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Dietary Intake of Various Lactic Acid Bacteria Suppresses Type 2 Helper T Cell Production in Antigen-Primed Mice Splenocyte

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Abstract Lactic acid bacteria (LABs) have been proposed as a potential oral allergy-therapeutic means of modulating immune phenotype expression *in vivo*, via promoting or reducing cytokine production. This study investigated the ability of LABs to suppress allergic response via modulating cytokine production in mice splenocytes. BALB/c mice were intraperitoneally primed with ovalbumin together with alum adjuvant to invoke antigen-specific Th1/Th2 cytokine-secreting cell populations in splenocytes. Spleen cells from mice fed with *Lactobacillus confusus* PL9001 (KCCM-10245), *L. fermentum* PL9005 (KCCM-10250), *L. plantarum* PL9011 (KCCM-10358), and *Bifidobacterium infantis* PL9506 (KCCM-10406) suppressed the levels of Th2 cell cytokines such as IL-4 and IL-5 during antigen sensitization. In addition, all mice fed with LABs induced secretion of Th1 cell cytokines such as IL-2 in splenocytes. These results suggested that LABs are anti-allergic agents, in view of their Th1/anti-Th2 immunoregulation.

Key words: Lactic acid bacteria, ovalbumin, cytokines, anti-allergy, mouse

Lactic acid bacteria are normal components of the healthy human intestinal microflora and are commonly used for fermentation of food products. Various strains of LABs have been shown to benefit a number of host physiological responses including immune functions [9, 11, 14, 17, 19]. Some LABs, such as *Lactobacillus casei* strain Shirota, suppressed IgE production via cytokine regulation and induced anti-allergic effects [20, 13]. However, the number of LABs with a beneficial anti-allergic effect is small, so it is important to find new strains of LABs that are able to inhibit allergic responses. Recently, the researchers of this study regarding isolated *Lactobacillus confusus* PL9001 (KCCM-10245), *L. fermentum* PL9005(KCCM-10250), *L. plantarum* PL9011(KCCM-10358), and *Bifidobacterium infantis* PL9506(KCCM-10406) from baby feces, and these strains showed various probiotic characteristics such as inhibitory activity on the growth of foodborne pathogens, the ability to adhere to intestinal epithelial cells, and resistance to acid and bile acid.

In this study, the effect of these LABs on immune regulation was investigated by examining patterns of cytokine production during ongoing antigen-specific immune responses in mice.

Materials and Methods

Animals

Six-week-old specific pathogen-free (SPF) female BALB/c mice were purchased from a vendor (Charles River, Tokyo, Japan). Animals were housed in cages at a controlled temperature (22±2°C, 55±10% humidity) with a 12-h light/dark cycle and fed with commercial mouse pellets and free access to water at all times throughout the study. Mice were acclimatized for 7 days prior to commencement of the experiments. All animal experimentation was performed in accordance with guidelines for the use and care of laboratory animals at Seoul National University.

Microorganisms

Stock cultures of *Lactobacillus confusus* PL9001 (KCCM-10245), *L. fermentum* PL9005 (KCCM-10250), *L. plantarum* PL9011 (KCCM-10358), and *Bifidobacterium infantis* PL9506 (KCCM-10406) were transferred to Tryptic soy broth (TSB, Difco, Sparks, U.S.A.) and subcultured in the same medium. Bacterial cells in the log-phase were harvested

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with centrifugation, washed, suspended in TSB, and incubated at 37°C overnight. The bacterial cell suspension for feeding mice was prepared daily by washing and suspending the bacterial cell pellets in phosphate buffered saline (PBS, pH 7.4) until the desired concentration was reached. The Colony Forming Unit (CFU) was calculated using the most probable method (MPn) using TSA.

Dietary Regimes and Antigen Sensitization

Mice (n=30) were divided into six groups. The negative control (NT) group was not ovalbumin primed (OVA, Sigma, St. Louis, U.S.A.) and not LAB-fed. The positive control (OA) group was OVA-primed and not LAB-fed. The treated group was OVA-primed and fed with each LAB. Mice were orally administered with each LAB (10^9 CFU suspended in PBS) using a stainless steel tube once a day for 21 days, while NT mice (n=5) and OA mice (n=5) received PBS during the same period. On days 0 and 14 of the feeding regime, all mice except the NT group were antigen-sensitized and boosted by the intraperitoneal injection of 50 µg of OVA absorbed onto 2 mg alum adjuvant. All mice were disposed of on day 21.

Preparation of Splenocytes

Spleens were removed aseptically from mice and placed individually into 3 ml of complete RPMI-1640 medium [RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (Sigma, St. Louis, U.S.A.), 100 U/ml penicillin (Sigma), and 100 µg/ml streptomycin (Sigma)]. A single-cell suspension was prepared by chopping the spleens into small pieces with sterile scissors and then forcing the spleen tissue up and down through a 1 ml syringe. The suspension was then transferred to a tube containing 5 ml of complete RPMI-1640 and centrifuged at 169 ×g for 15 min. Cells were suspended in ACK lysis buffer (Tris-NH_4Cl, pH 7.2) and incubated for 30 min with occasional mixing at room temperature to lyse erythrocytes. After cells were washed twice in complete RPMI-1640 medium, the concentration of cells was adjusted to 4×10^6 cells/ml in complete RPMI-1640 medium. Splenocytes (1.2×10^7 cells) were seeded to each well of a 12-well tissue culture plate and stimulated for 72 h with 100 µg/ml of OVA.

Cytokine Assays

The cell-free supernatant was harvested by centrifugation at 169 g and stored at -20°C until used. The concentration of each cytokine was determined by commercial sandwich ELISA detection kits for IL-2 and IL-5 (Bender Medsystems, Vienna, Austria), and for IL-4, IL-10, and INF-α (Chemicon International, Temecula, U.S.A.). Results were calculated against standard curves generated using known amounts of recombinant cytokines according to the manufacturer's instructions.

Statistical Analysis

The extent of significant differences between the experimental and control groups were determined by means of Duncan's Multiple Range Test (SAS ver. 8.1, SAS Institute Inc., Cary, U.S.A.). Values of $P<0.05$ were considered significant.

RESULTS AND DISCUSSION

Previously, it has been reported that murine helper T cells can be classified mainly into two categories according to the production pattern of cytokines [3, 11]. Th1 cells produce INF-γ, IL-2, and tumor necrosis factor-β there by augmenting cell-mediated immunity, whereas Th2 cells produce IL-4, IL-5, and IL-10, augment humoral immunity, and induce IgE, which is an important indicator of allergy [7, 15, 18]. The balance of cytokine derived from the two types of cells is considered to be important to maintain homeostasis of the host and defense against various immunological diseases, such as allergies and infections. Thus, the regulation of these two types of cells seems to be important in the preservation of the host's immune response, including IgE and cytokine production. Furthermore, LAB such as *L. casei* strain Shirota can promote Th1 cell cytokine production concomitant with a decrease in Th2 cell cytokine production [8, 12]. So it was hypothesized that some strains of LABs could regulate immune responses in a Th1/anti-Th2 manner.

In this study, antigen (OVA) sensitization invoked both Th1- and Th2-type immune responses among the OVA-primed mice, as signified by specific antigen-stimulated production of IL-2, IL-4, and IL-5 by splenocytes (Figs. 1A, 1C, 1D). The production of IL-4 and IL-5 by OVA-stimulated splenocytes was significantly lower in mice fed with LABs than in control-fed mice (Figs. 1C, 1D), while production of IL-2 was significantly higher in the mice fed with LABs (Fig. 1A). Th1 cell cytokines (IL-2 and INF-γ) play an important role in the down-regulation of Th2 cell cytokines that stimulate production of IgE secretion [4]. One of the important roles of IL-2 is to inhibit the production of IL-4 and IL-5 as well as the initiation and activation of T-cell growth. In addition, INF-α induces the development of Th1 cells such as IL-2 [16]. On the other hand, Th2 cell cytokines have a critical role in the initiation of the allergic response [1, 10]. IL-4 is important for IgE isotype switching, production, development of Th2 cells, and induction of adhesion molecules on endothelial cells that recruit eosinophils [5, 21]. And IL-5 is important for the production of IL-4 [6].

IL-10 was not lower in mice fed with LABs compared to mice treated with OVA, while the production of other Th2 cell cytokines were significantly lower (Fig. 1E). IL-10 has a down-regulation effect on Th1 cell cytokines in an anti-allergic manner [22]. In compensation, IL-10 might

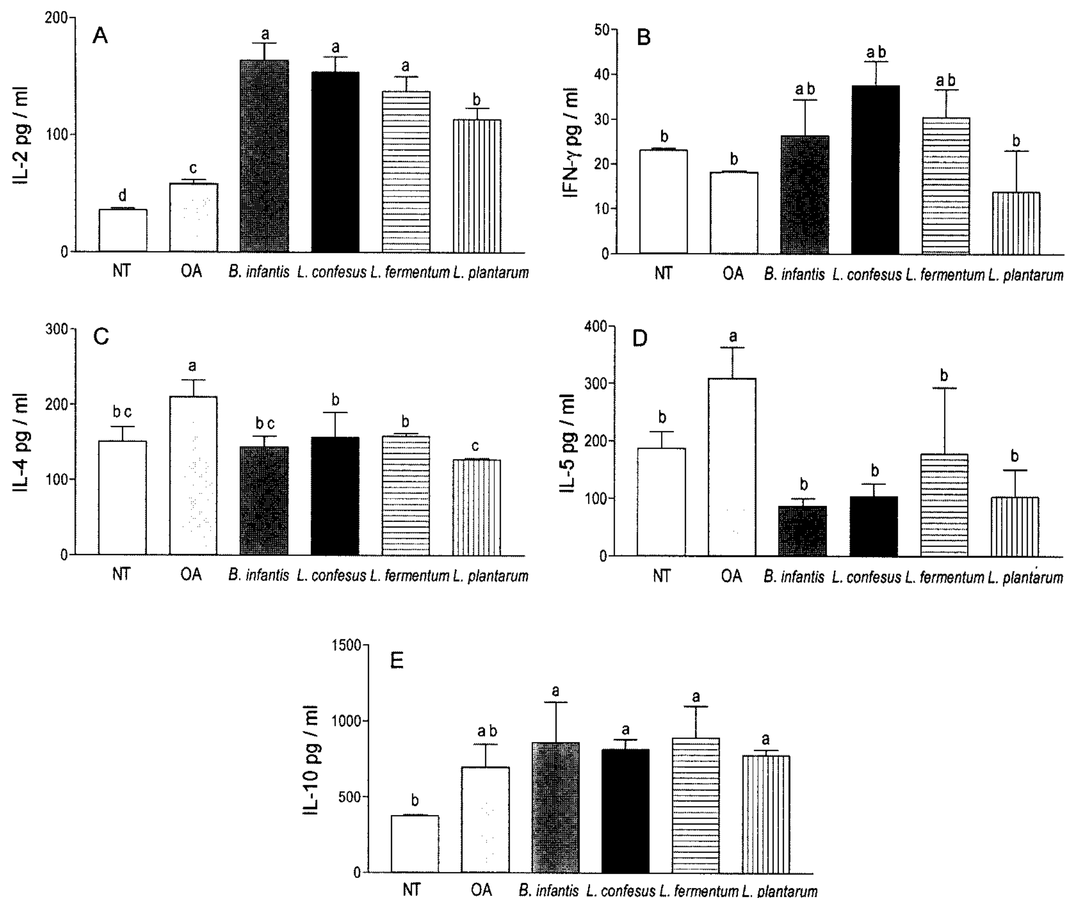

Fig. 1. Effect of LAB feeding on antigen-driven production of T-cell cytokines by splenic lymphocytes obtained from OVA-sensitized mice.

Splenocytes were cultured for 72 h with 100 μg/ml OVA in the complete RPMI medium. Concentrations of IL-2 (A), INF-α (B), IL-4 (C), IL-5 (D), and IL-10 (E) were determined by ELISA kits. Error bars indicate standard deviations. Any two means within each experiment not identified by the same letter are significantly different ($P<0.05$). NT, non OVA-primed and not LAB-fed mice group; OA, OVA-primed and not LAB-fed group.

increase to inhibit the overproduction of IL-2. Additionally, Th1 cell cytokine such as INF-γ did not increase in the mice fed with LABs, even though its amount was not significantly higher than the control (Fig. 1B). It was considered that IL-10 was more sensitive in inhibiting INF-γ production than IL-2 at this time. On the other hand, some LAB strains had various effects on cytokine production [2, 7]. Thus, it was possible that some LABs can decrease the levels of Th2 cytokines without increasing INF-γ levels [12].

In conclusion, the data presented in this study clearly indicate that LAB can act as an anti-allergic agent in a Th1/anti-Th2 manner in the OVA primed murine allergy model. It is important for human health to find these anti-allergic strains of LABs. These LABs may be beneficial for the prevention of allergic disorders and could be used in anti-allergy immunotherapy. Future research will be necessary to examine the inhibitory effect of oral feeding of these LABs on IgE production, and to characterize the effects of LABs for their relevance to human health and their anti-allergic activity.

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