Oral Administration Recombinant Bifidobacterium-LTB (B Subunit of Heat-Labile Enterotoxin) Enhances the OVA (Ovalbumin)-Specific sIgA in Jejunal Mucosa of Sprague-Dawley (SD) Rat

Yong-Ping Ma*, Ya-Ning Hao, Wei Tang, Rong-Rong Wang, Fa-Ping Yi, You-Quan Bu, Lu-Yu Zhang, Fang-Zhou Song

Key Laboratory of Biochemistry and Molecular Biology, The Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing, China.
Email: *ypma0909@yahoo.com.cn

Received June 19th, 2012; revised July 20th, 2012; accepted July 30th, 2012

ABSTRACT

The LTB of enterotoxigenic Escherichia coli (ETEC) expressed in Bifidobacterium infantis (BI) has been testified as mucosal adjuvant with co-vaccination BI-CfaB (the major fimbrial subunit) together in vivo in our previous study. In order to investigate the mucosal adjuvant effect of BI-LTB to purified antigens, we oral vaccinated SD rats with recombinant BI-LTB plus OVA (rBI-LTB + OVA), and wild type BI plus OVA (wBI + OVA), OVA and PBS (Phosphate buffered saline) were vaccinated as controls, respectively. The OVA-specific sIgA in jejunal mucosa and specific IgG in serum were measured with ELISA (Enzyme-linked immunosorbent assay) and the sIgA producing cells were detected with immunohistochemistry technology (IHC) and Qwin image manipulation tools subsequently. The results shown rBI-LTB could stimulate SD rats produce high titer OVA-specific sIgA in rBI-LTB + OVA group and the OVA-specific sIgA titer in rBI-LTB + OVA group was found significant greater than that of the wBI + OVA group or OVA single group (p < 0.05). However no such significant difference was detected between the group wBI + OVA and OVA. IHC results suggested that intestinal mucosa and submucosa was the main field of sIgA secretion. These results suggested that recombinant LTB expression in BI could be used as a wide range mucosal adjuvant with different form antigens.

Keywords: Bifidobacterium infantis; LTB; OVA; Mucosal Adjuvant

1. Introduction

The LTB of Escherichia coli is one of bacterial products with the greatest potential to function as mucosal adjuvant. LTB belongs to ADP-ribosylating enterotoxins (cholera toxin and the heat-labile enterotoxin of E. coli). Both LTB and CTB (cholera toxin B subunit) recognize and bind to mucosal cell surface via their receptor GM1-ganglioside existed ubiquitously on the surface of mammalian cells [1-3]. The adjuvanticity of LTB has been directly related to GM1-binding activity and the interaction between LTB and the receptor activates B and CD4+ T cells; and enhances antigen presentation by activating DCs (Dendritic cells) and other APCs (antigen presenting cells) through receptor-mediated endocytosis mechanisms [4]. Scientists explain that these molecules exert their adjuvant function by interacting with a variety of cell types, including epithelial cells and DCs, etc. [5]. LTB activates selective differentiation of lymphocyte populations and increases presentation on MHC (Major histocompatibility complex) class II, which may be the basis for its adjuvant effect [6-8].

LTB and cholera toxin B (CTB) proteins have been successfully expressed in different expression systems, such as intracellular production in Bifidobacterium infantis (BI), Mycobacterium bovis and Lactobacillus or Bacillus brevis surface-displaced on Staphylococcus xylosus and S. carnosus, as a secreted protein in a yeast expression system [9-15].

Lactic acid bacteria (LAB) have proved to be effective mucosal delivery vehicles that overcome the problem of delivering functional proteins to the mucosal tissues. BI, a special LAB, is a Gram-positive, probiotic and anaerobic bacterium belonging to Bifidobacterium in human intestinal [16]. We testified BI is suitable to human oral
vaccine development and the recombinant BI-LTB (rBI-LTB) co-vaccination with recombinant BI-CfaB of ETEC in vivo has mucosal adjuvant function in previous study [9]. However, we do not know whether rBI-LTB confers adjuvancy to purified antigens. The aim of the present study is to investigate the mucosal adjuvant effect of oral administration rBI-LTB on purified antigen OVA.

2. Materials and Methods

Bacterial strains and growth conditions. E. coli DH5α strain was propagated in LB medium (Luria-Bertani) and used as host cell for molecular cloning; pBEX-LTB was constructed as described previously [9].

BI strain was cultured in MRS broth (Difco) containing 0.25% l-cysteine-HCl (w/v, pH 7), at 37°C under anaerobic conditions. 50 μg·mL⁻¹ of ampicillin was added to both recombinant BI and E. coli strains when required.

Animals and Immunization. Sprague-Dawley (SD) rats of 3 - 4 weeks of age (male) were obtained from the experimental animal center (Chongqing Medical University, China) and divided into four groups as A, B, C and D. After treating with saturated sodium bicarbonate (0.3 μL each rat), the rats were intragastrically vaccinated three times on day 0, 7 and 14 with rBI-LTB + OVA, wBI + OVA, OVA and PBS, the BI dose was 1.0 × 10¹⁰ CFU mL⁻¹ and that of OVA was 10 μg per rat on 3 occasions (Table 1).

Immunological assay (IMA). Blood samples were individually collected from immunized rats by a tail bleed on days 0, 7, 14 and 21 for the analysis of systemic OVA-specific antibodies (10e10 bateria, on 3 occasions).

Fresh fecal pellets were individually collected lyophilized from the same rat groups on days 0, 7, 14 and 21. The samples were treated as previous described [9] (Ma et al., 2011). The supernatants were analyzed for OVA antibody (goat anti-OVA/HRP) at 37°C for 3 h and the fecal specific antibody titers were analyzed with goat anti-rat IgG and the fecal specific antibody titers were analyzed with goat anti-rat IgG or anti-rat slgA mAb conjugated to HRP (1.0 μg·mL⁻¹, BOSTER, China) was added and incubated again.

The OD₅₅₀ value was measured on a Molecular Devices SpectroMax Plus spectrophotometer. Endpoint titers were determined as the dilution of each sample showing a 2.1-fold higher level of absorbance at 450 nm than that of the negative control samples. Average OD₅₅₀ values for the animals were calculated.

Tissue samples preparation. Following collection of blood and fecal samples, two of vaccinated rats were randomly anaesthetised and sacrificed from each group and the jejunum samples were isolated from each rat on days 7, 14 and 21, respectively. Following wash with PBS, the jejunum samples were fixed with 4% polyoxyethylene for 24 h. Then the fixed jejunos were rinsed with 95% ethyl and successively dehydrated 10 μm with ethyl from concentration 90%, 95% and 100%, respectively. After treating with dimethylbenzene, the samples were embedded 4 h with paraffins at 56°C and cut into slices (<5 μm). Then the slices were treated with acetone containing 1% APES (3-aminopropyl triethoxysilane) and dried at 37°C.

Immunohistochemical staining sections (IHCS). 1) The prepared slice samples were twice treated 40 μm with dimethylbenzene to dewax and then successively dehydrated with ethyl from concentration 100%, 95%, 90%, 80% to 70 % for 30 s, respectively; 2) Following rinse 5 μm with PBS three times, each slice was incubated 30 μm with 50 μL peroxidase blocking solution (198 mL methanol + 2 mL 30 % hydrogen peroxide) at 25°C; 3) After rinsing 5 μm with distilled water, the slices were put into 0.01 M citric acid solution (pH 6.0) and heat 15 μm at 95°C. Then, the slices were naturally cooled to 25°C and rinsed with PBS three times; 4) Incubated with OVA (1.0 μg·mL⁻¹) at 37°C for 3 h and then rinsed 5 μm with PBS three times; 5) Following blocked with 5% skim milk, each slice was incubated at 37°C for 30 μm and then sucked up the serum with filter paper; 6) Incubated with OVA antibody (goat anti-OVA/HRP) at 37°C for 3 h and then rinsed 5 μm with PBS three times; 7) Incubated 3 μm with 100 μL DBA solution and rinsed with tap water; 8) Following counterstain 3 μm with hematine, then rinsed with tap water and overnight at 50°C; 9) Coverslip with neutral balsam.

ICH analysis (ICHA). All slices were surveyed and took pictures with Leica DM2000 microscope. Each slice randomly selected ten visual fields to statistical analysis.

Table 1. The SD rats and the vaccination groups.

<table>
<thead>
<tr>
<th>Groups (rats)</th>
<th>Vaccinations agents</th>
<th>Dose ((C.F.U. mL⁻¹) + μg OVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n=12)</td>
<td>rBI-LTB + OVA</td>
<td>1.0 × 10¹⁰ + 10 μg</td>
</tr>
<tr>
<td>B (n=12)</td>
<td>wBI + OVA</td>
<td>1.0 × 10¹⁰ + 10 μg</td>
</tr>
<tr>
<td>C (n=12)</td>
<td>OVA</td>
<td>10 μg</td>
</tr>
<tr>
<td>D (n=10)</td>
<td>PBS</td>
<td>/</td>
</tr>
</tbody>
</table>

Copyright © 2012 SciRes.
by Qwin image manipulation tools. The yellowish-brown value stands for the positive cell area.

**Statistical analysis.** The data was statistically evaluated by the SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL) and a value of \( p < 0.05 \) was considered significant.

3. Results

**IMA.** Serum and mucosal OVA-specific antibody levels were measured by ELISA. High levels of serum OVA-specific IgG were observed on day 14 after the secondary booster vaccination, with an endpoint titer of 206 and reached a novel increased level in following booster dose on day 21 with the titer of 566 in groups A (\( n = 12 \)). However, in group B (\( n = 12 \)), the OVA-specific IgG titer just reached to 95 on 14 d and to 183 on 21 d. In the other way, we did not find the OVA-specific IgG in group C (\( n = 12 \)) and D (\( n = 10 \)). Statistically, there was a significant difference in the IgG titer between the groups A and B for the adjuvant function of LTB (\( p < 0.05 \)) (Figure 1). The level of variation in the responses was 20.48 in group A and 20.18 in group B between individual animals on day 21.

Similar to the serum IgG titer variation, the fecal sIgA titer in group A was greater than that in group B. High levels of OVA-specific sIgA was observed on day 14 after the secondary booster vaccination, with an endpoint titer of 7.57 and reached a novel increased level in following booster dose on day 21 with the titer of 54.67 in groups A (\( n = 12 \)). However, in group B (\( n = 12 \)), the OVA-specific sIgA titer just reached to 3.02 on 14 d and to 9.70 on 21 d. However, we did not find the OVA-specific sIgA in group C (\( n = 12 \)) and D (\( n = 10 \)). The results suggest that rBI-LTB expressed in BI had mucosal adjuvant activity in group A. Statistically, there was a significant difference in the sIgA titer between the groups A and B for the adjuvant function of LTB (\( p < 0.05 \)) (Figure 2). The level of variation in the responses was 10.88 in group A and 0.61 in group B between individual animals on day 21.

**ICHA.** Examined under a microscope, the OVA-specific sIgA producing cells were stained in yellowish-brown and distributed in jejunal mucosa, submucosa, external and internal of intestinal crypt, intestinal lamina propria and serous membrane (Figures 3 (a)-(h)). However, the colored positive cells in serous membrane were obviously fewer than that in other type cells. So the results suggest that intestinal mucosa and submucosa was the main field of sIgA secretion.

Analysis the specific sIgA producing cells on 7 d post-vaccination statistically, we found that the positive cells were increased appreciably group A than that in group B, but there was no significant difference each other. However, the positive cells were increased significantly from secondary and third booster in group A than that in group B (\( p < 0.05 \)). As was expected, the positive cells did not find in group C and D (Figure 4).

4. Discussion

Appropriate mucosal adjuvant is essential for oral immunization to elicit immune response. Besides of the ADP-ribosylating enterotoxins (CT and LT), the other two bacterial products, synthetic oligodeoxy-nucleotides containing unmethylated CpG dinucleotides (CpG ODN), and mono-phosphoryl lipid A (MPL), were used as mucosal adjuvants. Both MPL and CpG act through

![Figure 1. The serum IgG titer in different groups post-immunization. The group A rats were immunized orally with rBI-LTB + OVA, group B with wBI + OVA, group C with OVA, and group D were treated with PBS (10^{10} bacteri, on 3 occasions). The group A showed significant difference, compared with group B (\( p < 0.05 \)). However, there was no statistically significant difference IgG titer between the group C and group D for no detectable specific IgG.](image1)

![Figure 2. The fecal sIgA titer in different immunization groups in SD rat. The group A rats were immunized orally with rBI-LTB + OVA, group B with wBI + OVA, group C with OVA, and group D were treated with PBS (10^{10} bateria, on 3 occasions). The group A shown significant difference, compared with group B (\( p < 0.05 \)). However, there was no statistically significant difference sIgA titer between the group C and group D for no detectable specific sIgA.](image2)
Oral Administration Recombinant Bifidobacterium-LTB (B Subunit of Heat-Labile Enterotoxin) Enhances the OVA (Ovalbumin)-Specific sIgA in Jejunal Mucosa of Sprague-Dawley (SD) Rat

Figure 3. Immunohistochemical evaluation of OVA-specific producing cells in jejunal mucosa. (a), (b) Jejunal mucosal sections on 14 d and 21 d in group A (magnification 200 and 400, respectively). (c), (d) Sections on 14 d and 21 d in group B (magnification 200 and 400, respectively). (e), (f) Sections on 14 d and 21 d in group C (magnification 200 and 400, respectively). (g), (h) Sections on 14 d and 21 d in group D (magnification 400 and 200, respectively). The OVA-specific sIgA producing cells were stained with goat anti-OVA/HRP in yellowish-brown and distributed in jejunal mucosa, submucosa (arrows indicating mucosal locations of sIgA producing cells).

Copyright © 2012 SciRes.
However, the positive cells did not find in group C and D.

LTB appears to be related with its capacity to: 1) enhance antigen presentation [8,9,15,17-19]. The adjuvant mechanism of immune response against co-administered antigen or co-adjuvant that induces a strong humoral and mucosal immune responses [5].

MyD88-dependent and -independent pathways and the adjuvant activities of CpG and MPL are due to several different effects they have on innate and adaptive immune responses [5].

LTB is a nontoxic molecule with potent biological properties and is a powerful mucosal and parenteral adjuvant that induces a strong humoral and mucosal immune response against co-administered antigen or coupled antigens [8,9,15,17-19]. The adjuvant mechanism of LTB appears to be related with its capacity to: 1) enhance antigen presentation via MHC class I and MHC II; 2) activate selective differentiation of lymphocytes; 3) influence DCs maturation and activation; 4) induce B7-2 expression on APCs for subsequent co-stimulatory signaling to CD4+ T cells and; 5) increase the expression of activation markers on B lymphocytes (MHC class II, B7, CD40, CD25 and ICAM-1) [4,20].

Oral vaccination is based on antigen delivery to the gastrointestinal tract, the largest mucosal surface and the central site of IgA secretion [21]. Since the intestinal mucosa is the natural site of BI colonization, rLTB producing in BI easily crosses the epithelial layer to the area rich in cells of the mucosal immune system. BI-LTB appears to be one of the best forms of LTB adjuvant delivery system [9]. Because of the same reason, the several strains LAB has been genetically modified as a promising oral recombinant vaccine delivery system for inducing efficient mucosal immunity as well as systematic immunity [9,22-26].

Bifidobacterial cell wall preparation (whole peptidoglycan, WPG) has been documented with adjuvant activity and the activity of WPG is related to their ability to induce a reduction in regulatory T cells (Tregs) activity [27,28]. A previous document was demonstrated that LAB might be promising adjuvants in vaccines due to their capability to reduce functional activity of Tregs, thereby speeding up vaccine-induced immune responses [29]. Another document suggests that WPG of bifidobacterium induces IL-12 secretion in DCs from bone marrow and rIL-12, as adjuvant, has been shown to augment both cellular and humoral immunity [30-33]. In this study, we find that wild type BI performed weak adjuvant activity to OVA oral administration, which suggests that WPG of BI might be promising adjuvant activity in OVA.

There are many more experiments have been done in mice where there is more immunological reagents for these types of investigation. In this study, we use of the rat model is novel for oral vaccination. One reason for choosing rat is that a report used of rat for investigation of the role of intestinal bifidobacteria on immune system development [34]. They found that intestinal bifidobacteria plays an important role of development of both the gut and systemic immunity in early life. Neonatal SD rats supplemented daily with bifidobacteria could promote the maturation of DCs and its expression of IL-12 locally in the gut, favour the development of Th1 response by increasing the local and systemic expression of IFNy and ensure the intestinal Treg response by promoting the local expression of IL-10 [34].

There is no purified LT-B plus OVA as a comparable control to compare the organism expressing LT-B in this study. One reason is that the nontoxic CT plus OVA or LT-B plus OVA confirmed that the enterotoxin B subunit acts as a mucosal adjuvant intranasally immunized [35,36]. Oral vaccination is not an economically affordable way for purified LT-B or other enterotoxin B subunit because it needs high dose enterotoxin B subunit. However, BI expressing LT-B can easily get over it.

5. Acknowledgements

This study was funded by the National Natural Science Foundation of China (No. 30972585).

REFERENCES


Oral Administration Recombinant Bifidobacterium-LTB (B Subunit of Heat-Labile Enterotoxin) Enhances the OVA (Ovalbumin)-Specific sIgA in Jejunal Mucosa of Sprague-Dawley (SD) Rat

doi:10.1016/j.vaccine.2010.11.091


Oral Administration Recombinant Bifidobacterium-LTB (B Subunit of Heat-Labile Enterotoxin) Enhances the OVA (Ovalbumin)-Specific sIgA in Jejunal Mucosa of Sprague-Dawley (SD) Rat


