Original Article
A bacterial extract of OM-85 Broncho-Vaxom suppresses ovalbumin-induced airway inflammation and remodeling in a mouse chronic allergic asthma model

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Abstract: Chronic allergic asthma is characterized by Th2-polarized inflammation, airway remodeling and fibrosis. Broncho-Vaxom (OM-85 BV) is an extract of eight different bacterial species, which has been used for airway infections in humans. However, the effects of microbial products on the prevention of chronic asthma remain unclear. In this study, we attempt to determine whether OM-85 BV inhibits chronic inflammation and airway remodeling in the ovalbumin (OVA)-induced mouse allergic asthma model. The oral administration of OM-85 BV before the sensitization markedly alleviated the OVA-induced airway hyperresponsiveness (AHR) and inhibited inflammatory cell infiltration, mucus hypersecretion and peribronchial collagen deposition compared with the model group. In addition, OM-85 BV reduced OVA-specific IgE levels in serum and decreased the level of Th2 cytokines in bronchoalveolar lavage fluid (BALF). All these findings in a murine model provide a new way for the immunoregulatory role of OM-85 BV to control the chronic asthma.

Keywords: OM-85 BV, allergic asthma, chronic airway inflammation, airway remodeling

Introduction
Asthma is an important chronic disease, affecting 334 million people of all ages around the world [1]. It is a Th2-skewed chronic inflammatory disease of the lung that is characterized by airway inflammation [2], airway hyperresponsiveness (AHR) [3] and remodeling [4]. Inflammation occurs due to the infiltration of eosinophils, neutrophils, and macrophages into the bronchial lumen and lung tissues [1]. The recruitment of these inflammatory cells is a critical event in the development of airway inflammation [1, 5]. The recruitment of leukocytes to the site of inflammation is mediated by the Th2 cytokines, such as IL-4, IL-5, and IL-13, which are crucial for Immunoglobulin E (IgE) synthesis, airway eosinophilia, mucus secretion, and ultimately AHR [4, 5]. Biological treatments targeting the Th2 cytokines such as anti-interleukin (IL)-4, anti-IL-5, and anti-IL-13 have been used but ineffective in asthma as a whole [6]. The growth, differentiation, recruitment, and survival of eosinophils are associated with IL-5, and IL-4 is the most important factor regulating IgE production by B cells [7]. IgE has been proved to play an important role in the development of allergic asthma [8] and recent studies suggest that anti-IgE therapy plays an important role in allergic diseases [9].

According to the hygiene hypothesis, the increased incidence of allergic diseases in the industrialized world is due to the lack of early childhood exposure to infectious agents [10]. Specific types of bacterial and viral infections during early life direct the maturing immune system toward a Th1 profile, which counterbalances the excessive responses of Th2 cells [11]. In our previous research, we investigated the immunomodulatory effects of oral administration of a bacterial extract, OM-85 Broncho-Vaxom (BV), with a low dose and general time course and we have found that OM-85 BV is a low-cost alternative candidate to prevent allergic rhinitis (AR) with simple oral administration.
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Considering the “One way, One disease” concept, the AR has been proved to be associated with the incidence of asthma [13], and the chronic asthma is a complex disease characterized by the airway remodeling, which makes it hard to control. No clear data regarding the effects of microbial products on the airway remodeling of chronic allergic asthma were reported in clinical and animal models.

Broncho-Vaxom (OM-85 BV) is an extract of eight different bacterial species that are frequently used in airway infections. It is an oral drug used to prevent and/or reduce the severity of acute attacks of chronic bronchitis and recurrent infections of the respiratory tract in both children and adults [14]. Pretreatment with OM-85 BV reduced asthma-associated allergic airway inflammation via the recruitment of regulatory T cells (Tregs) in mice and rats [15, 16]. And in our previous research, we found Broncho-Vaxom attenuates allergic airway inflammation by restoring GSK3β-related Tregs insufficiency in mice [17]. However, the effect of OM-85 BV on airway remodeling is still unclear. Furthermore, very large doses of OM-85 BV were used in previous studies examining asthma [11, 15, 16]. Thus, the safety of these drugs in a lower or rational dose is an important factor that must be considered, particularly in chronic asthma clinical therapy.

The time course of OM-85 BV to prevent airway infection was at least 3 months in clinic. In this study, we investigated the effects of OM-85 BV for preventing allergic chronic airway inflammation and airway remodeling in a mouse model of chronic asthma, using a similar time course (3 months) currently used and clinically tolerated by humans.

**Materials and methods**

**Animals**

Female Balb/c mice (4 weeks of age) were purchased from Medical Experimental Animal Center of Guangdong Province (Guangzhou, People’s Republic of China) and housed under specific pathogen free conditions. All the mice were provided free access to sterile water and ovalbumin (OVA)-free food. All procedures performed in studies involving animals were in accordance with the ethical standards according to protocols approved by the Institutional Animal Care and Use Committee, Sun Yat-sen University.

**Broncho-Vaxom (OM-85 BV)**

Each BV capsule (OM PHARMA, Meyrin/Geneva, Switzerland) contained 7 mg of lyophilized bacterial lysates of Hemophilic influenzae, Diplococcus pneumoniae, Klebsiella pneumoniae, Klebsiella ozaenae, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus viridans, and Neisseria catarrhalis.

**OVA-induced chronic allergic airways inflammation in mice and OM-85 BV administration**

A mouse model of chronic allergic airways inflammation and remodeling was induced with
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ovalbumin. The mice underwent three steps of inoculation, sensitization, and challenge, as shown in Figure 1. In the inoculation phase, bacterial extract 1.75 mg/mouse commercially available Broncho-Vaxom (BV) (OM-PHARMA, Meyrin/Geneva, Switzerland) dissolved in pyrogen-free phosphate-buffered saline (PBS) or PBS were intragastric (i.g.) administrated for 10 consecutive days and rested for 20 days as a circle. The 30-day treatment was considered as one course. The mice were orally administration with OM-85 BV for three courses according to the clinical application. Then mice were sensitized by intraperitoneal injection of 40 µg of ovalbumin (OVA, grade V, Sigma, Missouri, USA) in 2 mg of aluminum hydroxide (Sigma) in 200 µl pyrogen free phosphate-buffered saline (PBS) on days 1, 7, and 14. From days 21 to 53, mice were challenged every other day with aerosolized 5% OVA in a glass chamber and through an air-compressing nebulizer (403A, Yuyue, Danyang, Jiangsu, China) for 30 minutes. Then the mice were intranasally infused with 20 µl OVA (40 mg/ml). The mice were tested the airway hyperresponsiveness (AHR) at day 54 and sacrificed via cervical dislocation at day 55. The mice were divided into four different groups based on the different treatments of inoculation, sensitization, and challenge. Further detailed information for each group was as follows: (A) PBS/PBS/PBS mice, which were inoculated with PBS, sensitized, and challenged with PBS (n=5); (B) PBS/OVA/OVA mice, which were inoculated with PBS, sensitized, and challenged with OVA (n=5); (C) BV/OVA/OVA mice, which were inoculated with OM-85 BV, sensitized, and challenged with OVA (n=5) and (D) BV/PBS/PBS mice, which were inoculated with OM-85 BV, sensitized, and challenged with PBS (n=5).

Airway hyperreactivity measurement

Airway hyperresponsiveness (AHR) was measured in vitro 24 h after the last aerosol exposure. Briefly, mice were placed in a whole-body plethysmography chamber. The chamber was connected to the machine via a small polyethylene catheter. Increasing aerosolized methacholine doses (0, 6.25, 12.5, 25.0, 50.0 and 100 mg/ml) were administered from the machine to the chamber at 5-min intervals. The Penh were measured for 2 min after each dose using a Transducer Medlab (First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China). Results are expressed as the Penh after each dose of methacholine minus the baseline value.

Cells analysis of bronchoalveolar lavage fluid

After AHR measurements, the mice were sacrificed and bronchoalveolar lavage fluid (BALF) was collected. Briefly, BALF was obtained after lavage with 1 ml of cold PBS via a needle inserted through the upper airway. Then the bronchoalveolar lavage fluid was centrifuged (2500 rpm, 10 min) and the supernatants were collected for cytokine analysis. Cell pellets were resuspended in PBS and then cytopspinned onto glass slides and stained with Diff-Quick (Basso Diagnostics Inc. Zhuhai, Guangdong, China). The total cell number was determined using a hemocytometer and a total of 300 cells per slide were evaluated for different kinds of inflammatory cells at 400 magnifications.

Lung histology

Lung tissues were removed after the lavage and fixed in 10% neutral formalin. The lungs were then embedded in paraffin and the slides were prepared at a thickness of 4 um. The lung sections were then stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Masson staining. Inflammation score and Goblet cell (PAS positive cell) counts in the lungs were performed in a blinded way using a reproducible scoring system, as previously described [18, 19]. Briefly, for quantifying the lung inflammation, five sections across the main bronchus of each mouse were randomly selected and given scores ranging from 0 to 3 based on the level of peribronchial inflammation and perivascular inflammation. The values were given according to the following inflammatory parameters: 0. when no inflammation was detectable; 1. for occasional cuffing with inflammatory cells; 2. for most bronchi or vessels surrounded by a thin layer (1-5 cells) of inflammatory cells; and 3. when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells. For quantifying the goblet cell hyperplasia, the percentage of PAS-positive cells in epithelial areas was examined from 10 tissue sections per mouse. Mucus secretion was scored according to the percentage of PAS-positive cells in the total number of airway epithelial cells (0 score, less than 5% goblet cells; 1 score, 5-25%; 2 score,
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Masson trichrome staining was used to assess peribronchial collagen deposition. The staining area was shown and quantified using a light microscope (DM2500B, Leica, Bensheim, Germany) and the image analysis system (Image-Pro Plus 5.1, Media Cybernetics, Silver Spring, MD, USA). The lung fibrosis index was defined as the sum of the total area of collagen in the entire visual field divided by the sum of total connective tissue area in the entire visual field. At least 10 non-overlapping bronchioles were evaluated per slide [20].

Evaluation of the cytokines in the bronchoalveolar lavage fluids

The levels of IL-4 and IL-13 in the BALF supernatants were measured using sandwich enzyme-linked immunosorbent assay (ELISA) analysis following the manufacturer’s instructions (R&D Systems, Minneapolis, USA).

Mouse OVA-specific IgE ELISA

An ELISA experiment was conducted using an anti-mouse IgE-antibody to define serum levels of OVA-specific IgE. Briefly, microplate wells were coated with 1% OVA in coating buffer (0.05 M sodium carbonate-bicarbonate, pH 9.6) overnight at 4°C. Then the wells were incubated with blocking buffer (1% BSA in PBS, pH 7.2) at room temperature for 1 h and washed. The serum samples were diluted (1/10) and introduced to the microplate, then incubated at room temperature for 2.5 h. After washing, the microplate was incubated with Biotin anti-mouse IgE, followed by a step of extravidin-peroxidase incubation at room temperature for 30 min and with TMB substrate for 30 min. The enzymatic reaction was stopped with 2 M H₂SO₄, and absorbance was read at 450 nm. The units are reported as optical density at 450 nm.

Statistical analysis

The experimental data were expressed as the mean ± SEM. Statistical analysis was performed using a one-way analysis of variance followed by a Student-Newman-Keuls test for multiple comparisons of the data with Gaussian distribution. A Kruskal-Wallis rank sum test followed by a Mann-Whitney U test was performed for two-group comparisons of the data with abnormal distribution. Statistical analysis was performed using GraphPad Prism 5 software (San Diego, California, USA) and P<0.05 was considered statistically significant.

Results

Pretreatment with OM-85 BV attenuated the airway hyperreactivity (AHR) in OVA-induced mice chronic airway inflammation model

The airway hyperreactivity was assessed after the final challenge in a mouse model of chronic asthma. The Penh level of AHR were significantly higher in the PBS/OVA/OVA group compared with the PBS/PBS/PBS group (P<0.001; Figure 2). However, pretreatment with OM-85 BV (the BV/OVA/OVA group) significantly reduced the level of AHR in response to methacholine compared with the PBS/OVA/OVA group (P<0.001). There were no significant statistical differences of Penh levels between the BV/PBS/PBS group mice and the PBS/PBS/PBS group. The levels of AHR in the BV/OVA/OVA group were higher than those in both the BV/PBS/PBS and the PBS/PBS/PBS groups (P<0.05).
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Pretreatment with OM-85 BV suppressed OVA-induced airway inflammation and airway remodeling

The effect of inoculation of OM-85 BV on the histopathology of the lung was evaluated in a qualitative and quantitative way. Mice inoculated with PBS and then challenged and sensitized with OVA (PBS/OVA/OVA group) exhibited obvious inflammatory cells, especially eosinophilic infiltration in the bronchial lumen mucosa compared with PBS/PBS/PBS mice (P<0.01; Figure 3). Pretreatment with OM-85 BV resulted in a significant decrease in inflammatory cells infiltration in the lung (P<0.01). Furthermore, there was no allergen-driven airway inflammation in BV/PBS/PBS mice. The PBS/OVA/OVA groups with PBS inoculation had stronger eosinophilic infiltration compared with the two control groups, PBS/PBS/PBS and BV/PBS/PBS (P<0.01). The goblet cell hyperplasia and mucus hypersecretion into the lung tissue were also induced by OVA compared to those in the control group with PAS staining. OM-85 BV inhibited the OVA-induced goblet cell hyperplasia and mucus hypersecretion compared to those in the model group (P<0.01; Figure 3). The airway remodeling, staining by Masson, was developed in the model group (PBS/OVA/OVA group). The PBS/OVA/OVA groups with PBS inoculation had stronger eosinophilic infiltration compared with the two control groups, PBS/PBS/PBS and BV/PBS/PBS (P<0.01). Pretreatment with OM-85 BV resulted in a significant reduced level of collagen deposition in the lung (P<0.01; Figure 3). Representative images from the PBS/PBS/PBS, PBS/OVA/OVA, BV/OVA/OVA and BV/PBS/PBS groups are shown in Figure 3. These data indicate that pre-treatment with OM-85 BV significantly inhibited the OVA-induced inflammatory cells infiltration, goblet cell hyperplasia, mucus hypersecretion and airway remodeling in a murine model of asthma.

Figure 3. Oral administration of OM-85 Broncho-Vaxom (BV) attenuated ovalbumin (OVA) induced chronic airway inflammation and fibrosis in the lung. Mice pre-treated with the OM-85 BV showed a significant reduction in inflammatory cells and mucus secretion in the bronchial lumen mucosa and a lower level of collagen expression compared with phosphate-buffered saline (PBS)/OVA/OVA group mice. Representative photomicrographs of hematoxylin and eosin-stained, PAS-stained and Masson-stained lung sections from PBS/PBS/PBS, PBS/OVA/OVA, BV/OVA/OVA and BV/PBS/PBS mice (n=5 mice per group) are showed (original magnification, ×400). HE score, PAS score and Masson score are statistical analysis for the inflammation, PAS-positive cells and collagen deposition. The data are expressed as the mean ± SEM. PBS, phosphate-buffered saline; OVA, ovalbumin. *P<0.05, **P<0.01, ***P<0.001.
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As expected, we found a significant elevation in the serum level of OVA-specific IgE in PBS/OVA/OVA mice (Figure 5). OM-85 BV pre-treated significantly decreased the levels of specific IgE in mice serum (P<0.01). Moreover, there was no difference in the level of OVA-specific IgE between BV/PBS/PBS and PBS/PBS/PBS groups. Interestingly, we observed a significant difference for IgE level between BV/OVA/OVA and BV/PBS/PBS and PBS/PBS/PBS (P<0.05).

Our findings indicated that oral treatment with OM-85 BV was responsible for down-regulating the Th2 response in the allergic asthma model.

The Th2 cytokines IL-4 and IL-13 are involved in the pathogenesis of airway allergic diseases and contribute to inflammatory cell infiltration [4, 5]. We found high levels of IL-4 and IL-13 in the BALF in PBS/OVA/OVA mice (Figure 6, P<0.01). However, pretreatment with OM-85 BV reduced the levels of IL-4 and IL-13 (P<0.01). Furthermore, we observed higher levels of IL-4 and IL-13 in BV/OVA/OVA compared with PBS/PBS/PBS and BV/PBS/PBS groups (P<0.05). These data showed that OM-85 BV reduces the levels of cytokines involved in Th2 responses and influence the pathogenesis of allergic inflammation.

Microbes or their products are playing important role in dealing with allergic diseases according to the hygiene hypothesis. Experimental studies have shown that some types of microbes or their products inhibit asthma inflammation by directly suppressing local pulmonary Th2 cytokine responses [21] and pre-
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OM-85 BV, which is an extract of eight different bacterial species that are frequently responsible for respiratory infections, has the ability of anti-inflammatory. However, the effect of OM-85 BV on chronic airway inflammation of asthma and airway remodeling remain unclear. Our study is the first to provide experimental evidence demonstrating that OM-85 BV inhibits OVA-induced chronic airway inflammation and airway remodeling in a murine model of asthma. The oral administration of OM-85 BV significantly inhibited chronic asthmatic reactions such as AHR, inflammatory cells recruitment to the lungs, goblet cells mucus hypersecretion, collagen deposition, OVA-specific IgE levels in serum and the production of Th2 cytokines in BALF.

Moreover, it is believed that inflammatory mediators released during allergic inflammation play an important role in causing asthma symptoms, such as goblet cell hyperplasia, airway luminal narrowing, AHR, and subepithelial fibrosis [24-26]. In our study, we found that OM-85 BV markedly inhibited OVA-induced AHR in response to increasing concentrations of methacholine (Figure 2), the degree of inflammatory cell infiltration in the peribronchial areas, the goblet cell hyperplasia and collagen deposition (Figure 3). Consequently, it is likely that bacterial extract of OM-85 BV therapy improves AHR, airway inflammation and remodeling. Therefore, we conclude that the effect of OM-85 BV on AHR, airway inflammation and remodeling, and goblet cell hyperplasia may be attributed to the inhibition of Th2 cytokine production, eosinophilia, and serum IgE levels.

Based on previous studies, the immunological processes involved in airway inflammation of asthma are characterized by the proliferation and activation of Th2 cytokines, such as IL-4, IL-5, and IL-13 [1]. IL-4 causes B cells to synthesize IgE, which is involved in mast cell degranulation by cross-linking IgE receptors [8, 27]. IL-5 enables the terminal differentiation and proliferation of eosinophil precursors and the eosinophil activation effect [28]. IL-13 also contributes to symptoms of asthma such as AHR, airway inflammation, goblet cell hyperplasia, airway luminal narrowing, and subepithelial fibrosis [29]. In this study, we demonstrated that pretreatment with OM-85 BV significantly suppressed the levels of IL-4, IL-13 in BALF and OVA-specific IgE levels in serum (Figures 5 and 6). OM-85 BV also significantly reduced the numbers of total inflammatory cells and eosinophils in BALF (Figure 4). We conclude that OM-85 BV suppresses the number of inflammatory cells in BALF through the suppression of Th2 cytokines.

In conclusion, we have demonstrated that OM-85 BV alleviated AHR, reduced the number of inflammatory cells, down-regulated the excessive Th2 cytokines production in BALF and OVA-specific IgE levels in serum. Furthermore, goblet cell hyperplasia and airway remodeling were also attenuated. These results suggest that OM-85 BV exhibits inhibitory activities not only for allergen-induced AHR or airway eosinophilic inflammation but also for airway remodeling, probably due to the downregulation of allergen sensitization and/or decreased TGF-β cytokine amount [30]. Our results suggest that OM-85 BV might offer a new and safe therapeutic approach to chronic allergic asthma and a promising control to the airway remodeling.

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Disclosure of conflict of interest

None.

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