〈Original Article〉

Probiotics *Aspergillus oryzae* and *Lactobacillus sakei* reduce IgE and increase Th1 cytokines in an ovalbumin-induced murine model of allergy

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Summary Allergic inflammation is associated with an increased Type 2 helper T cell (Th2) response, including secretion of Th2 cytokines and IgE antibody. Reduction of allergic reaction is occurs due to an increase in Th1 immune responses to restrain Th2 via the secretion of the TNF-α and IL-12. Probiotics are believed to alleviate allergic reactions by improving intestinal microbial balance. We examined anti-allergenic effects of probiotics *Aspergillus oryzae* (AO) and *Lactobacillus sakei* (LS) in a murine macrophage cell line J774.1 and a mouse model of ovalbumin (OVA)-induced allergy. The treatment with AO and LS induced the secretion of Th1 response-related cytokines tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12) in macrophages and significantly reduced the production of anti-OVA IgE and proliferative activity of splenocytes in OVA-induced allergic mice. Our results suggest that the tested probiotics can reduce allergic immune reactions by upregulating Th1 and downregulating Th2 response via secretion of TNF-α and IL-12. These observations may be useful for the management of food and/or environmental allergic reactions in humans.

Key words: *Aspergillus oryzae*, *Lactobacillus sakei*, Immunoglobulin E, Macrophages, Cytokines

1. Introduction

Probiotic bacteria exert beneficial effects on human health through suppression of harmful microorganisms and immunomodulatory activity. Preclinical and clinical studies have accumulated evidence of the effect of orally administered probiotics in the management of allergic diseases, diabetes, obesity, heart disease, and other debilitating disorders. For example, oral administration of Bifidobacterium lactis modulates immune parameters and mitigates allergic symptoms[1]. Other probiotic bacteria exhibited cholesterol-lowering effects by interacting with bile acids[2], and could decrease lipid concentration in serum improving hemorheology[3]. The administration of probiotics to infants altered the composition of their

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intestinal microbiota reducing the risk of necrotizing enterocolitis[4].

The filamentous fungus *Aspergillus oryzae* (AO) has been used for more than thousand years for production of Japanese fermented food and beverages such as sake, soy sauce, and soybean paste. The approval level of AO is "generally recognized as safe" by the U.S. Food and Drug Administration[5]. AO is known to secrete high amounts of amylases and proteases, and a recent report has shown that AO cultures can be used to suppress the growth of human lung tumor cells[6].

Although lactic acid bacteria *Lactobacillus sakei* (LS) rarely resides on the surface of vegetables, anaerobic and low salt conditions stimulate rapid growth of LS, especially at the end of the fermenting process. In a recent study, LS has been shown to exhibit potent antibacterial activity by producing bacteriocins and activating murine macrophages[7].

Macrophages are active players in non-specific innate immunity and also facilitate the initiation of specific adaptive immunity in vertebrates. Antigen-presenting macrophages express pattern recognition receptors such as mannose receptors, scavenger receptors, and toll-like receptors (TLRs), which detect evolutionary conserved structures on invading pathogens[8-10]. TLRs primarily control the induction of T helper (Th)1-type immune response. Upon TLR activation with pathogen ligands, macrophages secrete pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6[11, 12], and IL-12. In the process of polarization of different Th subsets, IL-12 plays a critical role in inducing Th1 responses, most notably interferon (IFN)-γ, thus inhibiting Th2 immune response[13] involved in allergic reactions. Immunoglobulin E (IgE) antibody plays an important role in type I allergic reactions by mediating the binding of allergens to the surface of mast cells[14], which then secrete histamine in type I hypersensitivity[15]. The suppression of Th2 responses and decrease of IgE production inhibit the development of allergy.

In the present study, we examined the immunomodulatory effects of AO and LS on murine macrophages by measuring in vitro production of Th1-related cytokines IL-12 and TNF-α. We also used a murine model of ovalbumin (OVA)-induced allergy to assess the changes in IgE production in allergic mice induced by intragastric administration of AO or LS.

2. Material and methods

2.1. Microorganisms

Lyophilized AO and LS cultures used in this study were obtained from OTO Corporation (Odawara, Japan).

2. 2. Cell culture

Murine macrophage-like cell line J774.1 was purchased from Riken Gene Bank (Tsukuba, Japan) and grown in tissue culture flasks at 37℃ in a humidified atmosphere containing 5% CO_2. Cells were maintained in RPMI-1640 medium (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS) (Thermo Scientific Hyclone, Logan, UT, USA), and were passaged bi-weekly. For treatment experiments, adherent cells were detached and plated at the concentration of 5×10^5 cells/well in 24-well plates for 2 h at 37℃. After the removal of non-adherent cells, fresh medium containing 100 μg/mL AO or LS was added to cell monolayers; phosphate-buffered saline (PBS) and 100 μg/mL LPS were added to the wells used as negative and positive controls, respectively. After incubation for 24 h, cell supernatants were collected and stored at -80℃ until analysis. Four independent experiments in duplicate were performed.

2.3. Measurement of TNF-α and IL-12 secretion

The concentrations of TNF-α and IL-12 in J774.1 culture supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) using commercial kits (R & D Systems, Minneapolis, MN, USA).

2.4. Mice

Five-week-old female BALB/c mice were purchased from SLC (Hamamatsu, Japan) and housed under specific pathogen-free conditions. They were provided standard laboratory OVA-free rodent chow CRF-1 (Oriental Yeast, Tokyo, Japan) and water, and maintained on a 12:12 h light:dark cycle in an environ-

mentally controlled animal chamber. All procedures were conducted in accordance with the Guidelines for Animal Experiments in Kitasato University and approved by the Ethical Committee of Kitasato University.

2.5. Intraperitoneal sensitization to OVA and treatment with probiotics

Six-week-old female BALB/c mice were intraperitoneally injected on days 0 and 12 with 100 μl PBS (pH7.4) containing 50 μg of OVA (Sigma, St. Louis, MO, USA) and 2 mg alum; negative control mice received PBS. The animals received, by intragastric administration, 1 μg/g mouse weight of LS or 10 μg/g mouse weight AO cultures in PBS daily starting from day 1 to day 25. Negative control mice without sensitization were intraperitoneally injected with PBS without OVA-alum and were intragastrically administered PBS without the lyophilized microorganisms; positive control mice were intragastrically administered PBS without probiotics. Each group contained six animals.

On day 26, mice were sacrificed, and their sera were isolated and stored at -80℃ until the analysis of OVA-specific IgE and total IgE levels using commercial ELISA kits (Shibayagi, Gunma, Japan) according to the manufacturer's instructions. Splenocytes were isolated from the spleens, cultured at the concentration of 5×10^5 cells/mL with 1-10 μg/mL OVA for three days, and cell proliferation was estimated using the Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany). The assay was conducted in duplicate.

2.6. Statistical analysis

All data are presented as the means ± standard deviation (SD). The statistical significance of mean differences was evaluated by one-way ANOVA with the Dunnett's multiple comparison test using JMP (version 8.0.1). *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. The effect of probiotics on the growth of J774.1 cells

The level of TNF-α in culture supernatants of J774.1 cells treated with AO or LS was significantly increased ($P < 0.05$; Fig. 1A), in a dose-dependent manner, compared to untreated cells. However, no significant effect on the level of IL-12 secretion was

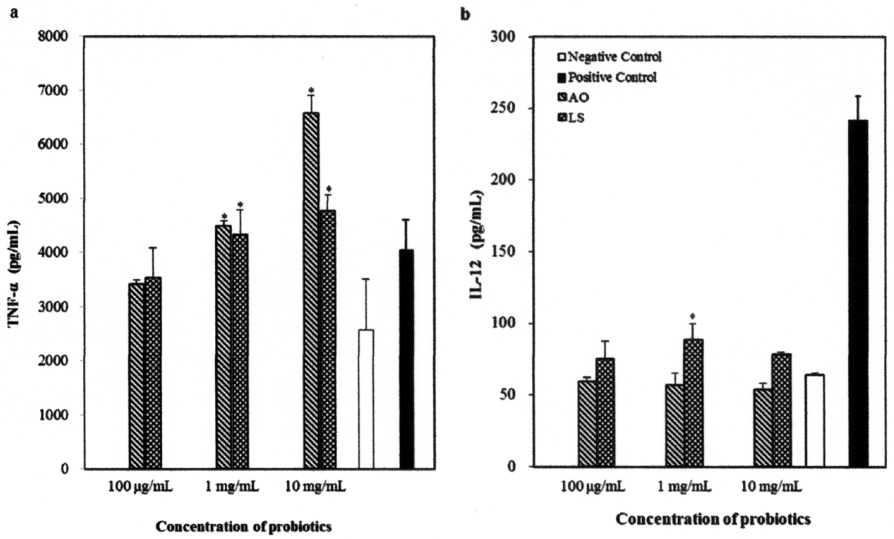

Fig. 1 Production of TNF-α and IL-12 in macrophage cell line treated with *Aspergillus oryzae* (AO) and *Lactobacillus sakei* (LS). Murine macrophage J774.1 cells were cultured with 100 μg/mL of AO, LS, or LPS (positive control), or PBS (negative control); after 24 h, culture supernatants were collected and analyzed for TNF-α (A) and IL-12 (B) levels by ELISA. The data represent the means ± SD of four independent experiments; *$P < 0.05$ vs. negative control.

detected (Fig. 1B).

3.2. IgE levels in OVA-induced allergic mice

OVA-alum-immunized mice were intragastrically administered AO, LS, or PBS for 25 days, and then analyzed for serum levels of total IgE and OVA-specific IgE (Fig. 2). The levels of both total and OVA-specific IgE were increased by OVA stimulation (data not shown). However, in OVA-induced mice fed the diet supplemented with AO and LS, serum concentration of OVA-specific IgE was decreased, but that of total IgE did not show significant changes.

3.3. Proliferation activity of splenocytes

We observed the changes in the proliferation activity of mouse splenocytes due to OVA-induced allergy. Negative control splenocytes cultured without OVA showed no significant proliferative activity, whereas the OVA-induced cells demonstrated increased proliferation in an OVA concentration-dependent manner. However, the proliferation of splenocytes from allergic mice fed with AO or LS decreased compared with those fed probiotic-free diet. The difference was even more pronounced when OVA concentration in the medium was increased to 10 μg/mL (Fig. 3).

4. Discussion

In this study, we demonstrated that AO or LS, used as probiotics, exert an immunostimulatory effect on mice. In macrophage-like cells, both probiotics dose-dependently stimulated the production of TNF-α, which is known as a proinflammatory cytokine produced by phagocytic cells in response to pathogenic bacteria and viruses.

TNF-α is secreted by macrophages in the early stages of inflammation in the same manner as IL-6 and IL-1[16]. TNF-α is released upon microbial binding to TLR2 on macrophages[17, 18], which then secrete IL-12, shifting Th1/Th2 balance towards Th1 and inhibiting antibody production[19]. It has been reported that gram-positive lactic acid bacteria induce secretion of Th1-type cytokines[20]; therefore, we hypothesized that allergic reactions associated with Th2 response could be suppressed by shifting the balance to Th1 response by using probiotics AO or LS. Our results indicate that TNF-α production in macrophages was increased

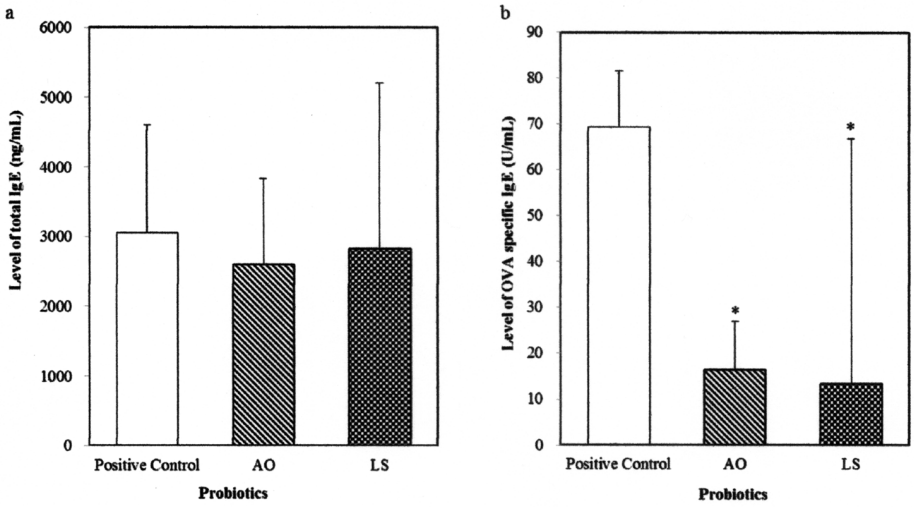

Fig. 2 Serum levels of total and OVA-specific IgE in OVA-induced allergic mice treated with probiotics. BALB/C mice were intraperitoneally injected on day 0 and day 12 with 100 μg of ovalbumin (OVA)-alum and fed a diet supple mented with *Aspergillus oryzae* (AO; 10 μg/g) and Lactobacillus sakei (LS; 1 μg/g) starting at day 1 for 25 days; PBS-treated and OVA-treated mice fed a probiotic-free diet were used as negative and positive controls, respectively. Serum levels of total IgE (A) and OVA-specific IgE (B) were determined by ELISA. The data represent the means ± SD (n = 6); * $P < 0.05$ vs. positive control.

by the addition of microbial cultures than addition of LPS using macrophage activator; moreover, a tendency for IL-12 upregulation was observed. Activated macrophages via TLR4 binding LPS is Th1 dominance by producing TNF-α [12]. AO and LS was shown that highly effective to produce TNF-α than LPS. In addition, it is known that IL-12 enhances the production of antibody isotypes associated with Th1-type immune response but inhibits that of Th2-related IgE. In our in vitro experiments, the level of antigen-specific IgE, but not total IgE, was decreased by AO and LS treatment, suggesting that IL-12-related immune effects were not markedly affected.

We also investigated immunomodulatory activity of AO and LS in vivo using OVA-induced allergy mouse model. The animals immunized with OVA, a potent egg allergen, received AO or LS by intragastric administration and were then analyzed for the levels of serum OVA-specific IgE. We observed that allergic mice treated with AO or LS had a significantly lower IgE antibody levels than the untreated control mice. In addition, OVA-re-stimulated splenocytes isolated from probiotic-treated mice demonstrated decreased proliferative activity. Stimulated Th1 lymphocytes inhibit a Th2-type immune response, which has been shown to play a role in alleviating Th2-mediated inflammatory diseases such as allergy[21]. The secretion of IL-12 and IFN-γ during immune response has resulted in inhibition of Th2 but not Th1 lymphocyte proliferation in vitro[22, 23]. Overall, these findings and our results suggest a possible role of AO and LS cultures in the suppression of Th2-type response and decrease of allergic symptoms. IL-12-inducing ability of lactic acid bacteria is an important factor in the selection of appropriate strains with the potential to prevent and treat allergic diseases[24]. IgE antibody plays an important role in the development of type I allergic reactions by mediating the binding of allergens to mast cells[2], which then degranulate and release a number of chemical mediators such as histamine,

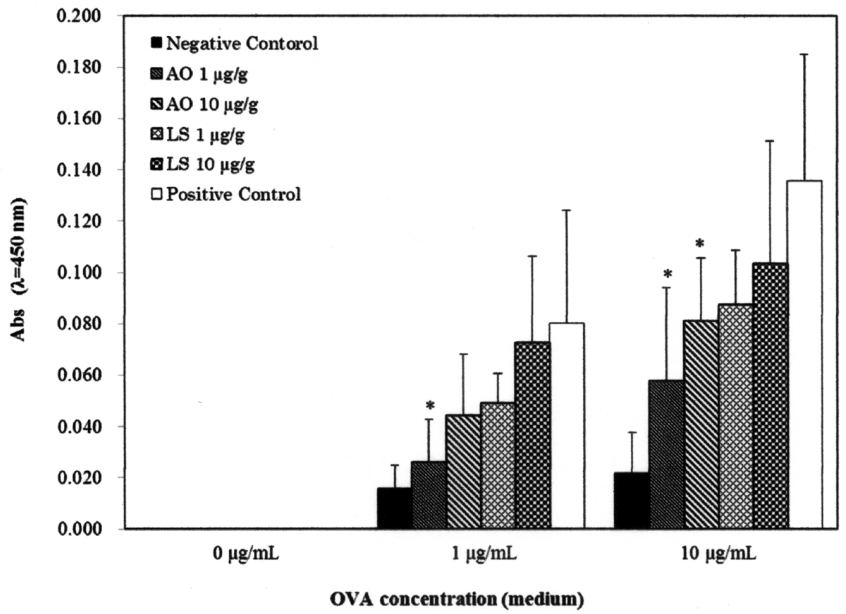

Fig. 3 Proliferation activity of splenocytes isolated from allergic mice fed with *Aspergillus oryzae* (AO) and *Lactobacillus sakei* (LS). BALB/C mice were intraperitoneally injected on day 0 and day 12 with 100 μg of ovalbumin (OVA)-alum and fed a diet supplemented with Aspergillus oryzae (AO, 10 μg/g) and Lactobacillus sakei (LS, 1 μg/g) starting at day 1 for 25 days; PBS-treated and OVA-treated mice on a diet without probiotics were used as negative and positive controls, respectively. Splenocytes were isolated and cultured in 96-well plates (5×10^5 cells/well) with 1 μg/mL or 10 μg/mL OVA or with PBS; after three days, culture supernatants were collected and cell proliferation activity was measured using the WST-1 Cell Proliferation kit. The data represent the means ± SD of six independent experiments; * $P < 0.05$ vs. positive control.

leukotrienes, and prostaglandins responsible for type I allergic symptoms[15].

In summary, we have shown that probiotics AO and LS have the ability to induce TNF-α production and IL-12 release in vitro, and to inhibit Th2 immune response associated with IgE production in vivo. To our knowledge, these results show, for the first time, that AO and LS can reduce allergic reactions by balancing Th1/Th2 immune responses, suggesting that these probiotic bacteria might prevent and/or suppress the development of allergic symptoms, and may be useful for the management of food or environmental allergy in humans. Further studies are needed to characterize the nature of Th1/Th2 balance shifting by AO and LS and its efficacy in humans.

Conflict of interest statement

This study was supported by OTO Corporation. Tsuyoshi Sugiyama is an employee of OTO Corporation. The sponsor had no control over the interpretation, writing, or publication of this work.

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