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Comparison of Nitric Oxide, Hydrogen Peroxide, and Cytokine Production in RAW 264.7 Cells by *Bifidobacterium* and Other Intestinal Bacteria

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Abstract Intestinal bacteria comprise one-third of the contents of the large intestine in humans. Their interactions with the gastrointestinal immune system induce characteristic immunological responses which stimulate or suppress the host's defense system. RAW 264.7 murine cell line was used as a macrophage model to assess the effects of the exposure to the isolated human intestinal bacteria, *Bacteroides, Bifidobacterium, Eubacterium, Streptococcus*, and *E. coli*, on NO (nitric oxide), H_2O_2 (hydrogen peroxide), and cytokines IL (interleukin)-6 and TNF (tumor necrosis factor)-α production. RAW 264.7 cells were cultured in the presence of heat-killed bacteria for 24 h at concentrations of 0~50 μg/ml. Our results showed that *Bacteroides* and *E. coli* stimulated IL-6, TNF-α, NO, and H_2O_2 production at high levels even at 1 μg/ml, whereas *Bifidobacterium, Eubacterium*, and *Streptococcus* showed a low level of stimulation at 1 μg/ml and a gradual increase as the cell concentration increased up to 50 μg/ml. This result suggests that gram-negative *Bacteroides* and *E. coli* are better able to stimulate macrophage than gram-positive *Bifidobacterium, Streptococcus*, and *Eubacterium*. The *in vitro* approaches employed here should be useful in further characterization of the effects of intestinal bacteria on gastrointestinal and systemic immunity.

Key words: *Bifidobacterium*, intestinal bacteria, macrophage, H_2O_2, nitric oxide, cytokine

There are at least 400 species and over 10^{14} bacteria amounting to several hundred grams of bacterial mass in the human intestinal tract [4]. Among them, anaerobic organisms such as *Bacteroides, Bifidobacterium*, and *Eubacterium* are the most predominant in the large intestine and facultative aerobic *E. coli* and *Streptococcus* are more prevalent in the small intestine [32]. After the weaning period, *Bacteroides* outnumber *Bifidobacterium* and comprise the greatest population throughout our life time. The role of *Bacteroides* in human intestinal physiology has been actively studied by various researchers [32]. They are known to produce several harmful metabolic products and enzymes in the intestine and are found most frequently among the anaerobic organisms in the extra-intestinal infection area [4].

Genus *Bifidobacterium* is a nonpathogenic, gram-positive and anaerobic bacteria which inhabits intestinal tracts of humans and animals. In breast-fed infants, bifidobacteria comprise more than 90% of the bacterial population [3, 32], however their numbers gradually decrease over the life time of the host. *Bifidobacterium* sp. are used in commercial 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 the synthesis of vitamins, and aiding anticarcinogenic activity [6, 21, 23, 26]. Gram-positive *Streptococcus* and gram-negative *E. coli* are the most predominant facultative aerobes in the human intestinal tract. However, their numbers exist at a 10^3-fold lower level than *Bacteroides* or *Bifidobacterium*.

Recently, there has been considerable interest in the importance of functional food in maintaining balanced microflora with beneficial functions. In this context, reinforcement of the immune function has been pursued based on the effect of bifidobacteria and lactic acid bacteria on the enhancement of immune functions [49, 52]. For instance, it has been shown that bifidobacteria enhance several immune functions, namely macrophage and lymphocyte activation [19, 41], antibody production [28, 29, 52, 53], and the mitogenic response in spleen and Peyer's patches [22, 24, 28, 46, 52]. Such stimulation of

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the immune response by bifidobacteria has been proposed to enhance resistance to infection by pathogenic organisms [10, 36, 51] and potentially prevent cancer [11, 35, 39, 42]. However, it has not been agreed what the optimum level is for various immune functions. Therefore, it is required to assess the role of various intestinal components including foods and bacteria in the immune function. Since macrophages play a major role in host defense against infection and tumor formation, the effect of various bacteria on the macrophage function were tested using the macrophage cell line, RAW 264.7. It is believed that macrophages regulate immunity through the production of several mediators such as TNF (tumor necrosis factor)-α and IL (interleukin)-6 [27]. Of particular interest, the production of NO (nitric oxide) and H_2O_2 (hydrogen peroxide) by macrophages mediates the killing or the growth inhibition of tumor cells, bacteria, fungi, and parasites [30, 37, 42]. While these mediators play key homeostatic functional roles, they are potentially capable of injuring host tissues [12]. Thus, regulation of these mediators is important for normal physiological immune states.

Characterization of the effects of intestinal bacteria on the production of macrophage mediators may contribute to the understanding of how *Bifidobacterium* and other intestinal bacteria affects the immune function at the cellular level in humans and animals. In this study, we used the RAW 264.7 murine macrophage model to evaluate the effects of several intestinal bacteria on NO and H_2O_2, IL-6, and TNF-α production. The results suggested that *Bacteroides* and *E. coli* act as better macrophage-activating bacteria than *Bifidobacterium*, *Eubacterium*, and *Streptococcus*.

MATERIALS AND METHODS

Bifidobacterium Cultures

The isolation of *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Streptococcus*, and *E. coli* from healthy volunteers for the supply of their feces were performed according to the protocol of Choi *et al.* [6] and identified according to Bergey's Manual of Systematic Bacteriology [38]. *Bif. longum* ATCC 15707 was obtained from American Type Culture Collection (Rockville, MD). *Bifidobacterium* Bf-1 was kindly supplied by Michelle Malone of Sanofi Bio-Industries (Waukesha, WI). All strains were cultured and sub-cultured anaerobically in MRS broth (Difco, Detroit, MI) containing 5% (wt/vol) lactose (MRSL) at 37°C until the late log phase. Cells were collected by centrifugation at 1000×g for 15 min at 4°C and washed twice with PBS followed by final washing with distilled water. They were dried (Speed-Vac Instruments, INC., N.Y.) and resuspended with Hanks' buffered salt solution (Sigma Chemical Co., St. Louis, U.S.A.) to the desired bacterial concentration on a dry weight basis. For introduction into tissue culture, bacterial cells were heat-killed by heating at 95°C for 30 min. Heat-killed cultures were aliquoted and stored at -80°C until used.

Chemicals and Reagents

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, U.S.A.). 2,7-Dichlorofluorescin diacetate (DCF-DA) and dichlorofluorescein (DCF) were purchased from Molecular Probes (Eugene, U.S.A.). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Chagrin Falls, U.S.A.). Tetramethylbenzidine (TMB) was from Fluka Chemical Corp. (Ronkonkoma, 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 incubated at 37°C in a humidified atmosphere with 5% CO_2. Cell number and viability were assessed by the trypan blue dye exclusion method [45] on a Neubauer hemacytometer (American Optical, Buffalo, U.S.A.). Cells were grown to confluence 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, U.S.A.). Cultures containing bacterial cells were incubated for various time intervals and analyzed for H_2O_2, NO, TNF-α, and IL-6.

H_2O_2 Determination

The methods described by Tiku *et al.* [47] were used for H_2O_2 determination. After culturing in 96-well bottom plates, cell culture supernatants were removed and attached cells were washed twice with 0.01 M phosphate (pH 7.2) buffered saline (PBS) and then resuspended in PBS containing 4 μM DCF-DA. The intracellular production of H_2O_2 was measured after 1 h incubation at 37°C by fluorometric detection of DCF oxidation using a Cytofluor II Model (Bioresearch Inc., Bedford, U.S.A.) set at 484 nm extinction/530 nm emission. A DCF calibration curve was generated to quantitate the amount of DCF oxidized.

NO Determination

Nitrite accumulation was used as an indication of NO production. This procedure for NO determination was based on the Griess reaction [17]. One hundred μl of culture supernatant or sodium nitrite was mixed with an equal volume of Griess reagent (0.1% (w/v) naphthyl ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v)

phosphoric acid) in microtiter wells. After 5 min at room temperature, the optical density at 550 nm (OD_{550}) was measured by using a microplate reader (Menlo Park, U.S.A.). Samples of the culture media incubated without macrophage were assayed for background levels of nitrite and these values were subtracted from the values measured in the culture supernatant.

TNF-α and IL-6 Quantitation

Production of TNF-α and IL-6 was quantitated by ELISA using modification of the procedure of Dong *et al.* [9]. Briefly, microtiter strip wells (Immunolon IV Removawell; Dynatech Laboratories Inc., Chantilly, 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 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 TNF-α, 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 TNF-α or IL-6 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 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 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, U.S.A.). TNF-α and IL-6 were quantitated using Vmax Software (Molecular Devices).

Statistical Analysis

Data were analyzed by the Student-Newman-Keuls (SNK) test following one way analysis of variance (ANOVA) using the Sigmastat Statistical Analysis System (Jandel Scientific, San Rafael, U.S.A.).

RESULTS

Cytokine Production by RAW 264.7 Cells

To assess the effects of *Bifidobacterium* and other intestinal bacteria such as *Bacteroides, E. coli, Eubacterium*, and

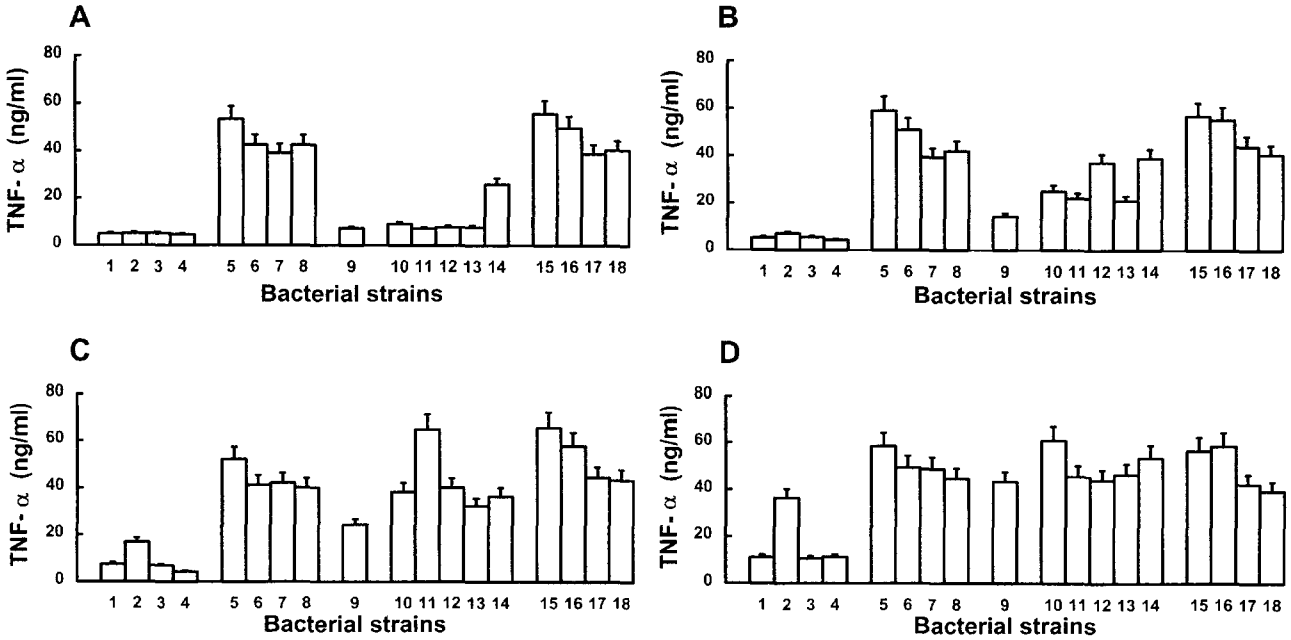

Fig. 1. Effect of various intestinal bacteria on TNF-α production by murine macrophage cell line RAW 264.7 cells.

Macrophage cells (5×10^5 cells/ml) were cultured for 24 h in the presence of various bacterial concentrations of 1 (A), 5 (B), 10 (C), and 50 μg/ml (D). Data are means (±SD) of triplicates. TNF-α production in the absence of bacterial cells (control, data not shown) was below 4.0 ng/ml. Bacterial strains tested were 1. *Bifidobacterium longum* ATCC15707; 2. *Bifidobacterium* Bf1; 3. *Bifidobacterium* NO3; 4. *Bifidobacterium* JS8; 5. *Bacteroides* YV101; 6. *Bacteroides* WV102; 7. *Bacteroides* MV103; 8. *Bacteroides* BV104; 9. *Eubacterium* BN101; 10. *Streptococcus* YS101; 11. *Streptococcus* MS102; 12. *Streptococcus* BS103; 13. *Streptococcus* TT104; 14. *Streptococcus* WS105; 15. *E. coli* YD101; 16. *E. coli* WD102; 17. *E. coli* MD103; 18. *E. coli* BD104.

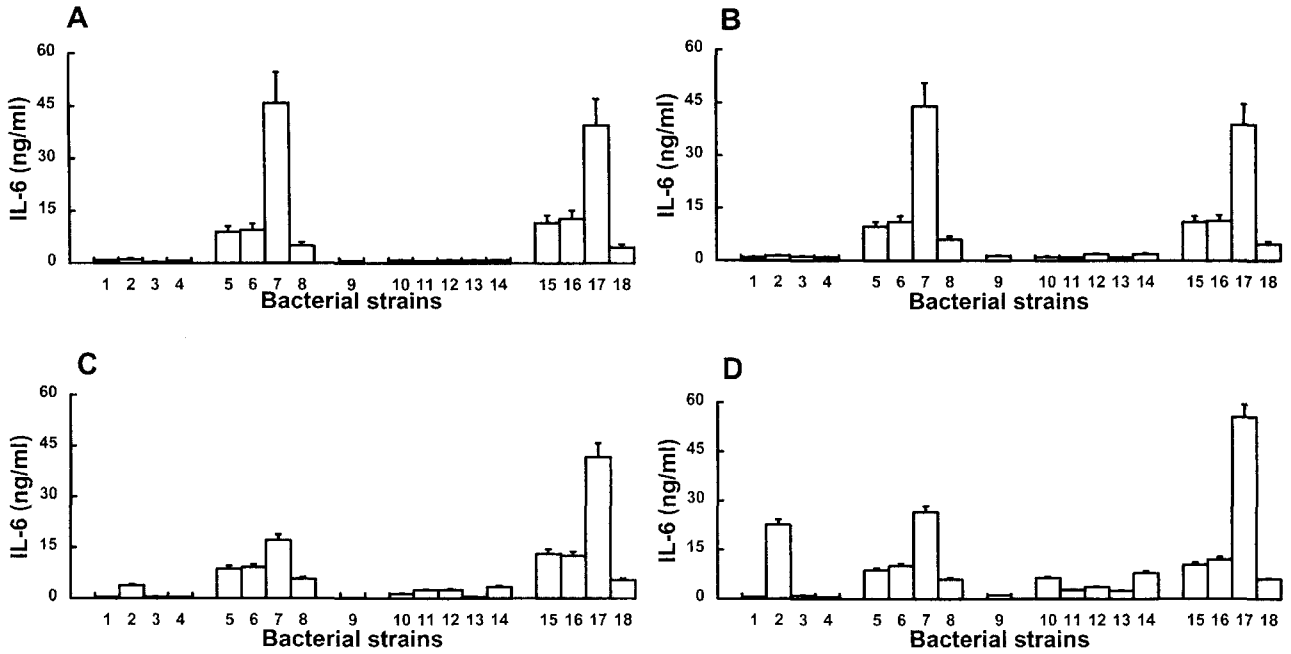

Fig. 2. Effect of various intestinal bacteria on IL-6 production by murine macrophage cell line RAW 264.7 cells.

Macrophage cells (5×10^5 cells/ml) were cultured for 24 h in the presence of various bacterial concentrations of 1 (A), 5 (B), 10 (C), and 50 μg/ml (D). Data are means (±SD) of triplicates. IL-6 production in the absence of bacterial cells was below 1.0 ng/ml. Bacterial strains used and their numberings are the same as in Fig. 1.

Streptococcus on TNF-α and IL-6 production of macrophages, RAW 264.7 cells were incubated in the presence of 0~50 μg/ml bacterial cells. Cytokine secretion in culture supernatants was monitored by ELISA. The results for TNF-α and IL-6 production are shown in Figs. 1 and 2, respectively. A marked difference between

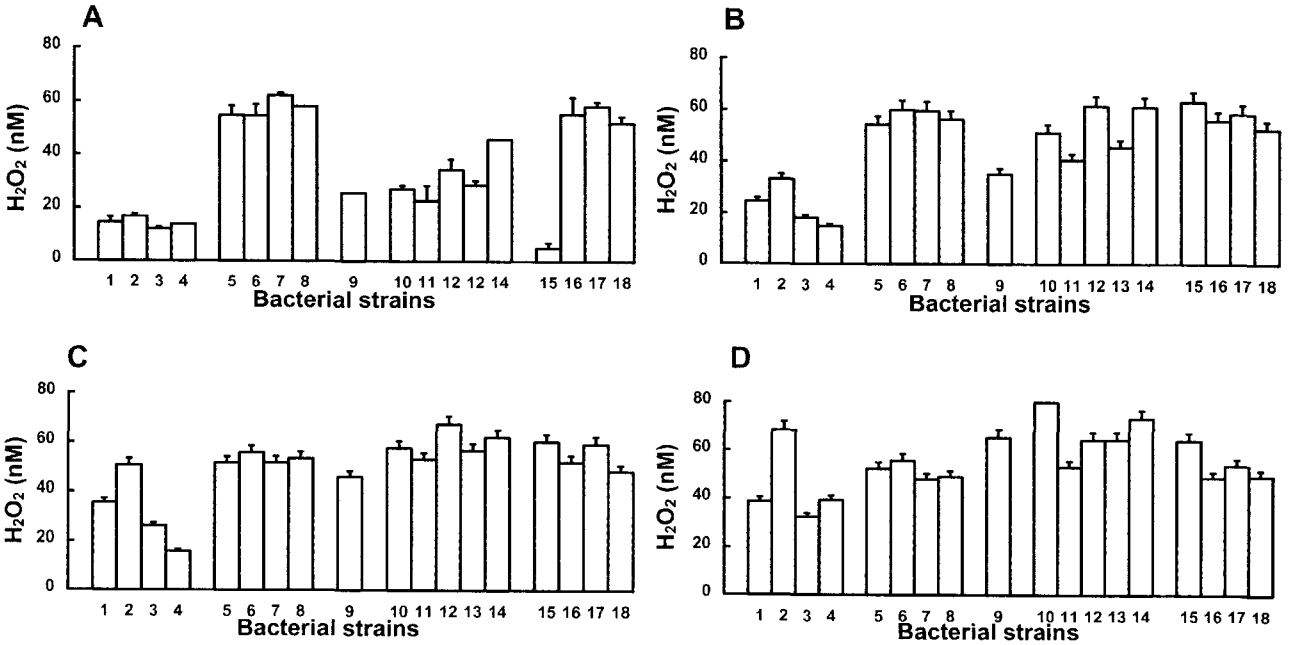

Fig. 3. Effect of various intestinal bacteria on H_2O_2 production by murine macrophage cell line RAW 264.7 cells.

Macrophage cells (5×10^5 cells/ml) were cultured for 24 h in the presence of various bacterial concentrations of 1 (A), 5 (B), 10 (C), and 50 μg/ml (D). Data are means (±SD) of triplicates. H_2O_2 production in the absence of bacterial cells was below 7.0 nM.

different genera for TNF-α production was observed. At all concentrations of 1~50 μg/ml, *Bacteroides* and *E. coli* showed high levels of TNF-α and IL-6 production suggesting that the level of stimulation almost reached its peak at 1 μg/ml. *Bacteroides* MV103 was highest and *Bacteroides* BV104 was lowest among the *Bacteroides*, while *E. coli* MD103 was highest and *E. coli* BD104 was lowest in the production of IL-6. For the production of TNF-α, the difference between strains of *Bacteroides* and *E. coli* was not pronounced. *Bifidobacterium* strains except Bf1 were weak in the production of TNF-α and IL-6. *Bifidobacterium* Bf-1 showed a noticeable increase at 25 μg/ml compared to other *Bifidobacterium* strains. Overall, *Streptococcus* showed a higher level of stimulation than *Bifidobacterium* and *Eubacterium* but a lower level than *Bacteroides* and *E. coli*. The production of TNF-α and IL-6 tended to increase as the concentration of *Bifidobacterium*, *Eubacterium*, and *Streptococcus* increased in the range of 0~50 μg/ml bacterial cell concentrations.

Effect of *Bifidobacterium* on H_2O_2 and NO Production

Hydrogen peroxide and NO are important mediators of macrophage phagocytosis because they act as reactive oxygen and nitrogen intermediates during oxygen-dependent phagocytosis [25]. The effects of *Bifidobacterium* and other intestinal bacteria such as *Bacteroides*, *Eubacterium*, *E. coli*, and *Streptococcus* on H_2O_2 and NO production in RAW 264.7 cells were evaluated using fluorometric H_2O_2 analysis and Griess assay, respectively. Their results are shown in Figs. 3 and 4. A marked difference between genera for H_2O_2 and NO production was observed. Even at bacterial cell concentration as low as 1 μg/ml, *Bacteroides* and *E. coli* showed peak values. On the other hand, production of H_2O_2 and NO increased with increasing concentrations of *Bifidobacterium*, *Eubacterium*, and *Streptococcus* in the range of 5~50 μg/ml. Among *Bifidobacterium* strains, Bf-1 showed significantly higher H_2O_2 production than other *Bifidobacterium* strains tested at the cell concentrations of 1~50 μg/ml. All tested strains of *Streptococcus* and *Eubacterium* showed higher levels of production of H_2O_2 and NO than *Bifidobacterium* except Bf1, but lower than *Bacteroides* and *E. coli*. However, as the cell concentration increased gradually, the difference among different genuses became less marked.

DISCUSSION

Bifidobacteria and other lactic acid bacteria have been previously shown to stimulate the immune function [15, 24, 28] and improve antitumor activity of the host [11, 35, 40, 42]. These activities may arise from their ability to stimulate macrophages and T cells [19, 39, 40]. Many of the macrophage functions are mediated through the release of different cytokines, TNF-α and IL-6 [5]. There is extensive evidence that cytokines play pivotal roles in host defense, inflammatory responses, and autoimmune disease [1, 2] and alter these functions by a variety of

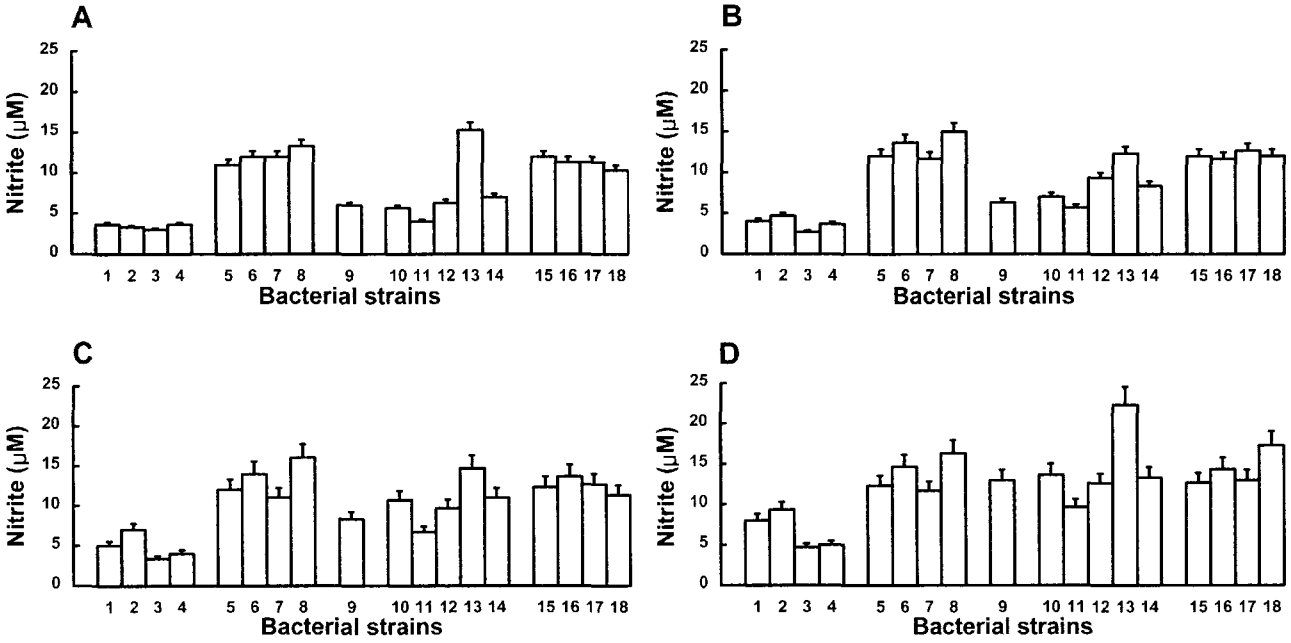

Fig. 4. Effect of various intestinal bacteria on NO production by murine macrophage cell line RAW 264.7 cells.

Macrophage cells (5×10^5 cells/ml) were cultured for 24 h in the presence of various bacterial concentrations of 1 (A), 5 (B), 10 (C), and 50 μg/ml (D). Data are means (±SD) of triplicates. NO production in the absence of bacterial cells was below 1.5 μM. Bacterial strains used and their numberings are the same as in Fig. 1.

stimulatory or suppressive signals depending on environmental factors. Cell components of *Bifidobacterium* which function as immuno-modifiers of the host were reported to include peptidoglycan, intra- and extra-cellular polysaccharide products, cell free extracts, cell wall preparation [15, 19, 22, 33, 39, 42]. However, at the present time, there is not yet a clear understanding of the molecular and cellular basis for bifidobacterial immunostimulation.

In the present study, exposure of RAW 264.7 cell line to various intestinal bacteria, including predominant large intestinal bacteria such as *Bacteroides, Bifidobacterium, Eubacterium*, and small intestinal bacteria such as *Streptococcus* and *E. coli*, resulted in marked differences in the extent of IL-6, TNF-α, NO, and H_2O_2 production between genera. The degree of enhancement was markedly higher with *Bacteroides* and *E. coli* compared to *Bifidobacterium* and *Streptococcus*. Therefore, gram-negative strains showed higher stimulatory activity than gram-positive strains. The high stimulatory activity of *Bacteroides* and *E. coli* may be due to the presence of lipopolysaccharide in these strains. The lipopolysaccharides of *Bacteroides* and *E. coli* have been reported as strong macrophage activators [7]. The fimbrae and capsular components of these strains may also be contributing factors for the observed activation [13, 18]. In notobiotic mice, *E. coli* showed a higher production of IL-6 by peritoneal cells and immunogenic activity [41] than *Bifidobacterium*. Also, the immunogenicity of *Bif. breve* was weaker than that of *Bac. thetaiotamicron* examined in mice serum fed these strains and *in vitro* Peyer's patch cells [50]. Our results are consistent with the above-mentioned references and contrast to Solis Peyera's report [44] that *Bifidobacterium* and *Streptococcus* induced a higher level of TNF-α than intestinal *E. coli* when human blood monocytes were incubated in the presence of these bacteria. The major resident flora comprise about 10^7 cfu/g of *E. coli* and *Streptococcus* in the small intestinal tract and about 10^{10} cfu/g of *Bacteroides* and *Bifidobacterium* in the large intestinal tract [32].

Our results suggest *E. coli* has more influence on macrophage activity than *Streptococcus* in the small intestine and *Bacteroides* have a stronger influence than *Bifidobacterium* in the large intestinal tract. Since *Bacteroides* and *E. coli* are harmful to the human host [26, 31], the high level of macrophage activation may be due to the necessity of the human host to defend from these challenging organisms. However, *Bifidobacterium* does not act as a pathogen in healthy hosts. Therefore, the relatively low degree of activation may not lead to immune toxicity for the host but rather contribute to the enhancement of the host immune function. The effect of these organisms is also dependent on the cell concentration reacting with the macrophage cells. Therefore, the degree of infiltration of the different bacteria into the immune system needs to be assessed, especially in the Peyer's patch which is exposed to a constant influx of intestinal bacteria and act as an active sampling tissue through the M cell component system [34].

The successful colonization of *Bifidobacterium* may decrease the *Bacteroides* population [14]. This might lead to the lowering of the macrophage cytokine level if our *in vitro* results occur also *in vivo*. However, most of the previous reports showed an increased level of macrophage cytokine production when bifidobacteria were administered orally into the human model [8]. The reported stimulated cytokine production of the orally-administered *Bifidobacterium* may be from their interaction with the small intestinal tract immune system during their passage from the stomach to the large intestine, whereas indigenous organisms may act on the lower part of the small intestine and large intestine where the immune reaction may be different from the small intestine. The induction of immune tolerance by the indigenous bacteria may be another contributing factor. Administered organisms may have strong influence on the immune activity whereas a transient change of the indigenous flora may not show a noticeable change in the immune activity due to the already established tolerance.

TNF-α mediates a wide variety of biological activities [48]. The induction of NO and H_2O_2 by bacteria may be a consequence of TNF-α stimulation as TNF-α is known to be an inducer for NO and H_2O_2 [20, 31]. Moderate production of appropriate levels of cytokines, H_2O_2, and NO may play a role in the immune defense for the tumoricidal and microbicidal activities, while large amounts of TNF-α, H_2O_2, and NO for a long period of time causes inflammation as well as tissue injury [1]. Therefore, the control of those macrophage mediators *in vivo* is very important.

In conclusion, the results reported here suggest *Bacteroides* and *E. coli* increase the secretion of several mediators by macrophage greater than gram-positive *Bifidobacterium* and *Streptococcus* and thus could potentially modulate the host immune response more strongly than *Bifidobacterium* and *Streptococcus*. The *in vitro* approaches employed here should be useful in the future mechanistic characterization of the effects of bifidobacteria and intestinal bacteria on gastrointestinal immunity and their influence for enhancing, hyperactivating, or suppressing the immune function of the host and the mechanism underlying immune-modulation.

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