ORIGINAL RESEARCH

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The effect of *Lactobacillus johnsonii* Ncc533 (La1) on the balance of Th1/Th2 cells in BALB/c mice

Abstract

Purpose: To determine the effect of *Lactobacillus johnsonii* Ncc533 (La1) on Th1/Th2 balance, the production of IL-4 and IFN- γ by splenocytes was evaluated following its administration to mice from newborn to adult. Changes in IL-4 and IFN- γ expression and serum levels of OVA-specific-IgE were then investigated in an asthma model.

Methods: Using flow cytometry (FCM) and ELISA, the percentage of IL-4 and IFN- γ expressing splenocytes and serum levels of OVA-specific-IgE were measured in different groups of mice.

Results: The percentages of IL-4 and IFN- γ expressing splenocytes in the offspring and in the adults of the La1-treated group were not significantly different when compared with the water-treated group. In the asthma model, the percentages of IL-4 expressing cells and the serum levels of OVA-specific-IgE in the La1-treated and water-treated group were significantly increased compared with those in the control group. The percentage of IFN- γ expressing cells was significantly lower in the La1-treated and water-treated groups. The percentage of IL-4 expressing cells and the serum levels of OVA-specific-IgE in the La1-treated group were significantly lower compared with those in the water-treated group, whereas the percentage of IFN- γ expressing cells was significantly higher.

Conclusion: Administration of La1 had no effect on the immune system from the neonate to the adult in the normal mice. It did, however, significantly alter the percentages of IL-4 or IFN- γ expressing CD4⁺ T lymphocytes in the asthma model, suggesting that administration of La1 might regulate the immune response.

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With the improvements in sanitation and the development and aggressive use of vaccines and antibiotics, the incidence of infectious diseases is gradually declining. In comparison, allergic diseases, such as bronchial asthma and atopic dermatitis, are progressively increasing. Bronchial asthma is a disease that is characterized by hyperresponsiveness and chronic inflammation in the airway. One hypothesis for the disease's etiology is the "hygiene hypothesis": with improvements in sanitation, the chance of infection and exposure to the microorganisms in childhood is decreased, especially for the neonate. The immune system is thus immature and disordered and the incidence of allergic disease is increased [1]. One of the immunological characteristics of asthma is a Th1/Th2 imbalance [2]. The quantity and activity of Th1 cells are decreased, and those of Th2 cells are increased. The cytokines, including IL-4, IL-5 and IL-13, which are produced by Th2 cells, can enhance IgE levels and increase proliferation of eosinophils, basophils and mastocytes. All of these changes result in airway hyperresponsiveness. One potential method of treating bronchial asthma is to balance the quantity and activity of these T-helper cells through administration of probiotics.

According to the hygiene hypothesis, moderate stimulation with microorganisms is necessary for the formation of a normal immune system. *Lactobacillus johnsonii* Ncc533 (La1), a probiotic, was used in this study. Probiotics are "live microorganisms which when administered in adequate amounts confer a healthy benefit on the host". There are three commonly used probiotic bacterial types: bifidobacteria, lactic acid bacteria and facultative anaerobic bacteria, and each are thought to exert their effects in different ways and via different mechanisms. A number of clinical trials have been done on *Lactobacillus rhamnosus* and *Bifidobacterium longum* [3-7]. Some trials suggest that lactic acid bacteria might improve immune function by increasing the number of IgA-producing plasma cells [10], by stimulating phagocytosis [11] or by increasing the proportion of T-lymphocytes and natural killer cells [6-7]. It has been shown that the levels of IL-4, IL-5, IL-6 and IL-10 decreased in a murine diabetic model in response to treatment with lactobacillia [12].

In this study, mice were treated with *Lactobacillus johnsonii* Ncc533 (La1). Levels of IL-4 and IFN- γ , produced by splenocytes, were compared in newborn and adult mice. Serum levels of OVA-specific-IgE, in addition to IL-4 and IFN- γ , were then measured in a murine model of bronchial asthma with and without La1 treatment.

Materials and Methods

Mice

Twenty female and five male matured BALB/c mice were obtained from the animal center of Shandong University (Shandong, China). The weight of the female mice was from 24 g to 28 g and the mean weight was 26.0 g. The weight of the male mice was from 26 g to 30 g and the mean weight was 27.9 g. Animals were mated and the date of pregnancy was calculated from the date of observing the vaginal plug.

Reagents

Monoclonal antibodies of FITC-CD4, PE-IL-4 and PE-IFN- γ were purchased from Immunotech Laboratories (Glendale, CA). Fix and Perm was obtained from Caltag (Shanghai, PRC). Ovalbumin (OVA, grade V), phorbol ester (PMA), ionomycin and monensin were obtained from Sigma-Aldrich Co. (Shanghai, PRC). OVA-specific IgE was purchased from Do-Bio (Shanghai, PRC). The suspension of La1 (1×10^{12} CFU/L) was obtained from the Nestle Research Center (Lausanne, Switzerland).

Grouping

Groups 1 and 2: Pregnant BALB/c mice were randomly divided into two groups. Group 1 were treated with La1, 2 ml via gastric tube, b.i.d., for 2 to 3 weeks and Group 2 were treated with sterile, distilled water 2 ml via gastric tube, b.i.d., for 2 to 3 weeks.

Groups 1' and 2': 7 mice were randomly selected from offspring of groups 1 and 2 and assigned to groups 1' and 2', respectively.

Groups a and b: Groups a and b included 16 mice randomly selected from the offspring of groups 1 and 2 respectively. For group a, La1 was administered, 1 ml via gastric tube, b.i.d., from the time of birth. For group b, sterile, distilled water was administered, 1 ml via gastric tube, b.i.d., from the time of birth.

Groups A and B: After 4 weeks of age, 7 mice were randomly selected from groups a and b, and assigned to groups A and B, respectively.

Groups A' and B': These represented the remaining 9 mice of groups a and b.

Group C': Seven 4- to 6-weeks old mice were used as healthy controls.

FIGURE 1. *IL-4* Expressing *CD4+* *T* cells: La1-treatment of neonatal and 4-week-old BALB/c mice did not induce significant differences in the percentage of splenocyte derived IL-4 expressing *CD4+* *T* cells. Data are shown as mean $\pm$ SD (n=7).

Asthma model

All the mice of group A' and B' were sensitized at the 1st and 14th day of life with 0.2 ml sensitizing solution (20 μ g OVA and 2 mg Al(OH)₃ dissolved in 0.2 ml PBS and administered via intraperitoneal injection). Starting at the 21st day of life, all mice were put into a closed glass container (30 cm $\times$ 18 cm $\times$ 13 cm) to breathe in aerosolized OVA PBS for 20 min, *q.d.*, for 5 days. The mice of group C' were injected with the same volume of sterile, distilled water and were not exposed to aerosol OVA PBS.

Isolation of splenocytes

All the spleens, which were obtained from the mice of groups 1', 2', A, B, A', B' and C', were ground with a 300 mesh sieve. Then the splenocyte suspensions were centrifuged at 1800 $\times$ g for 7 min, and the resultant cells washed with erythrocyte lysis buffer (10 ml) and resuspended at 1 $\times$ 10⁶/ml in RPMI with 10% FBS.

Isolation of *CD4+* *T* cells

For every 10⁷ cells, 90 μ l buffer (PBS with 5 g/L OVA and 2 mM EDTA) was added to the splenocytic suspension. Ten μ l of *CD4+* magnetic beads were added to the solution and incubated at 4-8 $^{\circ}$ C for 15 min then resuspended with 500 μ l buffer. The MiniMACS system was used and splenocyte suspension was passed through separation column. After rinsing three

FIGURE 2. *IFN- γ* Expressing *CD4+* *T* cells: La1-treatment of neonatal and 4-week-old BALB/c mice did not induce significant differences in the percentage of splenocyte derived IFN- γ expressing *CD4+* *T* cells. Data are shown as mean $\pm$ SD (n=7).

times with 500 μ l buffer, *CD4+* *T* lymphocytes were collected in 1 ml of buffer.

Antibody staining

Lymphocytes were incubated with PMA (50 μ g/L), ionomycin (1 mg/L) and monensin (2 mg/L) for 4 to 6 hours. Cells were collected and centrifuged at 7000 $\times$ g for 5 min, cultured in the dark at room temperature for 10 min and then processed with 10 ml Fix and Perm. PE labeled monoclonal anti-IL-4 was added to a half of the cells and PE labelled anti-IFN- γ was added to the other half. Cells were cultured in dark at room temperature for 30 min and then washed and prepared for flow cytometry.

Flow cytometry

(FCM) Expression of IL-4 or IFN- γ by *CD4+* lymphocytes was assayed with FCM at 400 nm. An isotype control IgG was used as the negative control.

Serum levels of OVA-specific IgE

Serum levels of OVA-specific IgE in all the groups were determined using ELISA.

Results

To assess the function of La1, expression levels of IL-4 and IFN- γ were compared in the offspring of La1-treated BALB/c pregnant mice and water-treated pregnant mice. The percentages of IL-4 and IFN- γ expressing *CD4+* lymphocytes in the

FIGURE 3. IL-4 and IFN- γ Expressing CD4+ T cells in the asthma model: In mice with OVA-induced asthma, the percentage of IL-4 expressing cells in La1-treated and water-treated animals was significantly higher than that in the control mice. And the percentage of IL-4 expressing cells in La1 mice was significantly lower than that in the water-treated mice. The percentage of IFN- γ expressing cells in La1-treated and in the water-treated mice was significantly lower than that in the control mice. And the percentage of IFN- γ expressing cells in La1 mice was significantly higher than that in the water-treated mice. Data are shown as mean $\pm$ SD (n=6,5,7 respectively) * $p < 0.001$.

offspring of La1-treated pregnant mice did not show significant differences in comparison with the water-treated mice (Fig. 1 and 2).

In addition, following 4 weeks of administration of La1, neither %IL-4 nor %IFN- γ was significantly different from water-treated mice (Fig. 1 and 2).

Some of the mice died due to complications associated with asthma, so there were 6, 5 and 7 mice in group A', B' and C', respectively. The percentage of IL-4 expressing cells in both the La1-treated and water-treated mice were significantly increased compared with that in the control animals (Fig. 3; $p < 0.001$ and $p < 0.001$ respectively) and the percentage of IL-4 positive cells in the La1-treated mice was significantly decreased compared with that in the water-treated mice (Fig. 3; $p < 0.001$). The percentage of IFN- γ expressing cells in both the La1-treated and water-treated mice were significantly lower than that in the control animals (Fig. 3; $p < 0.001$ and $p < 0.001$, respectively) but the percentage of IFN- γ expressing cells in the La1-treated mice was significantly different when compared with that in the water-treated mice (Fig. 3; $p < 0.001$). The serum levels of OVA-specific IgE in the La1-treated mice and the control mice were higher compared with the water-treated mice (Fig. 4; $p < 0.05$ and $p < 0.05$, respectively) and there were

FIGURE 4. The serum levels of OVA-specific-IgE in BALB/c mice in the asthma model: Serum levels of OVA-specific-IgE (ng/mL) in the La1-treated and water-treated mice were significantly higher compared with that in the control mice. There was also a significant difference in the serum levels of OVA-specific-IgE between La1-treated mice and water-treated mice. Data are shown as mean $\pm$ SD (n=6,5,7 respectively) * $p < 0.05$.

significant differences in the serum levels of OVA-specific IgE between La1-treated mice and water-treated mice (Fig. 4; $p < 0.05$).

Discussion

Studies on the function of the probiotics from the neonate to the adult are limited. In our present study, the administration of La1 in pregnant mice did not change the immune system in the neonate, nor did the continuous administration of La1 after birth. The ratio of IL-4 and IFN- γ was altered in both La1-treated and water-treated group. Thus, the administration of La1 during pregnancy does not appear to induce changes in the immune system. In contrast, in the asthma model, administration of La1 significantly decreased the serum level of OVA-specific IgE, decreased the percentage of IL-4 expressing CD4+ T cells and increased the percentage of IFN- γ expressing CD4+ T cells in the spleen, suggesting a potential new approach to the management of bronchial asthma.

Previous studies have shown that the function of T cells is immature in the neonate, and this study confirms that the percentages of IL-4 and IFN- γ expressing cells were lower in newborn mice in comparison with those in the adult. In both the neonate and in the adult, the percentage of IL-4 and IFN- γ

CD₄⁺ T cells did not significantly differ between La1-treated mice and water-treated mice. These results are different from those reported in other studies, which showed that the matured B lymphocytes and monocytes increased with such stimuli [13-15].

Bronchial asthma is a result of chronic inflammation leading to airway hyperresponsiveness, and the imbalance of Th1/Th2 responses could play an important role in its pathogenesis. In our asthma model, the percentage of cells expressing IL-4 and the serum level of OVA-specific IgE were both increased and the percentage of cells expressing IFN- γ was decreased in water-treated mice. These results are consistent with the pathogenesis of asthma as we currently understand it. The percentage of cells expressing IL-4 was increased in La1-treated mice compared with the control, but the magnitude of the increase was lower than that seen in water-treated mice. The same trend was seen in the serum levels of OVA-specific IgE. Taken together, these results suggest that the administration of La1 could decrease the function of Th2 lymphocytes. On the other hand, the percentage of cells expressing IFN- γ was decreased in La1-treated mice compared with the control, but the magnitude of the decrease was lower than that seen in water-treated mice. These data, therefore, suggest that administration of La1 could enhance the function of Th1 lymphocytes.

In summary, the administration of *Lactobacillus johnsonii* Ncc533 (La1) had no effect on the immune system in healthy neonatal or adult in mice. In contrast, in the asthma model, administration of La1 significantly changed the percentage of CD₄⁺ T lymphocytes expressing IL-4 and IFN- γ . These data suggest a possible role for the clinical application of La1 in the treatment of patients with allergic disease, such as bronchial asthma.

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