10^5 cells/96 well B3Z cells to evaluate antigen presentation. After 4 hrs, the supernatant was removed, and the cells were lysed by a lysis buffer (0.1% Triton X-100, 250 mM Tris, pH 8.0) and kept in -70°C for 10 min. The plates were thawed at room temperature for 10 min and added 50 μl of PBS containing 0.5 %

BSA. Overlaid with 100 μl of substrate (1 mg/ml of chlorophenolred-β-D-galactopyranoside in β-galactosidase buffer: 60 mM sodium dibasic phosphate buffer (pH 8.0), 1 mM magnesium sulfate, 10 mM KCl, 50 mM β-mercaptoethanol. The plates were incubated in humidified air with 5.5% CO_2 for 12 hrs at 37°C and were measured by an ELISA reader at 580 nm.

TNF-α and IL-1β quantitation

TNF-α and IL-1β were quantified by ELISA using a modification of the protocol of Kim *et al.* (2003). Briefly, microtiter strip wells (Immunoion IV Removawell; Dynatech Laboratories, Chantilly, VG) were coated overnight at 4°C or room temperature with 100 μl of 1 μg/ml purified antibodies to TNF-α and IL-1β antibodies (rat anti-mouse) in 0.1 M sodium bicarbonate buffer (pH 8.2). Wells were incubated with 200 μl of 3% (v/v) bovine serum albumin (BSA) in 0.01M PBS (pH 7.2) containing 0.2% (v/v) Tween 20 (PBST) at room temperature for 1 hr to block nonspecific protein binding. Standard recombinant murine TNF-α, IL-1β and samples, in 10% (v/v) FBS DMEM-1640 were added in 100 μl aliquots to appropriate wells and incubated at room temperature for 1 hr. After washing four times with PBST biotinylated rat anti-mouse TNF-α or IL-1β antibodies were diluted in BSA-PBST to 1 μg/ml and 1.5 μg/ml respectively, and 50 μl were added and incubated at room temperature for 1 hr. Plates were washed six times and incubated with 50 μl of streptavidin-horseadish peroxidase conjugate (1.5 mg/ml in BSA-PBST) at room temperature for 1 hr. After washing eight times, bound peroxidase conjugate was detected by adding 100 μl per well solution of substrate consisting of 0.1 mg/ml (3, 3', 5, 5' tetramethylbenzidine) TMB, and 100 ml of 1% H_2O_2 in 25 ml of 0.1 M citric phosphate buffer (pH 5.5). 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 Microplates Reader (Molecular Devices, Menlo Park, CA). TNF-α and IL-1β were quantitated using Vmax Software (Molecular Devices).

Nitric oxide assay

Flat-bottomed 96 well, LPS (10 ng/ml), two *bifidobacterium* strains, media only (DMEM-10), RAW 264.7 cell line, and Griess reagent (stock-I: 0.2% naphylenediamine HCl, stock-II: 2% sulfanilamide in 5% H_2PO_4) were used as materials in this study. NO production was carried out according to the method reported by Stuehr and Nathan (Stuehr and Nathan 1989). LPS, cells only (1×10^6 cells/ml), supernatant (12.5, 25, 50, 100 μl/ml) of two *Bifidobacterium* strains, were prepared as the treated groups. 6 wells per each group were used and 200 μl of the cells (1×10^6 cells/ml) was added to each well. The plates were incubated overnight and 100 μl from the surface of each well was transferred into new plate and added the equivalent amount of Griess regent. The new plate was then incubated for 10 min at room temperature and was measured by an ELISA reader at 540 nm. Standard calibration curves were prepared using sodium nitrite as a standard.

Western blot analysis of TNF-α and IL-1β

The RAW 264.7 cells were washed with phosphate buffered saline and lysed by boiling with a lysis buffer (1% SDS, 1.0 mM sodium vanadate, 10 mM Tris-Cl buffer, pH7.4) for 5 min. 112 μg protein from the cell lysates was applied to 12% SDS-polyacrylamide gels. The membrane was blocked with a solution containing 5% BSA for 1 hr. It was then incubated with anti-TNF-α monoclonal antibody and anti-IL-1β monoclonal antibody for 2 hrs and washed 3 times with phosphate buffered saline. After incubation with alkaline phosphates-labeled anti-antibody for 1 hr, the bands were visualized using Western Blot Kit substrate for phosphatase (LumiGLO System, KPL, U. S. A.).

Cell staining

To determine the effects of *Bifidobacterium* strains on the macrophage morphology, the cells were cultured in sterile glass-slide chambers at a density of 1000 cells/well for 48 hrs. The culture medium was removed, and the cells were treated with either LPS (100 ng/ml), two *Bifidobacterium* strains (3 μl/well) only for 2 days. Following the treatment, the culture supernatant was removed. The cells were fixed and stained in a Diff-quick Solution (Baxter, Houston, TX).

Statistical analysis

Nitrite and cytokine production is expressed as means ± SD of 2 to 6 independent experiments. The statistical significance was estimated using Student-t tests.

RESULTS

Identification of *Bifidobacterium pseudocatenulatum* SPM 1204 16s rRNA & PCR-RAPD assay

Selected Bifidobacteria was finally identified through 16s rRNA sequence and PCR-RAPD assay. According to the results of 16s rRNA sequenry, strain SPM1204 and SPM1205 were identified as *B. pseudocatenulatum* and *B. longum* respec-

tively. Electrophoresis procedures confirmed that *Bifidobacterium pseudocatanulatum* SPM1204 showed different aspects of pattern among the *Bifidobacterium species* (data not shown).

Bifidobacteria strains enhance presentation of exogenous particulate OVA in association with class I MHC

The efficiency of exogenous antigen presentation with class I MHC was evaluated using a K^b/OVA peptide-specific T cell hybridoma reporter system. B3Z is a T cell hybrid that expresses the *lacZ* gene upon recognition of an OVA peptide with the class I MHC molecule K^b. Activated B3Z cells turn red in the presence of X-Gal substrate, allowing simple determination of the number of activated B3Z. For exogenous particulate antigen, we first used OVA-conjugated beads, because this system has been well characterized (Shen *et al.*, 1997). Treatment of DC with supernatant of *Bifidobacterium* strains resulted in a population of cells with an enhanced ability to present particulate OVA (Fig. 1). SPM1204 and SPM1205 were higher than the ATCC *Bifidobacterium* spp. based on the presentation of exogenous particulate antigen in association of MHC class I. We wished to determine whether presentation of exogenous OVA (10 μg/ml) could be enhanced by the culture supernatant of two bifidobacteria strains. These strains could enhance by 131.7 and 127.6% compared with cross-presentation to exogenous p-OVA only as a control.

Effect of bifidobacteria strains on NO production in murine macrophage cell line, RAW 264.7

LPS was used as a positive control for macrophage activation. In the LPS (10 ng/ml) stimulated RAW 264.7 cell culture system, NO production was examined through i-NOS expression by western blot (data not shown). When various amounts of supernatants (12.5, 25, 50, 100 μl/ml) of two different bifidobacteria strains were added to the culture media and cultured for 12 hrs, nitric oxide as the form of nitrite was produced significantly compared to LPS-stimulated cultures as a positive control. In the case of SPM1204, production of nitric oxide was exceeded (73 μM respectively) that of control group of LPS-treated (10 ng/ml) cultures (48.5 μM) at a concentration of 25 μl and increased in a dose dependent manner up to 100 μl of each supernatant of SPM1204 and SPM1205 which gave 118 and 85 μM of nitrite products respectively (Fig. 2). When those active supernatants of bifidobacteria strains were cultured with combination of LPS did not give synergic effect of NO production on the macrophage cell line (data not shown).

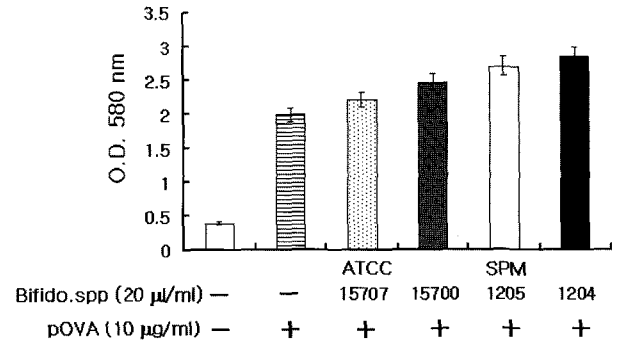

Fig. 1. Effects of *Bifidobacterium pseudocatenulatum* SPM 1204 on DCs for cross presentation of an exogenous antigen (p-OVA: 10 μg/ml). p-OVA were added to cultures of DC 2.4 cells and treated with supernatants (20 μl/ml) of ATCC *Bifidobacterium* spp. (ATCC15700, 15707), SPM1204, SPM1205 and then the amounts of cross-presented OVA peptides were measured by the *lacZ* T cell activation assay. The results are reported as a mean±S.D. of three.

Cytokines production by unstimulated and LPS-stimulated in response to bifidobacteria strains

To determine whether two *Bifidobacterium* strains have a direct effect on cytokine production, a biological assessment of TNF-α and IL-1β activation was measured using the macrophage cell line. Co-stimulated of macrophages with both LPS (100 ng/ml) and *Bifidobacterium* increased the production of IL-1β synergistically (Fig. 3b). ATCC *Bifidobacterium* spp. and two samples (SPM1204 and SPM1205) increased TNF- α productions in the absence of LPS in a dose dependent manner (Fig. 3c). Bifidobacteria increased TNF-α productions synergistically in a dose dependent manner in the presence of LPS (Fig. 3d). Western blot analysis (Fig. 4) showed that TNF-α

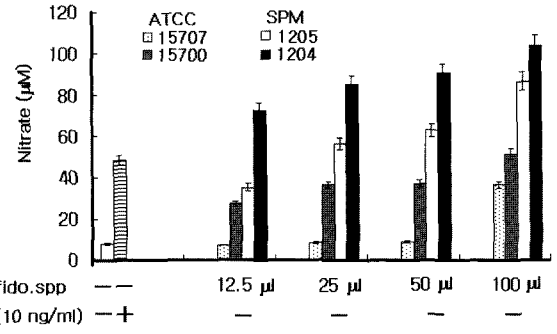

Fig. 2. Effects of *Bifidobacterium pseudocatenulatum* SPM 1204 or 1205 on RAW 264.7 cells for NO production. NO production was determined by the accumulation of nitrite using an ELISA reader at 540 nm. The results are reported as a mean ±S.D. of three independent experiments.

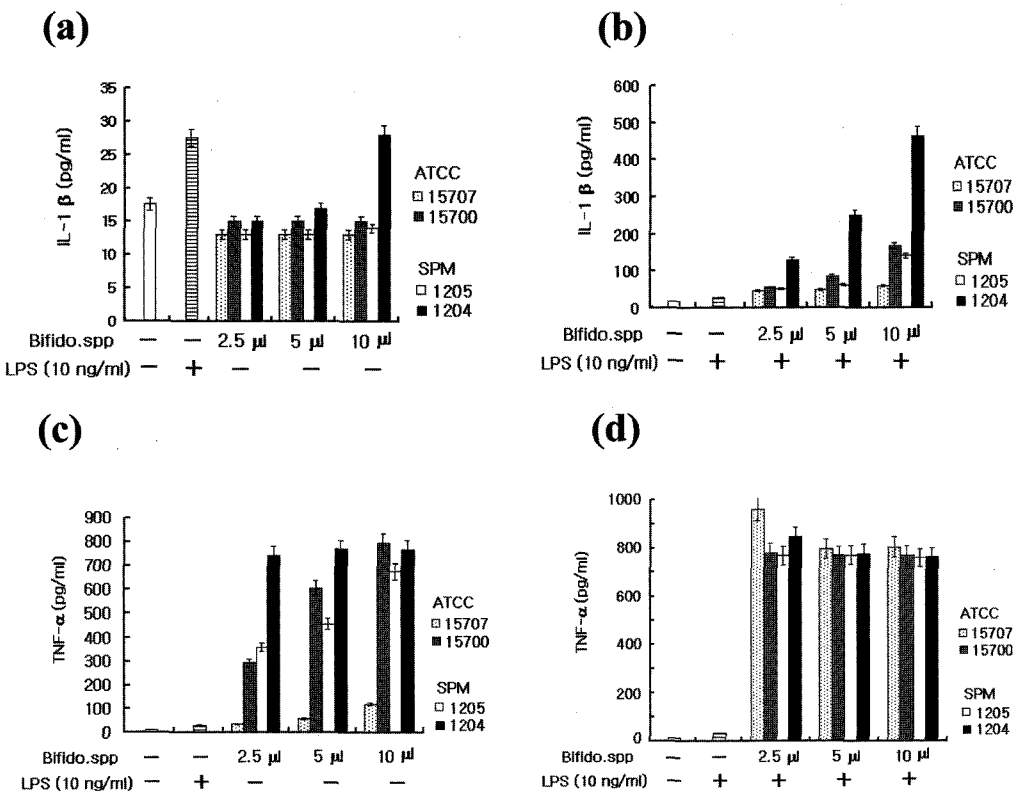

Fig. 3. TNF-α and IL-1β production by *Bifidobacterium pseudocatenulatum* SPM 1204 or ATCC *Bifidobacterium* spp. in the murine macrophage cell line. Supernatant (2.5, 5, 10 μl/ml) of four bifidobacteira strains on RAW cells. (a) and (c) without LPS, (b) and (d) with LPS.

was produced by the supernatants of these two bifidobacteria strains. SPM1204 differentially stimulated had a great effect on macrophage activation according to the western blot analysis (Fig. 4). Among them, much greater production of TNF-α and IL-1β were generated by *Bifidobacterium pseudocatanulatum* SPM1204 compared to other control bifidobacteria strains.

Effect of *bifidobacterium* strains on macrophage morphology

Normal RAW cells, when cultured in a medium alone, tend to be round in morphology (Fig. 5a). None appeared to spread over the surface. The gradual changes of morphology in different groups of cells, which were treated with LPS (100 ng/ml) (Fig. 5b) alone, the supernatants of SPM1204 (3 μl/well) (Fig. 5c), ATCC15707 (3 μl/well) (Fig. 5d), were examined. Those treated with the supernatants of bifidobacteria were larger and rougher than those exposed to medium or LPS only as well as in a dose dependent manner (data not shown). These results suggested that cells treated with the supernatants of *Bifidobacterium* strains were more activated than those exposed to either media or LPS (100 ng/ml) alone.

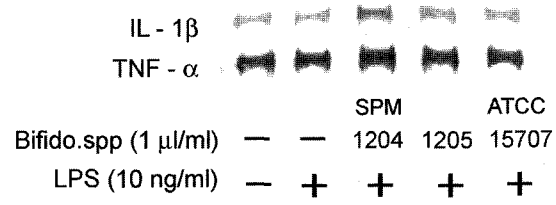

Fig. 4. Western Blot Analysis of the TNF-α and IL-1β in lysates of RAW 264.7 cells (112 μg Protein/Lane). The untreated cells and the cells incubated with LPS alone and with supernatants of *Bifidobacterium pseudocatenulatum* SPM 1204 or 1205 or two bifidobacteira strains (1 μl/ml) were separated by SDS-PAGE, transferred to a nitrocellulose and blotted with a mouse monoclonal anti-TNF-α or IL-1β antibodies. 1;cells, 2; LPS (10 ng/ml), 3;Bifido.SPM1204, 4; Bifido.SPM1205, 5; AT CC15707.

DISCUSSION

Bifidobacteria and other lactic acid bacteria have been previously shown to stimulate immune function (Lee *et al.*, 1993). Furthermore, bifidobacteria and other lactic acid bacteria can improve antitumor activity of the host (Rafter, 1999). It has been suggested that these activities may arise from their ability

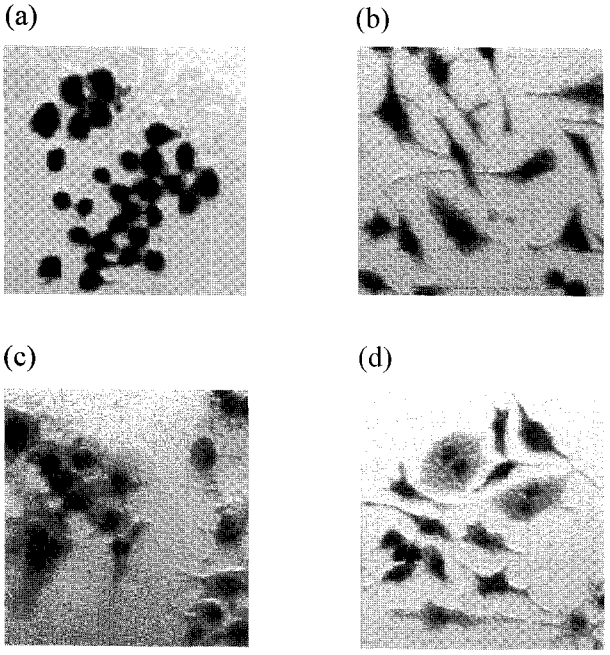

Fig. 5. Macrophage morphological changes in response *Bifidobacterium pseudocatenulatum* SPM 1204 or ATCC *Bifidobacterium* spp. Cells only (a), LPS (b), SPM1204 (c), ATCC15707 (d). The cells were fixed and stained in Diff-quick. Observed under the light microscope at X 400.

to stimulate macrophage and T cell (Sekine *et al.*, 1994) and has been proposed to enhance immunity such as macrophage and lymphocyte activation (Hatcher and Lambrecht 1993; Sekine *et al.*, 1994) and antibody production (Yasui and Ohwaki 1991, Lee *et al.*, 1993; Link-Amster *et al.*, 1994) against infection by various pathogenic organisms. However, the mechanism whereby bifidobacteria may modulate the immune responses remains unclear. Since cross presentation emerges to be required for the effective generation of CTL responses, it is interesting to see whether modulation of cross presentation capability could be a feasible way of immunoregulators on the cross presentation capability of DCs. This report demonstrates that culture supernatants of two bifidobacteria isolated from the feces of healthy adults can enhance presentation of exogenous particulate antigen in the association of class I MHC by dendritic cell line (DC2.4).

Macrophages are important regulatory and effector cells that play a central role in cell-mediated immunity because they present antigen and mediate inflammatory, tumoricidal and microbiocidal activity (Kovacsovics-Bankowski *et al.*, 1993). These functions can be altered by a variety of stimulatory or suppressive signals and are influenced by many environmental factors. Numerous macrophage functions are mediated through the release of different cytokines (Abbas *et al.*, 1994; Cavaillon 1994). Therefore, cytokine production is likely to be a good indicator of the degree of macrophage activation. In the present study, exposure of RAW 264.7 cell line to human bifidobacterial isolates resulted in remarkable increases of NO, TNF-α production. The culture supernatant of bifidobacteria was found to stimulate macrophages to release TNF-α and NO production. The increased production of NO, TNF-α by bifidobacteria observed in this study suggests that these may be key factors for the increased phagocytosis and inhibition of tumor cell proliferation observed by various investigators. Therefore, these results demonstrate the ability of bifidobacteria to activate macrophage and increase APC function through MHC class I. This paper showed the other beneficial effects of the intake of bifidobacteria are reported to include the reinforcement of immune function. It was found that SPM1205 could activate macrophage and enhance cross-presentation strongly. Further research is needed to identify the effective components of this *Bifidobacterium*.

Although bifidobacteria are now routinely incorporated into dairy foods or pharmaceuticals, statements of specific benefits from these products need to be further backed up by mechanistic and clinical studies. This study provides some insights into the mechanisms by which these organisms may provide health benefits. The results reported here suggest that both human and commercial isolates of bifidobacteria increase the secretion of several macrophage mediators and thus could potentially modulate the host immune response.

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