Article

Bifidobacteria DNA Induces Murine Macrophages Activation *in vitro*

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Previous studies have shown that oligodeoxynucleotides containing unmethylated CpG motifs were used as adjuvants for immunoregulation and immune response. This study was to explore the activation effects of Bifidobacteria DNA containing unmethylated CpG motifs (CpG DNA) on murine macrophage J774A.1 cells. The genomic DNA of Bifidobacteria was extracted and purified, and the methylation degree of CpG motifs was tested. The phagocytic ability of the macrophages was detected by flow cytometry. The cytokines (IL-1β, IL-6, IL-12p40 and TNF-α) levels in the culture supernatants of Bifidobacteria DNA treated J774A.1 cells were assayed by ELISA. The content of nitric oxide (NO) was detected by Griess reagent. After treated with Bifidobacteria DNA for 24h, Nile Red stain increased in J774A.1 macrophage, which suggested that the lipid metabolism increased in the macrophages. The phagocytic ability and levels of NO and cytokines of IL-1β, IL-6, IL-12p40 and TNF-α were significantly higher than PBS group and CT DNA group. The results indicated that Bifidobacteria DNA could activate murine macrophages J774A.1, which could provide scientific basis for the research and application of microorganism DNA preparation. *Cellular & Molecular Immunology*. 2005;2(6):473-478.

Key Words: Bifidobacteria DNA, murine J774A.1 macrophage, phagocytic ability, cytokine, nitric oxide

Introduction

Recognition of conserved molecular characteristic patterns of pathogens by pattern recognition receptor has been suggested to be a property of the mammalian immune system. Bacterial lipopolysaccharide (LPS or endotoxin) is one of the activating components from the Gram-negative bacteria outer membrane, and it plays an important role in the pathogenic mechanism of Gram-negative bacteria. Bacterial DNA and oligodeoxynucleotides containing unmethylated CpG motifs have similar effects and mechanisms on LPS, as they can activate antigen presenting cells to release proinflammatory cytokines and other inflammatory mediators, affecting their differentiation, survival and migration activity. Thus they can induce many types of pathological changes even septic shock, so they were danger signals for mammalian immune system (1, 2). Besides that, they were always used as adjuvants to elicit immunoregulation and immune response. CpG-oligodeoxynucleotides (CpG-ODNs), which contain unmethylated CpG dinucleotides in the context of particular base sequences, have gained attention because of their stimulating effects *via* NF-κB. Leelawongtawon R and others reported that CpG-DNA and liposome are effective mucosal adjuvants for an oral cholera vaccine prepared from refined V. cholerae antigens and their combination seems to be synergistic (3). Bacterial DNA containing unmethylated CpG motifs is a pathogen-associated molecular pattern (PAMP) that interacts with host immune cells *via* a Toll-like receptor (TLR) to induce immune responses (4). Garbi Ne and others evaluated the effectiveness of immunostimulatory oligodeo-xynucleotides (ODN) with cytosine-guanine-rich (CpG) motifs (CpG-ODN). Their data demonstrated that systemic application of CpG-ODN reagents drastically enhanced extravasation of effector cells into tumor tissue, an observation that was of general importance for immuno-therapy of solid tumors in a clinical setting (5). The gut micro-biota is a complex ecosystem composed of hundreds of different bacterial species that altogether play an important role in the physiology of their host. Bifidobacteria are among the first

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Abbreviations: CpG, unmethylated CpG dinucleotides within specific sequence contexts; LPS, lipopolysaccharide; NO, nitric oxide; FCS, fetal calf serum; B DNA, Bifidobacteria DNA; CT DNA, calf thymus DNA.

species to colonize the human gastrointestinal tract and as such are believed to play an important role in gut homeostasis and normal development (1). Bifidobacteria are Gram-positive prokaryotes that naturally colonize the human gastrointestinal tract (GIT) and vagina. Although not numerically dominant in the complex intestinal microflora, they are considered as key commensals that promote a healthy GIT (4). Le Leu RK and others reported that the synbiotic combination of resistant starch (RS) and Bifidobacterium lactis (B. lactis) significantly facilitated the apoptotic response to a genotoxic carcinogen in the distal colon of rats (6). It has been found that the Bifidobacteria cell wall can effectively activate immunocytes, evoke these effector cells to release immune active substance, and inhibit tumor growth and development. Sekine K and others studied the mechanisms of Bifidobacteria in antitumor activity using a cell wall preparation (WPG) of B. infantis. Their study suggested that WPG enhanced the *in vitro* antitumor activities of mouse peritoneal exudate cells elicited with proteose-peptone (P-PEC) and thioglycollate broth (TG-PEC) (7). To date, little is known about the immunoregulation of Bifidobacteria DNA to the murine J774A.1 macrophages. We observed the effective activity of Bifidobacteria DNA to the murine J774A.1 macrophages.

Materials and Methods

Strains and cells

Bifidobacteria were purchased from Shandong Sanitary Epidemic Prevention Station. Murine macrophage J774A.1 was kindly provided by Dr. Xuetao Cao (Second Military Medical University).

Reagents

DMEM (Dulbecco's modified eagle's medium) and fetal calf serum (FCS) were purchased from Hyclone Company. Nile Red and FITC-conjugate Dextran-10000 were purchased from PharMingen. mIL-1β, mIL-6, mIL-12p40 and mTNF-α ELISA kits were obtained from Endogen Company. LPS was purchased from Beijing Bangding Company. Calf thymus DNA (CT DNA), lysozyme, naphthylethylenediamide-dihydrochloride and sulfanilamide were purchased from Sigma Company. Restriction endonulease Hpa II was purchased from Youyi Company of Chinese Academy of Sciences.

Preparation and identification of Bifidobacteria DNA

Bifidobacteria were inoculated into sulfoglycolate broth, and cultured in anaerobic incubator at 37°C for 72 h. The bacteria were then harvested. Lysozyme (1 mg/ml) and 10%SDS (final concentration 1%) were added to lyse the bacteria, and then extracted with saturated phenol to remove the bacterial protein. The concentration of Bifidobacteria DNA was OD260/OD280 1.823 detected by ultraviolet spectrometry. There was no LPS in the prepared Bifidobacteria DNA which was detected by thin-layer chromatography. Electrophoresis showed that Bifidobacteria DNA could be digested into fragment by restriction enzyme Hpa II, which suggested that the prepared Bifidobacteria DNA contained unmethylated CpG motifs. The prepared Bifidobacteria DNA was frozen and dried for use.

Cell cuture

The murine J774A.1 macrophages were adjusted to the concentration of 5×10^5/ml with DMEM containing 10% FCS. Then PBS, Bifidobacteria DNA (10 μg/ml), LPS (100 ng/ml) CT DNA (10 μg/ml) were added respectively. After incubation at 37°C for 24 h inside a humidified 5%CO2 incubator, cells and supernatants were collected for detection, respectively. Lipid was detected by Nile Red stain, and morphological changes were observed by light microscopy.

Phagocytic ability analysis

J774A.1 cells were incubated at 37°C for 2 h with fluorescein isothiocyanate (FITC)-conjugated Dextran at a final concentration of 25 μg/ml in RPMI 1640 medium containing 10% FCS, washed twice with ice-cold PBS (pH7.2, containing 0.1% NaN_3 and 0.5% BSA) and resuspended in chilled PBS for immediate flow cytometry (FACS caliber, BD Bioscience), analyzed with WinMDI 2.8 software.

ELISA for cell supernatants cytokine determination

For the assay, the supernatants prepared from treated J774A.1 cells were evaluated using mouse IL-1β, IL-6, IL-12p40 and TNF-α specific ELISA kits according to the manufactures' instruction respectively.

NO measurement

NO production was assayed by measurement of the nitrite concentration with the Griess assay. Briefly, 100μl supernatants were placed in 96-well plate, and then mixed with equal volume of Griess reagent containing 1% sulfanilamide, 0.1% naphthylethylene-diamine-dihydrochloride, 2.5% H_3PO4. Plates were incubated for 10 min at room temperature, and absorbance at 550nm was measured with a microplate reader (BioRad). Each sample was repeated in three wells, and then the mean value was counted. Nitrite concentrations were calculated with a sodium nitrite standard curve as reference.

Statistical analysis

All results were expressed as the arithmetic mean ± SD of triplicate determinations performed under the same conditions. The differences between the groups were analyzed by the Student's *t*-test and $p < 0.05$ was taken to imply statistical significance.

Results

The prepared Bifidobacteria DNA contained unmethylated CpG motifs

Bifidobacteria DNA could be digested into small fragment by restriction enzyme Hpa II. But if either ^1C or ^2C site in the $^1C^2CGG$ motif of Bifidobacteria DNA was methylated, Hpa

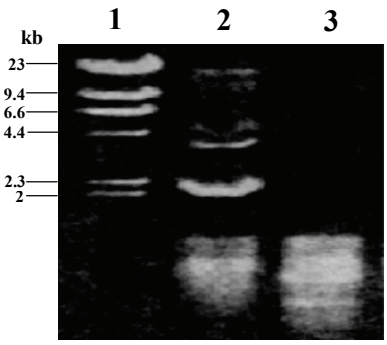

Figure 1. Identification of methylation of Bifidobacteria DNA by restriction enzyme. Lane 1, λDNA/Hind III marker; Lane 2, Bifidobacteria DNA digested by restriction enzyme Hind III; Lane 3, Bifidobacteria DNA digested by restriction enzyme Hpa II.

II could not digest the site. Therefore the sensitivity of Bifidobacteria DNA digested by Hpa II could reflect the CpG motif methylation. Results showed that Bifidobacteria DNA contained some unmethylated CpG motifs (Figure 1).

Effect of Bifidobacteria DNA on the morphology of murine J774A.1 macrophages

After treated with PBS or CT DNA for 24 h, J774A.1 cells change rarely. While treated with Bifidobacteria DNA or LPS, J774A.1 cells became obviously bigger, and their surface became crude and crapy. The morphological changes indicated that the cells were activated (Figure 2). Nile Red

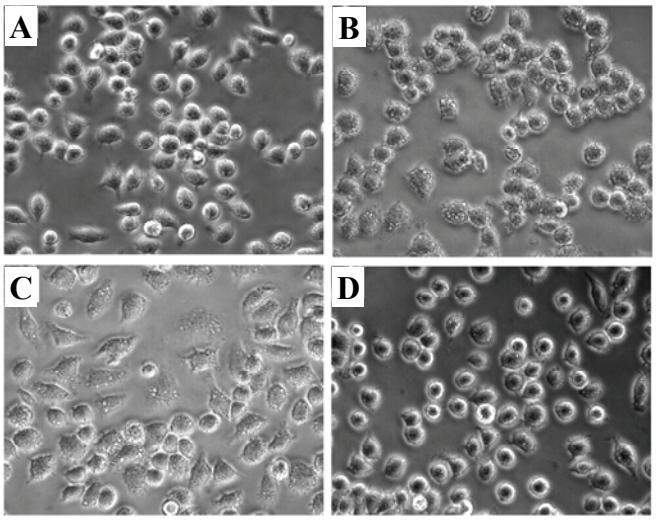

Figure 2. Effect of Bifidobacteria DNA on the morphology of J774A.1 macrophage. The J774A.1 macrophages were adjusted to the concentration of 5×10^5/ml with DMEM (10% FCS). Then PBS (A), Bifidobacteria DNA (B), LPS (C) and CT DNA (D) were added respectively, and cultured at 37°C for 24 h. Cells were collected and morphological changes were detected by light microscopy (× 200).

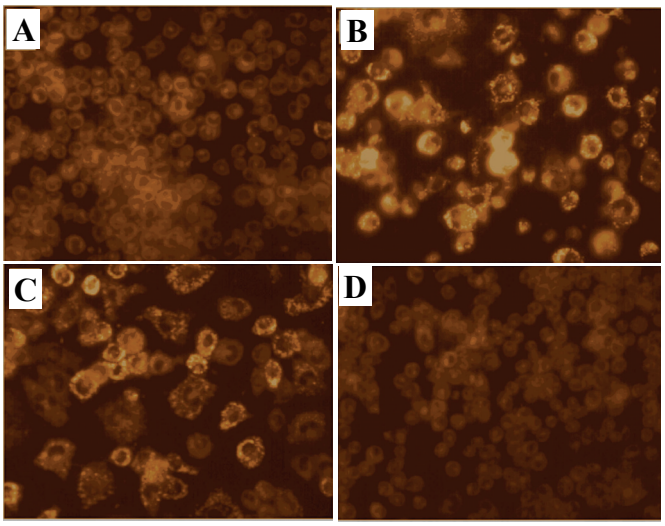

Figure 3. J774A.1 macrophage stained by Nile Red. The J774A.1 macrophages were adjusted to the concentration of 5×10^5/ml with DMEM (10% FCS). Then PBS (A), Bifidobacteria DNA (B), LPS (C), CT DNA (D) were added respectively, and cultured at 37°C for 24 h. Cells were harvested and lipid was detected by Nile Red stain (× 200).

influorescence stain showed that in the PBS and CT DNA treated groups, only the cell membranes were positively stained, while in the Bifidobacteria DNA and LPS treated group, Nile Red stain increased. The results suggested that the lipid metabolism increased (Figure 3).

Bifidobacteria DNA promoted the phagocytic ability of J774A.1 macrophages

Phagocytic ability of macrophages was measured by their ability to engulf the FITC-conjugated Dextran-10000. The mean influorescence intensity of each group was detected by flow cytometry. Phagocytic ability of J774A.1 macrophages was enhanced treated by Bifidobacteria DNA and LPS (Figure 4).

Bifidobacteria DNA promote inflammatory cytokines production of J774A.1 macrophages

J774A.1 cells treated by LPS secreted the highest level of cytokines in all these groups. J774A.1 cells treated with Bifidobacteria DNA secreted higher level of cytokines, IL-1β, IL-6, IL-12p40 and TNF-α than the control group. There was significant statistical difference ($p < 0.01$, Figure 5). There was no significant difference between the CT DNA group and the control group.

Bifidobacteria DNA induced NO production of J774A.1 macrophages

Since nitric oxide has been shown to play an important cytotoxic role in the host defense against invading microorganisms (8), the NO production of J774A.1 macrophages treated wich Bifidobacteria DNA was

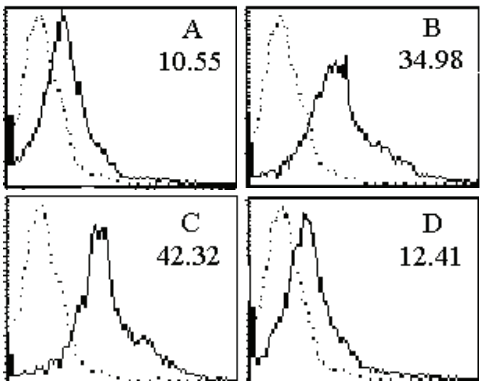

Figure 4. Flow cytometric analysis the phagocytosis of J774A.1 machrophages in vitro. FITC-conjugated Dextran-10000 (final concentration 25 μg/ml) was added into each of the treated J774A.1 cells groups (A, Control; B, Bifidobacteria DNA; C, LPS; D, CT DNA group), and cultured at 37°C for 2 h, then the cells were washed twice by PBS. Phagocytic ability of macrophages was measured by their ability to engulf the FITC-conjugated Dextran-10000 and the mean fluorescence uptake was analyzed by flow cytometry.

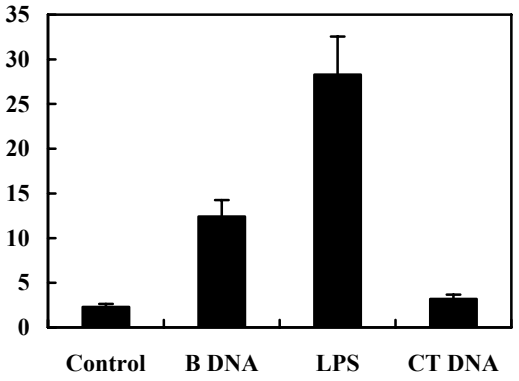

Figure 6. The amounts of NO in the culture supernatants of J774A.1 murine macrophages. The supernatants prepared from treated J774A.1 cells were obtained and the amounts of NO were determined by the colorimetric Griess reaction (n = 3, $^{**}p<0.01$).

investigated. The NO secretion of J774A.1 cells treated with LPS was the highest in all these groups. J774A.1 cells treated wich Bifidobacteria DNA secreted higher than the control group. There was significantly statistical difference ($p < 0.01$, Figure 6). There were no significant difference between the CT DNA group and the control group.

Discussion

Bacterial DNA and oligodeoxynucleotides containing unmethylated CpG motifs have similar effects and mechanisms on LPS. On one hand, they are non-self and danger signals for mammalian immune system. After recognized by antigen presenting cells (APCs), they will elicit APCs to synthesize and secrete many kinds of cytokines and other inflammatory mediators, and induce many types of pathological changes even septic shock. On the other hand, they were always used as adjuvants to elicit immunoregulation and immune response (9-11). Adjuvants can be broadly separated into two classes, based on their principal mechanisms of action, "vaccine delivery systems" and "immunostimulatory adjuvants". Vaccine delivery systems are generally particulate formulations e.g. emulsions, microparticles, iscoms and liposomes, and mainly function to target associated antigens into antigen presenting cells (APC). In contrast, immunostimulatory adjuvants are predominantly derived from pathogens and often represent pathogen associated molecular patterns (PAMPs), e.g., LPS, MPL and CpG DNA, which activate cells of the innate immune system (12).

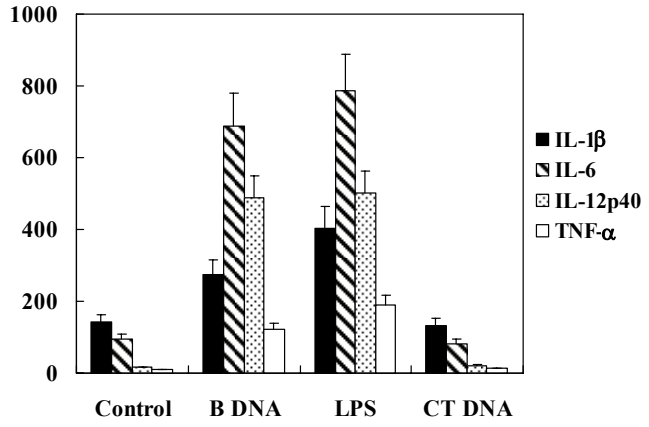

Figure 5. The concentrations of CKs in the culture supernatants of J774A.1 murine macrophages. The supernatants prepared from treated J774A.1 cells were obtained and the levels of CKs (IL-1β, IL-6, IL-12 (p40) and TNF-α) were determined by ELISA study (n=3, $^{**}\ p < 0.01$).

Recent studies showed that murine peritoneal macrophages could discriminate bacterial genomic DNA from the mammalian DNA, and high degree methylation of mammalian DNA couldn't activate the immune system (13). Pisetsky and others reported that the mammalian DNA has the ability to suppress immune response, as they can neutrolize IL-12 produced by bacterial genomic DNA activated macrophages (14). Bacterial DNA containing unmethylated CpG motifs is a pathogen-associated molecular pattern (PAMP) that interacts with host immune cells *via* a Toll-like receptor (TLR) to induce immune responses. McCoy SL and others reported that treatment of RAW264.7 macrophages with *Escherichia coli* DNA or LPS resulted in a significant increase in DNA binding. They speculated that increased DNA binding and internalization following interaction with bacterial PAMPs may provide a mechanism to limit an ongoing immune inflammatory response by enhancing

clearance of bacterial DNA from the extracellular environment (15). Our results suggested that phagocytic ability of J774A.1 macrophages was enhanced after treated with Bifidobacteria DNA containing unmethylated CpG. Bifidobacteria belong to the intestinal normal flora, and the effects were similar to *Escherichia coli* DNA or LPS, so it can be used as adjuvant to elicit immunoregulation and immune response.

Apart from phagocytosis, nitric oxide production is another parameter indicating macrophage activation. In this study, we found that Bifidobacteria DNA containing unmethylated CpG promoted NO production by macrophage. NO has been recognized as a major effector molecules involved in the destruction of tumor cells and pathogenic micro-organism by activated macrophages (16). NO can also suppress growth of tumor cells by many pathways, such as inducing tumor cells apoptosis and inactivating the rate-limiting enzyme and reductase of synthesizing tumor cells DNA.

Cytokines, such as IL-1β, IL-6, IL-12p40 and TNF-α are proinflammatory cytokines usually secreted by activated macrophage. IL-12 is usually secreted by macrophages and dendritic cells, and it can evoke $CD4^+$ T cells to differentiate into Th1 cells. Th1 cells could release IL-2, IFN-γ and other cytokines which could promote cellular immunity and induce the production of LAK (lymhokine activated killer cells) and CTL (cytotoxic T cells). Both of the two type cells have the activity of broad-spectrum killing tumor cells *in vivo* and *in vitro* (17). Joseph A and others reported that synthetic oligo-deoxynucleotides (ODNs) containing immunostimulatory sequences (ISS-ODN, also known as CpG-ODNs) have been shown to display in experimental models potent Th1-biassed immunoadjuvant activity upon parenteral or mucosal co-administration with a variety of antigens (18). In this study, we found that Bifidobacteria DNA containing unmethylated CpG promoted IL-12 production of J774A.1 macrophage, which is consistent with those studies.

Peroxisome proliferation is a kind of cell modified reaction aimed at pathophysiological phenomenon such as cell morphological change and enzyme activity change. Recent evidence suggested that peroxisome proliferator-activated receptor (PPAR) regulatory genes involved in lipid metabolism (19). Our findings showed that Nile Red stain increased in J774A.1 macrophages treated with Bifidobacteria DNA for 24 h, which suggested that the lipid formation were enhanced, which may be related to PPARs activation and peroxisome proliferation (20).

Collectively, our present study suggested Bifidobacteria DNA primed macrophages for an enhanced phaocytosis, directly stimulated NO production, and induced macrophages to secrete inflammatory cytokines IL-1, IL-6, TNF-α, and IL-12. The study could provide scientific basis for the research and application of microorganism DNA preparation.

Acknowledgements

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