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World J Gastroentero, 2001;7(3):440 - 443
World Journal of Gastroenterology
 ISSN 1007 - 9327

Influence of whole peptidoglycan of bifidobacterium on cytotoxic effectors produced by mouse peritoneal macrophages

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Subject headings bifidobacterium; whole peptidoglycan; macrophage; laser scanning confocal microscopy

Wang LS, Zhu HM, Zhou DY, Wang YL, Zhang WD. Influence of whole peptidoglycan of bifidobacterium on cytotoxic effectors produced by mouse peritoneal macrophages. ***World J Gastroenterol***, 2001;7(3):440-443

INTRODUCTION

Bifidobacteria are physiologically beneficial bacteria which are predominant in human intestine, and possess the most important functions. They play an important role in maintaining microbial balance of the intestine. Furthermore, their presence is thought to be an important indication of health of the body[1-4]. Whole peptidoglycan (WPG) is the major component in the cell wall of bifidobacterium, which is also a biological response modifier with nontoxic side effects. At present, many scholars have demonstrated that bifidobacteria can activate macrophages, T lymphocytes and natural killer cells to secrete many kinds of cytokines and important mediators[5-8]. In our report, the level of IL-6, IL-12 and TNF-α produced by macrophages is detected by employing laser scanning confocal microscopy, when these cells are stimulated by WPG of bifidobacteria bifidum. Simultaneously, the content of nitric oxide (NO) secreted from the macrophages is investigated by utilizing Griess reagent. Our goal is to explore the roles of these cytotoxic effector molecules on adjusting immune reaction of bifidobacterial WPG.

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Supported by the Natural Science Foundation of Guangdong Province, No.994066
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Received 2001-02-06 **Accepted** 2001-04-12

MATERIALS AND METHODS

Materials

Whole peptidoglycan It was extracted from the cell wall of bifidobacteria bifidum according to the method described by Sekine and his colleagues,donated by Dr. Hu, who worked at department of examination of Zhongjing Medical University. Simultaneously, it had been evaluated[9].

Experimental animals BALB/c nude mice were purchased from the Experimental Animal Center of the First Military Medical University, 6-8 weeks in age, 18 g-22 g in weight, and housed in the SPF animal room.

Methods

Division of the experimental group and management of WPG The experimental animals were divided into two groups: the WPG injection group: 10 nude mice, 2 mL of 100 $g \cdot L^{-1}$ thioglycollate was injected into the nude mice intraperitoneally on d1, 0. 25 mg WPG (equivalent to 1×10^9 bifidobacteria) was injected intraperitoneally from d 2-6 continuously; and the control group: the number of animals and procedures were similar to the WPG injection group on d1, 0.2 mL of PBS was injected intraperitoneally from d 2-6 continuously.

Collection and culture of peritoneal macrophages of nude mice All animals were killed on d7. Abdomen skin of the nude mice was sterilized routinely. Cells were obtained by sterile lavage of the peritoneal cavity with cold D-hanks balanced salt solution. The lavage fluid was centrifuged at 1000 rpm for 10 min. The supernatant was decanted. The cells thus obtained were twice washed, resuspended in RPMI 1640 culture medium containing free fetal calf serum, then adjusted to be $1 \times 10^9 \cdot L^{-1}$, and plated ($2 \times 10^5$ cells/well) in 96-well tissue culture plates. Nonadherent cells were removed by repeated washing after 2 h incubation at 37℃ in 50 $mL \cdot L^{-1}$ CO_2 in air. Adherent cells were macrophages. These cells were stimulated with 10 $\mu g \cdot L^{-1}$ LPS in RPMI 1640 containing 100 $mL \cdot L^{-1}$ FCS for 24 h. Supernatant was stored at -30℃ until assayed.

Detection of IL-6, IL-12 and TNF-α contents of peritoneal macrophages of nude mice Laser scanning

confocal microscopy was utilized to detect the content of IL-6, IL-12 and TNF-α. The concrete protocol was as follows: ① The peritoneal cell suspension of nude mice was harvested and added to the modified Petri plate, and the desired peritoneal exudate cells were adjusted to 1×10^5 in 100 μL·plate^{-1}. After incubated in a 50 mL·L^{-1} CO_2 incubator at 37℃ for 2 h, these plates were washed vigorously to eliminate nonadherent cells, and macrophages were obtained. Then 500 μLof RPMI 1640 medium containing 10 μg·L^{-1} LPS was added to each plate. After 24 h, the supernatants were discarded, and the macrophages were washed with phosphate buffered saline (PBS) 3 times for 5 min; ② 100 g·L^{-1} non-specific bovine serum album was added to the plates which were incubated for 10 min at room temperature; ③ The plates were rinsed with PBS 3 times for 5 min;④ 100 μL of rabbit anti-mouse IL-6, IL-12 and TNF-α monoclonal antibody (Genda Technology Corp. Canada) was added to the plates which were incubated for 60 min at 37℃ respectively; ⑤ The plates were rinsed with PBS 3 times for 5 min; ⑥ 100 μL of FITC labeled goat anti-rabbit IgG (Santa Cruz) was added to the plates which were incubated for 30 min at 37℃. The antibody was used at a dilution of 1 in 100. ⑦ The plates were rinsed with PBS 3 times for 5 min; ⑧ The fluorescent intensity of macrophages was detected by employing laser scanning confocal microscopy with an arousement wavelength of 488 nm and a radiation wavelength of 475 nm. For each plate, average fluorescent value from beyond 100 macrophages in different fields was investigated, which was chosen to be quantitative parameter.

Griess reagent Naphthylethylenediamine hydrochloride (0.1%) (Sigma) was prepared with distilled water, and 1% sulfanilamide (Sigma) was prepared with 5% H_3PO_4. The two reagents were mixed equally before utilization.

Detection of the level of NO After 1 mmol·L^{-1} sodium nitrite ($NaNO_2$) was diluted by means of doubling series, it was mixed with equal Griess reagent, then shaken for 10 min lightly at room temperature. At last, absorbance value (A) of triplicate samples was read on Bio-Rad 550 type microelisa reader using a test wavelength of 550 nm. One hundred microliter of each supernatant which was to be determining was mixed with 5 μL of 300 g·L^{-1} $ZnSO_4$ to remove protein, then centrifuged at 5000 rpm for 10 min. The supernatant was collected, and mixed with equal Griess reagent, then shaken lightly for 10 minutes at room temperature. Absorbance value (A) was read on Bio Rad 550 type Microelisa Reader using a test wavelength of 550 nm.

Statistic analysis

The statistical analysis between WPG injection group and control group was made by means of Student's *t* test.

RESULTS

NO content of produced by peritoneal macrophages of nude mice stimulated by WPG

A standard curve was made by analyzing the relationship between different concentrations of standard $NaNO_2$ and the corresponding A value assayed. Simultaneously, linear correlation and regression analysis was performed, and the linear regression equation was as follows: Y = 0.013 + 0.022X, r = 0.999. It suggested that there existed a fine correlation between the content of standard $NaNO_2$ and the corresponding A value. The content of NO was obtained correspondingly when the A value of different samples substituted the linear regression equation. By means of the statistical analysis,we found that the content of NO produced by peritoneal macrophages of nude mice in WPG injection group was significantly higher than in the control group (P<0.01, Table 1).

WPG influence on IL-6, IL-12 and TNF-α production of peritoneal macrophages of nude mice

The content of IL-6, IL-12 and TNF-α produced by peritoneal macrophages of nude mice was detected by employing laser scanning confocal microscopy, when WPG of bifidobacteria bifidum was injected into nude mice intraperitoneally. The results suggested that the macrophages present different color. The macrophages in WPG injection group appeared white, red, yellow and blue which was narrow in area relatively, while, the macrophages in control group were mainly blue and less yellow. Different color reflected different fluorescent intensities. Analyzing with the ACAS software of laser scanning confocal microscopy, showed that the fluorescent intensity, which reflected the content of IL-6, IL-12 and TNF-α emitting from peritoneal macrophages of nude mice in WPG injection group, was markedly higher that in control group (P<0.01, Table 1 and Figures 1,2).

Table 1 Influence of WPG on IL-6, IL-12, TNF-α and NO production by peritoneal macrophages of nude mice ($\bar{x} \pm s$, n = 10)

Groups	IL-6 / A	IL-12 / A	TNF-α / A	NO (μmol·L^{-1})
WPG	1956.48±265.32[b]	2603.24±395.72[b]	813.42±106.77[b]	53.21±6.40[b]
Control	931.56±189.70	1054.33±184.50	318.90±76.35	30.67±12.83

[a]P<0.01, *vs* control group.

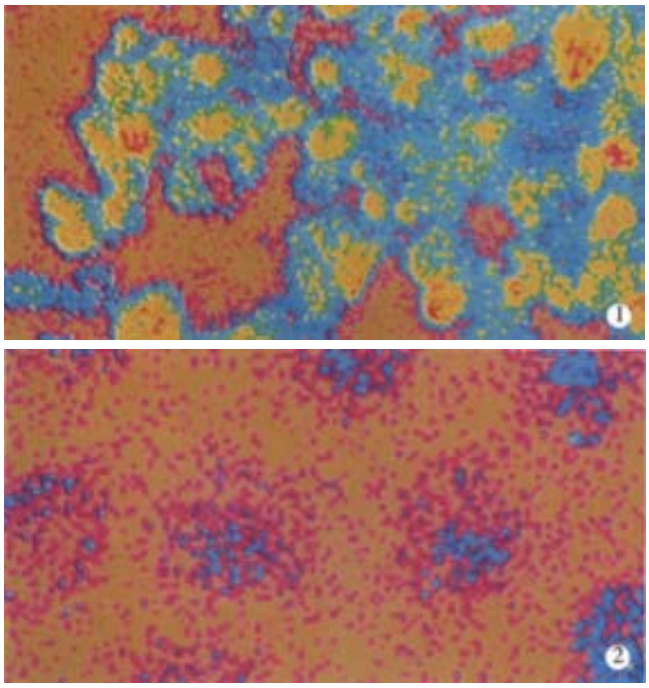

Figure 1 The fluorescence picture of IL-12 derived from the macrophages in WPG injection group.
Figure 2 The fluorescence picture of IL-12 derived from the macrophages in control group.

DISCUSSION

WPG of bifidobacteria bifidum is a bag-form structure which consists of polysaccharides and peptidoglycans. It preserved the integral structure of whole cells, and possessed some important biological characteristics, such as relaxation of senescence, antitumor, control of infection, antimutation, etc[10-14]. Furthermore, it could also activate macrophages of immune system of the body. Tejada-Simon and his colleagues demonstrated that WPG of bifidobacteria infantis could activate RAW 264.7 macrophage cell line to produce a lot of TNF-α and NO *in vitro*[8]. In our report, the fluorescence intensity of IL-1, IL-6 and TNF-α and the content of NO derived from the peritoneal macrophages of nude mice was significantly elevated when WPG of bifidobacteria bifidum was injected into nude mice intraperitoneally. It was indicated that WPG of bifidobacteria bifidum could activate macrophages to secrete a large amount of cytotoxic effector molecules.

IL-1, IL-6 and TNF-α was the important cytokines produced by activated macrophages. They could act many aspects of immune system. IL-6 could promote the differentiation and maturation of B lymphocytes, and stimulate these cells to secrete antibodies. Furthermore, it also could induce proliferation and activation of resting T lymphocytes directly[15,16]. IL-12 could induce the production of IFN-γ by resting and activated T lymphocytes and natural killer (NK) cells, and possess the ability to act as a LAK cell growth factor[17-20]. TNF-α could augment the antitumor ability of NK, CTL and LAK cells, and play an important role in adjusting the activation of T lymphocytes[21,22]. These cytokines had broard antitumor and antimetastatic activities *in vivo* and *in vitro* markedly[23-35]. NO was the signal molecules and effector molecules which had broard biological activities. It was also the important effector molecules that activated macrophages killing tumor cells and pathogenic micro-organisms[36-41]. The induction of these cytotoxic effector molecules may play an important role in antitumor immune reaction of WPG. It was widely acknowledged that WPG could obviously inhibit the growth of many kinds of tumors *in vivo*. Rhee and his colleagues demonstrated that WPG of bifidobacteria spp. Exhibited markedly antitumor activity against subcutaneously transplanted sarcoma 180 in mice [42]. Ishihara, *et al* reported that the volume of metastatic skin melanoma obviously decreased, when intralesional administration of WPG of bifidobacteria infantis was prepared. We had also found that WPG of bifidobacterium bifidum could induce apoptosis of the colorectal carcinoma transplantation neoplasms of nude mice, and inhibit its proliferation simultaneously[43]. In our report, WPG could activate macrophages to secrete a lot of IL-6, IL-12, TNF-α and NO. Because these important mediators present obvious antitumor activity, the cytotoxic effector molecules produced by activated macrophages may mediate the effect on antitumor of WPG.

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Edited by Ma JY