Microbial Pathogenesis 100 (2016) 112–118

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Effects of clostridium butyricum and bifidobacterium on BTLA expression on CD4$^+$ T cells and lymphocyte differentiation in late preterm infants

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ARTICLE INFO

Article history:
Received 18 July 2016
Received in revised form
8 September 2016
Accepted 9 September 2016
Available online 10 September 2016

Keywords:
Late preterm infants
Live clostridium butyricum and bifidobacterium combined powder
BTLA
Lymphocyte subsets

ABSTRACT

Background & aims: Probiotics is recognized to promote growth performance and immune function via balancing the intestinal microflora. Live clostridium butyricum and bifidobacterium combined powder (LCBBCP) has been widely to treat intestinal dysbacteriosis in newborns in China. This study was undertaken to investigate the effects of the combined probiotics on the expression of B and T lymphocyte attenuator (BTLA) on CD4$^+$ T cells and the differentiation of lymphocyte subsets in late preterm infants.
Methods: Eighty eligible late preterm infants were equally randomized into LCBBCP therapy group (oral LCBBCP dissolved in formula milk before intake) and control group (treated with simple formula milk for preterm infants) by random digit table. Flow cytometry was used to determine the expression level of BTLA on CD4$^+$ T cells and the percentage of individual subpopulation of lymphocytes in peripheral-blood mononuclear cells (PBMCs) obtained from the late preterm infants in both groups.
Results: BTLA protein expression on CD4$^+$ T cells showed no significant change in LCBBCP therapy group before and after intervention, yet was rapidly and significantly down-regulated in the controls. The percentage of increased CD4$^+$ T cells, decreased CD8$^+$ T cells and increased ratio of CD4$^+$/CD8$^+$ T cell proportion were seen in both groups after treatment, yet the increasing or decreasing extent in LCBBCP therapy group was more obvious than in control group. The proportion of NK cells and B lymphocytes remained no significant difference between the two groups before and after therapy.
Conclusions: LCBBCP appears capable of facilitating the activation, proliferation and differentiation of T lymphocytes, which is beneficial to improving immunity in late preterm infants. The continuous high expression of BTLA on CD4$^+$ T cells in LCBBCP therapy group may be involved in the inhibiting of excessive activation of T lymphocytes. Our findings may lay a basis for further clinical evaluation of the efficacies and wider clinical recommendation of probiotics containing live clostridium butyricum and bifidobacterium for late preterm infants.

1. Introduction

In clinic, compared with term infants, later preterm infants have similar Apgar score and birth weight, but the development and function of various systems in their bodies are immature [1,2]. In the immune system, the immune organs and immune cells in late preterm infants are close to that in full-term infants, whereas the former have lower immune response to pathogenic conditions [3].

Some studies have shown that the percentage of CD3$^+$, CD4$^+$ lymphocytes and the ratio of CD4$^+$/CD8$^+$ T cells in peripheral blood are significantly higher in premature infants than in the mature infants, however, whereas the former have lower the percentage of CD8$^+$, CD19$^+$ and NK lymphocytes [4,5]. In humoral immunity, the ability to activate B cells to synthesize antibodies is lower due to the lack a variety of signal stimuli from the antigen and T cells in premature infants. In short, the function of T and B lymphocytes in late preterm infants is immature, making them more susceptible to

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http://dx.doi.org/10.1016/j.micpath.2016.09.008
0882-4010/

pathogen invasion, more serious infection and longer duration, and more prone to immune imbalance [6]. Therefore, it has become a new hot spot in the research of perinatal medicine to improve immune function of premature infants and maintain the body's immune balance.

Recent research further demonstrated that the establishment of intestinal normal flora in premature infants is essential for the development and maturation of the immune system [7]. The delayed colonization of intestinal bacteria in premature infants is mainly a result of immature development of intestinal mucosa and the easy accompanying infection, leading to antibiotics requirement to limit the survival of normal flora [8]. Therefore, the most current clinical practice to effectively promote the establishment of normal intestinal flora in premature infants relies on oral probiotics [9].

LCBBCP is a new, mixed intestinal probiotics independently developed by China. At present, in China, LCBBCP is mainly used for the clinical treatment of premature infants with diarrhea, indigestion, constipation and other diseases [10,11]. Clostridium butyricum or bifidobacterium, normal human intestinal flora, not only has the role of nutrition and protection to the human body, but also has the inhibitory effect on the pathogenic bacteria, and is of great value for the establishment of the normal flora in intestine [12,13]. There are no bacteria in the intestinal tract of newborn infants, but with the increase of day age and the increased exposure to external antigens, intestinal tract can be invaded by the pathogenic bacteria while the normal flora is being established [14]. Early oral LCBBCP can promote the early colonization of normal flora in guts, establish a perfect micro-ecological balance, and build a biochemical barrier [11]. Meanwhile, clostridium butyricum and bifidobacterium can be taken as antigens to activate the moderate local immune response, and promote the maturation of the immune function. Although LCBBCP preparation is a safe agent in clinical treatment of premature infants with intestinal dysfunction, few reports are available on effects of LCBBCP on the immunoregulatory functions.

The present work was undertaken to better understand the expression of BTLA on $CD4^+$ T cells and the differentiation of lymphocyte subsets changed in the peripheral blood of late preterm infants after oral LCBBCP administration. We hypothesized that LCBBCP may produce similar protection as a “vaccine” that is capable of leading to increased ratio of $CD4^+/CD8^+$ T cells and improving the immune function in the late preterm infants.

2. Materials and methods

2.1. Study subjects

Eighty late preterm infants, admitted to neonatal intensive care unit (NICU) at the department of Pediatrics, Yijishan Hospital of Wannan Medical College, were recruited from June 2013 through January 2015. The admission was within 24 h after birth, and the late preterm infants were defined as infants born at gestational age between $34^{0/7}$ weeks and $36^{6/7}$ weeks. The eligible criteria were absent of neonatal comorbidities (including asphyxia, infection, congenital malformation, respiratory distress syndrome, pneumorrhagia, congenital immunodeficiency and other related conditions) as well as maternal infectious diseases during pregnancy and autoimmune disorders. Then the 80 neonates were equally randomized into LCBBCP therapy group and control group by random digit table. All test subjects were treated by conventional support therapy or expectant treatment and fed with the same formula milk. LCBBCP therapy group received oral LCBBCP (420 mg powder dissolved in the formula milk), twice a day, for consecutive 7 days. The control group were only given equivalent amount of formula milk at the same time point. The test would be immediately terminated to either requirement of special medication (such as use of antibiotics, transfusion of plasma or whole blood, use of gamma globulin or glucocorticoid) because of sudden changes of conditions in process or disagreement from any of the closest relatives. Finally, 32 late preterm infants completed whole process of treatment and detection, including 15 in LCBBCP therapy group and 17 in the control group. There was no significant difference regarding gender, gestational age, birth weight, feeding mode and delivery mode between the two groups. The study was approved by the Ethical Committee of Yijishan Hospital, Wannan Medical College and parents of all subjects were given written informed consent.

2.2. Medicine

LCBBCP (trade name: Changlekang, a product manufactured by Shandong Kexing Bioproducts. Ltd, Shandong, China) was approved by China's Food and Drug Administration (batch number: S20020015) for the treatment of diarrhea, dyspepsia intestinal disorders and intestinal flora alteration.

2.3. Fluorescent antibodies and reagents

Anti-CD272 (BTLA) phycoerythrin (PE) and isotype control antibody were purchased from Biolegend (America). Anti-CD4 fluorescein isothiocyanate (FITC) and Anti-CD3 PE-Texas Red (ECD) were products of eBioscience (America). Monoclonal antibody CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5 (PE-Cyanin 5), CD3-FITC/CD19-PE and CD3-FITC/CD (16 + 56)-PE were from Beckman Coulter (America). Human lymphocyte separation medium were obtained from HaoYang Biological Manufacture (Tianjin, China). OptiLyse C lysing solution, an erythrocytic reagent intended for the lysis of red blood cells to acquire PBMCs, was purchased from Beckman Coulter (America).

2.4. Isolation of PBMCs

1 ml of peripheral venous blood was respectively collected from individual late preterm infant with EDTA-anticoagulant tube on the day 1 and the day 8 after admission. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation or lysing red cells according to the manufacturer's instructions.

2.5. Determining of BTLA expression on $CD4^+$ T cells and percentage of lymphocyte subsets by flow cytometry

For investigating the BTLA expression on $CD4^+$ T cells, PBMCs, which were isolated by Ficoll-Hypaque density gradient centrifugation, were stained with Anti-CD3 ECD, Anti-CD4 FITC, Anti-CD272 PE and isotype control fluorescent antibody. For determining the percentage of lymphocyte subsets, PBMCs, which were isolated by lysing red cells, were stained with monoclonal antibody CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5, CD3-FITC/CD19-PE and CD3-FITC/CD (16 + 56)-PE, respectively. Events were acquired with Cytomics™ FC 500 flow cytometry (BeckmanCoulter, America) and analyzed with Flowjo software (FlowJo, LLC, America).

2.6. Statistical analysis

Data were analyzed using software GraphPad Prism (version 6.0). Frequency differences of lymphocyte subsets and $BTLA^+CD4^+$ T cells in LCBBCP therapy group or control group before and after intervention were compared by the Wilcoxon paired test. The differences of the elevated frequency ratio of lymphocyte subsets and $BTLA^+CD4^+$ T cells before and after treatment between LCBBCP