Oral Administration of Heat-Killed *Lactobacillus brevis* SBC8803 Ameliorates the Development of Dermatitis and Inhibits Immunoglobulin E Production in Atopic Dermatitis Model NC/Nga Mice

Shuichi SEGAWA,*a, a Atsushi HAYASHI,b Yasukazu NAKAKITA,a Hirotaka KANEDA,a Junji WATARI,a and Hisako YASUIb

a Frontier Laboratories of Value Creation, Sapporo Breweries Ltd.; 10 Okatohme, Yaizu, Shizuoka 425–0013, Japan; and b Sciences of Functional Foods, Graduate School of Agriculture, Shinshu University; 8304 Minami-minowa, Kami-ina, Nagano 399–4598, Japan. Received November 22, 2007; accepted February 13, 2008; published online February 25, 2008

We have previously shown that the oral administration of heat-killed *Lactobacillus brevis* (L. brevis) SBC8803 strain inhibits IgE production in ovalbumin (OVA)-sensitized BALB/c mice through improvement of the type-1 helper T (Th1)/Th2 balance toward Th1 dominance. Atopic dermatitis is one of the most common skin diseases and is frequently associated with elevated immunoglobulin E (IgE) antibodies against many kinds of allergens. In this study, the inhibitory effect of oral administration of *L. brevis* SBC8803 on the development of dermatitis and IgE elevation using the NC/Nga atopic dermatitis model mice. Male 8-week-old NC/Nga mice were sensitized by the topical application of picryl chloride to foot pads and shaved abdomen. These mice were boosted with picryl chloride by topical application onto the ears once a week for 9 weeks. The mice (n=10 per group) were fed a diet containing 0%, 0.05% or 0.5% of heat-killed *L. brevis* SBC8803 from 2 weeks before the first sensitization to the end of the study. Total IgE concentration in serum, clinical score, and ear thickness were periodically examined throughout the study. Finally, cytokine (interleukin (IL)-4, IL-5, IL-6, IL-10, IL-12, IFN-γ and transforming growth factor (TGF)-β) productions from splenocytes and Peyer’s patch (PP) cells of mice were measured. Oral administration of *L. brevis* SBC8803 significantly inhibited IgE production and ear swelling, and suppressed the development of dermatitis in a dose-dependent manner. Immunosuppressive cytokines such as IL-10 and TGF-β production from PP cells significantly increased in the 0.5% group compared to the control group although Th1-type and Th2-type cytokines production was not affected.

Key words *Lactobacillus brevis*; atopic dermatitis; allergic disease; immunoglobulin E; cytokine

Atopic dermatitis (AD) is one of the most common skin diseases and its prevalence is increasing in industrialized countries. This disease is frequently associated with elevated immunoglobulin E (IgE) antibodies against many kinds of allergens.1) IgE antibodies are secreted from IgE producing plasma cells, which are differentiated from B cells. The proliferation and differentiation of B cells into IgE producing plasma cells are mainly regulated by type-2 CD4+ helper T (Th2) cells. CD4+ T cells can be classified into two subtypes, type-1 helper T (Th1) cell and Th2 cells.2—4) Th1 cells producing interferon (IFN)-γ play an important role in cellular immunity. Th2 cells, on the other hand, producing interleukin (IL)-4 promotes B cell proliferation, IgE class switching and then augments IgE secretion.5—7) The IgE production induced by IL-4 is modulated by several cytokines. IFN-α, IFN-γ and IL-12, which are Th1-type cytokine, have inhibitory effects on IgE production. IL-10 and transforming growth factor (TGF)-β, which is an immunosuppressive cytokine, have inhibitory effects on IgE production and the development of AD-like skin disease too. On the other hand, IL-5 and IL-6, which are Th2-type cytokine, enhance IL-4-induced IgE production.

Several studies have reported that some lactic acid bacteria (LAB) inhibit IgE production through improvement of the Th1/Th2 balance toward Th1 dominance.8—13) Moreover, regulatory T (Treg) cells, which have an immunosuppressive function, are able to inhibit the development of the Th2 response.14,15) Treg cells exert the immunosuppressive properties through the production of IL-10 and TGF-β. Oral administration of *Lactobacillus rhamnosus* GG was reported to alleviate the clinical symptoms of atopic dermatitis through the enhanced IL-10 generation.16) Subcutaneous injection of rTGF-β1 was reported to suppress the development of the AD-like skin lesions through the reduction of IgE production.17) Accordingly, the regulation of these T cell populations is important for controlling IgE production.

NC/Nga mice, an inbred strain established from fancy Japanese mice, are to develop AD-like skin lesions under conventional care, or upon treatment with repeated challenge with 2,4,6-trinitrochlorobenzene (picryl chloride) under specific pathogen free (SPF) condition.18—20) The induced dermatitis is accompanied by elevated serum IgE levels, increased expression of Th2 cytokines, eosinophil accumulation in the lesions and frequent scratching behavior, which are characteristic features of human AD.19)

We have previously shown that the oral administration of heat-killed *Lactobacillus brevis* (L. brevis) SBC8803 strain inhibited IgE production in ovalbumin (OVA)-sensitized BALB/c mice through improvement of the Th1/Th2 balance toward Th1 dominance. In this study, we investigated the inhibitory effect of oral administration of *L. brevis* SBC8803 on the development of dermatitis and IgE elevation, which were induced by the topical application of picryl chloride under SPF condition, using AD model NC/Nga mice. The inhibitory mechanism of the development of dermatitis and IgE elevation by the oral administration of heat-killed *L. brevis* SBC8803 was investigated through the measurement of Th1-type (IL-12 and IFN-γ), Th2-type (IL-4, IL-5 and IL-6), and regulatory (IL-10 and TGF-β) cytokines production from splenocytes and Peyer’s patch (PP) cells.

* To whom correspondence should be addressed. e-mail: syuichi.segawa@sapporobeer.co.jp © 2008 Pharmaceutical Society of Japan
MATERIALS AND METHODS

**Animals**  Male 5-week-old NC/Nga mice were purchased from Charles River Japan, Inc (Kanagawa, Japan). The animals were housed in an air-conditioned room maintained at 23±2°C with a relative humidity of 55±15%. They were given standard laboratory rodent chow MF (Oriental Yeast, Tokyo, Japan) and water ad libitum. All procedures were conducted in accordance with the Guidelines for Animal Experiment at Shinshu University and approved by the Ethical Committee at the Shinshu University.

**Microorganisms**  The L. brevis SBC8803 strain used in this study was collected from Sapporo Breweries Ltd. L. brevis SBC8803 was inoculated in 10 ml of DeMan-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MN, U.S.A.) and cultivated for a day at 37°C under anaerobic conditions (N2 : CO2 : H2 = 90 : 5 : 5, Tabai Spec, Tokyo, Japan), collected by centrifugation, and then washed three times with phosphate buffer saline (PBS). Then, the microorganisms were killed by heating at 121°C for 20 min and then lyophilized.

**In Vivo Experiments**  AD-like skin lesions were induced by repeated topical application with picryl chloride. Male 8-week-old NC/Nga mice were sensitized with 150 µl of 5% picryl chloride dissolved in ethanol/acetone (4 : 1) by topical application onto foot pads and shaved abdomen. Four days after the first sensitization, 15 µl of 1% picryl chloride dissolved in olive oil was applied to each side of the ears once a week for 9 weeks. The mice (n=10 per group) were fed an MF diet containing 0%, 0.05% or 0.5% of *L. brevis* SBC8803 from 2 weeks before the first sensitization (Day 0) to the end of the study (Day 82) (Fig. 1). Total IgE concentration in serum, clinical skin severity score, and ear thickness were periodically examined throughout the study. Dermatitis symptoms were evaluated according to the scoring method described by Matsuda et al. Edema was evaluated by measuring ear thickness, skin lesions were evaluated by macroscopic observation. Total clinical skin severity score was calculated from the sum of the individual scores grades as 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of the five symptoms (redness/hemorrhage, edema, acomia/excoriation, dryness and anthema). Ear thickness was measured using a micrometer (Mitsutoyo Corp., Kanagawa, Japan). Finally, cytokines (IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ and TGF-β) and IgE production from splenocytes and PP cells of mice were measured. Splenocytes were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, U.S.A.), which contained 10% FBS (Equitech-Bio, Kerrville, TX, U.S.A.), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen, Carlsbad, CA, U.S.A.), to be 2.5×10^6 cells/ml on a 96-well culture plate. PP cells were prepared from PP by treating with 2.25 mg/ml of dispase (Invitrogen) dissolved in Joklik modified MEM medium (Sigma-Aldrich), they were then cultured in RPMI-1640 medium, which contained 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin, to be 5×10^6 cells/ml on a 96-well culture plate. These cells were cultured at 37°C at a 5% CO2 concentration. Supernatants were collected on Day 2 or Day 3 to measure the cytokines (Day 2: IL-4, Day 3: IL-12, IFN-γ, IL-10 and TGF-β) production, and on Day 14 to measure total IgE production. The concentrations of cytokines and total IgE in the supernatants were determined by sandwich ELISA.

**ELISA**  The concentration of IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, and TGF-β in the culture supernatant, and total IgE in serum was measured using a sandwich ELISA assay. Fifty microliters of the first antibodies dissolved in 50 mM sodium carbonate buffer was added to each well of a 96-well microtiter plate and incubated overnight at 4°C. The concentration of the first antibodies for IL-4, IL-5, IL-6, IL-10, IL-12, and IFN-γ measurement was as follows, 1 µg/ml of monoclonal anti-mouse IL-4 antibody (R&D Systems, Minneapolis, MN, U.S.A.), 1 µg/ml of purified anti-mouse IL-5 antibody (Biologend, San Diego, CA, U.S.A.), 1 µg/ml of purified anti-mouse IL-6 antibody (Biologend), 0.1 µg/ml of purified anti-mouse IL-10 antibody (Biologend), 1 µg/ml of purified anti-mouse IL-12 (p40/p70) monoclonal antibody (BD Pharmingen, San Diego, CA, U.S.A.), and 2 µg/ml of rabbit anti-mouse/rat IFN-γ (Biosource, Camarillo, CA, U.S.A.). Each well was washed with wash buffer (PBS containing 0.05% Tween 20) three times. One-hundred microliters of 1% BSA in 50 mM sodium carbonate buffer was added to each well, the plate was incubated for 90 min at 37°C and then each well was washed with wash buffer five times. Fifty microliters of the culture supernatant or the standard solution was added to each well, the plate was incubated for 90 min at room temperature and then each well was washed with wash buffer five times. The following recombinant cytokines were used in this study as standard, recombinant mouse IL-4 (R&D systems), recombinant mouse IL-5 (Biologend), recombinant mouse IL-6 (Biologend), recombi-
nont mouse IL-10 (Biolegend), recombinant mouse IL-12 (R&D systems) and recombinant murine interferon-γ (Biosource). Fifty microliters of the biotinylated second antibody dissolved in wash buffer containing 1% BSA was added to each well of a 96-well microtiter plate and incubated for 90 min at room temperature. The concentration of the second antibodies for IL-4, IL-5, IL-6, IL-10, IL-12, and IFN-γ measurement were as follows, 0.1 μg/ml of biotinylated anti-mouse IL-4 antibody (R&D Systems), 0.5 μg/ml of biotinylated anti-mouse IL-5 antibody (Biolegend), 0.5 μg/ml of biotinylated anti-mouse IL-6 antibody (Biolegend), 0.5 μg/ml of biotinylated anti-mouse IL-10 antibody (Biolegend), 0.5 μg/ml of biotinylated rat anti-mouse IL-12 (p40/p70) monoclonal antibody (BD Pharmingen) and 0.1 μg/ml of mouse anti-rat/mouse IFN-γ biotin conjugate (Biosource). Each well was washed as before. Fifty microliters of 0.1 μg/ml of streptavidin-horseradish peroxidase (Biosource) was added to each well, the plate was incubated for 30 min at room temperature in the dark and then each well was washed as before. Substrate solution 3,3′,5′,5′-tetramethylbenzidine (Sigma-Aldrich) was added to each well, and the plate was allowed to develop at room temperature in the dark. The color reaction was stopped by the addition of 0.5 n H2SO4. Absorbance at 450 nm was measured using a microplate reader (MTP-800 AFC, Hitachi High-Technologies, Tokyo, Japan). The concentration of TGF-β1 and IgE was measured using mouse TGF-β kit (Bender MedSystems, Vienna, Austria) and a mouse IgE ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX U.S.A.).

Histological Analysis Ears of mice, which were induced by repeated topical application with a picryl chloride, were removed at the final day of the experiment (Day 82). Each ear was fixed in Bouin’s solution (composition; saturated picric acid solution/formalin/acetic acid = 15/5/1), embedded in paraffin, cut in 3 mm, and stained with hematoxylin and eosin.

Statistical Analysis All values are expressed as mean±S.D. Statistical evaluation of the clinical score was performed by Mann–Whitney’s U test. Statistical evaluation of the results for the change in the ear thickness and total IgE in serum was performed by one-way analysis of variance (ANOVA) followed by Tukey’s test. A probability value of less than 0.05 was considered statistically significant.

RESULTS

Effect of Oral Administration of Heat-Killed L. brevis SBC8803 on Clinical Skin Severity Score and Ear Thickness of NC/Nga Mice Induced by Topical Application of Picryl Chloride

Each value represents the mean±S.D., n=10. ∗ ∗∗ Significantly different from the corresponding control group at p<0.05 and p<0.01, respectively.

Effect of Oral Administration of Heat-Killed L. brevis SBC8803 on the Development of Dermatitis Figure 2 shows changes in the clinical skin severity score and ear thickness in NC/Nga mice with aging. The clinical skin severity score and ear thickness of the control group, which were sensitized with picryl chloride under SPF condition, increased from the beginning of the sensitization. Oral administration of heat-killed L. brevis SBC8803 significantly inhibited the increase of the clinical skin severity score and the ear thickness in comparison with the control group throughout the experimental period. This inhibitory effect of heat-killed L. brevis SBC8803 on the development of dermatitis in NC/Nga mice was dose-dependent. Figure 3 shows the symptom of dermatitis in mice at Day 82. The development of dermatitis was suppressed by the oral administration of heat-killed L. brevis SBC8803 in a dose-dependent manner. In the control group, fully developed dermatitis was observed on the face and ears. On the other hand, in the 0.5% L. brevis SBC8803 fed group, macroscopically observed dermatitis was almost completely suppressed. The histological analysis of ear skin was performed, and typical photographs are shown in Fig. 4. In comparison with the control group, the L. brevis SBC8803 containing diet fed group showed less ear thickening, hyperplasia of the epidermis, and infiltration of inflammatory cells in dermis. These results clearly show that oral administration of L. brevis SBC8803 is effective to prevent the development of dermatitis in NC/Nga mice induced by the topical application of a picryl chloride under SPF condition.

Effect of Oral Administration of Heat-Killed L. brevis SBC8803 on Total IgE Level in Serum The clinical severity of dermatitis in NC/Nga mice is reported to be associated with elevated serum IgE levels.18 Therefore, IgE concentration in serum was measured. As shown in Fig. 5, total IgE level in serum of the control group increased by the periodical topical application of a picryl chloride. Total IgE level in serum had significant correlation with the onset and progression of dermatitis. Oral administration of heat-killed L. brevis SBC8803 dose-dependently inhibited the increase of total IgE in serum.

Effect of Heat-Killed L. brevis Ingestion on Cytokine Production from Splenocytes and Peyer’s Patch Cells of NC/Nga Mice To clarify the inhibitory mechanism of L.
brevis SBC8803 on the development of dermatitis in NC/Nga mice, spontaneous cytokine production from splenocytes and PP cells, which were prepared from picryl chloride-sensitized NC/Nga mice, was investigated. At Day 82, splenocytes and PP cells were prepared from each group, and then these cells were cultured for 2 d to measure IL-4 production, and for 3 d to measure IL-5, IL-6, IL-10, IL-12, IFN-γ, and TGF-β1. As shown in Table 1, Th1-type cytokines such as IFN-γ, and Th2-type cytokines such as IL-5, IL-6 production from splenocytes and PP cells were not affected by the oral administration of L. brevis SBC8803, although IL-12 production from PP cells was slightly increased. IL-4 production from splenocytes and PP cells and IFN-γ production from splenocytes was under the detection limit in our ELISA system. As shown in Fig. 6, the production of immunosuppressive cytokines such as IL-10 and TGF-β1 from PP cells dose dependently increased by the oral administration of heat-killed L. brevis SBC8803, although the production of these cy-

Table 1. Cytokine Production from Splenocytes and Peyer's Patch Cells Isolated from NC/Nga Mice

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>Splenocytes</th>
<th>Peyer's patch cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>ND</td>
<td>0.05%</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.42±1.66</td>
<td>1.73±0.41</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.31±0.01</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>IL-12</td>
<td>2.84±0.22</td>
<td>2.79±0.21</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D., n=10. **Significantly different from the corresponding control group at p<0.01. ND: not detected.
High sensitivity to IL-4 and CD40L signal through enhanced less IL-4, and splenic B cells isolated from this strain had BALB/c mice, T cells isolated from NC/Nga mice produced 4 production from splenocytes and PP cells was extremely however, in comparison with BALB/c mice, spontaneous IL-4 due to its spontaneous secretion without antigen stimulation. The reason for this lower production of IL-4 was probably switching to IgE. In this study, IL-4 production from splenocytes did not change (data not shown). DISCUSSION The results of this study demonstrated that the oral administration of heat-killed L. brevis SBC8803 prevented the development of AD-like skin lesions in NC/Nga mice, which were induced by the periodical topical application of a picryl chloride under SPF condition. The development of dermatitis in this strain is reported to be associated with elevated serum IgE levels. In this study, the periodical sensitization of NC/Nga mice with picryl chloride caused a significant increase of IgE concentration in serum. The increase of IgE concentration in serum and clinical skin severity score was also significantly inhibited by the oral administration of heat-killed L. brevis SBC8803 (Figs. 2, 5). IL-4 is an important factor for immunoglobulin class-switching to IgE. In this study, IL-4 production from splenocytes and PP cells was under the detection limit (Table 1). The reason for this lower production of IL-4 was probably due to its spontaneous secretion without antigen stimulation. However, in comparison with BALB/c mice, spontaneous IL-4 production from splenocytes and PP cells was extremely low level. Matsumoto et al. reported that, in comparison with BALB/c mice, T cells isolated from NC/Nga mice produced less IL-4, and splenic B cells isolated from this strain had high sensitivity to IL-4 and CD40L signal through enhanced tyrosine phosphorylation of JAK3. Therefore, hyperproduction of IgE in spite of undetectable IL-4 production in this study might be due to these characteristics of NC/Nga mice. The IgE production induced by IL-4 is strongly blocked by IFN-γ. However, the production of IFN-γ from PP cells was not affected by the oral administration of heat-killed L. brevis. Moreover, IFN-γ production from PP cells did not increase although IL-12, which is an important mediator to stimulate IFN-γ production by T cells, production from PP cells slightly increased (Table 1). Matsumoto et al. reported that in comparison with BALB/c mice, NC/Nga mice showed defective production of IFN-γ when stimulated by IL-12 because of its weak phosphorylation in STAT4. On the contrary, many studies have reported that the development of AD-like skin lesions in NC/Nga mice was associated with not only Th2 cell-mediated responses but also Th1 cell-mediated responses. Intraperitoneal administration of rIL-12 or rIFN-γ showed an exacerbating effect on the progression of skin lesions in conventional NC/Nga mice, and had no ability to suppress IgE level in serum. STAT6-deficient NC/Nga mice were also able to develop AD-like skin lesions, and IFN-γ-secreting T cells were involved in its development. For these reasons, in the present experiment, the inhibition of IgE synthesis by the oral administration of heat-killed L. brevis SBC8803 is probably not attributable to the increased production of IFN-γ.

IL-10 and TGF-β production from PP cells increased in a dose-dependent manner by the oral administration of heat-killed L. brevis SBC8803 (Fig. 6). These cytokines are reported to have immunosuppressive properties. Allergen-specific Th1- and Th2-type responses are down-regulated by these cytokines. IL-10 is reported to suppress e transcript expression and IgE production induced by IL-4. Moreover, subcutaneous injection of rTGF-β1 was reported to suppress the development of AD-like skin lesions in NC/Nga mice, which was accompanied by the reduction of IgE level in serum and down-regulation of IFN-γ production from splenocytes. In our previous study, the oral administration of L. brevis SBC8803 suppressed not only Th2-type cytokines such as IL-4, IL-5 and IL-6 but also Th1-type cytokines such as IL-12 and IFN-γ production from OVA-sensitized mouse splenocytes. Accordingly, prevention of the development of AD-like skin lesions and inhibition of IgE synthesis in NC/Nga mice by the oral administration of heat-killed L. brevis SBC8803 might be due to the down-regulation of Th1 and Th2 response through the increased secretion of IL-10 and TGF-β1.

In this study, the increase of IL-10 and TGF-β1 production was observed in PP cells but not in splenocytes by the oral administration of heat-killed L. brevis SBC8803. The normal mucosal immune system has hyporesponsiveness to enteric indigenous bacteria. Homeostasis is achieved by the induction of immunologic tolerance through the activation of immunosuppressive TGF-β/Smad signaling. Colonization of gram-positive Enterococcus faecalis, which is normally non-pathogenic enteric bacteria, was reported to initiate and perpetuate experimental colitis in IL-10-deficient mice because of its failure to inhibit TLR2-mediated proinflammatory gene expression in intestinal epithelial cells. IL-10 and TGF-β1 play a critical role in maintaining homeostasis under the coexistence with commensal enteric bacteria. The increase of IL-10 and TGF-β1 production from PP cells by the oral administration of heat-killed L. brevis SBC8803 might be attributable to the activation of this immunologic tolerance. However, the precise mechanism of IL-10 and TGF-β1 production by orally ingested heat-killed L. brevis SBC8803 re-
mains to be elucidated.

In conclusion, we investigated the inhibitory effect of oral administration of heat-killed *L. brevis* SBC8803 on the development of AD-like skin lesions induced by the periodical topical application of picryl chloride in NC/Nga mice. Ingestion of heat-killed *L. brevis* SBC8803 prevented the development of dermatitis and the increase of IgE production in a dose-dependent manner. In this study, improvement of the development of dermatitis and the increase of IgE production in a dose-dependent manner. These results clearly demonstrate that the intake of *L. brevis* SBC8803 strain is effective in preventing and alleviating the development of AD-like skin disease.

REFERENCES