**Tetragenococcus halophilus MN45 Ameliorates Development of Atopic Dermatitis in Atopic Dermatitis Model NC/Nga Mice**

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We investigated the inhibitory effects of oral administration of *Tetragenococcus halophilus* MN45 (MN45) isolated from miso on the development of atopic dermatitis (AD) using NC/Nga AD model mice. NC/Nga mice were fed a diet containing 0.05% or 0.5% MN45 (0.05% or 0.5% MN45 groups) or lacking MN45 (control group). Mice were sensitized and boosted with picryl chloride by topical application once per week. IgE production in serum, clinical score and ear thickness in both MN45 groups were significantly suppressed. In addition, IgE and IL-17 production from splenocytes from mice in both MN45 groups were significantly decreased. IL-4 production from splenocytes in the 0.5% MN45 group decreased significantly, while IL-10 production from splenocytes in the 0.05% MN45 group increased significantly. These results demonstrate that intake of MN45 is effective in preventing and alleviating the development of type-1 allergic symptoms in humans.

Keywords: *Tetragenococcus halophilus*, oral administration, anti-atopic dermatitis, IgE suppression, immune modulation

**Introduction**

Atopic dermatitis (AD) is a type-1 allergic skin disease with an incidence that has steadily increased over the last several decades worldwide (Horii *et al.*, 2007; Segawa *et al.*, 2008b; Segawa *et al.*, 2008c; Van Bever and Llanora, 2011). Type-1 allergies are caused by immunological hypersensitivity to a common substance, an allergen, and are associated with excess production of IgE antibodies against the allergen. Immune responses regulated by helper T (Th) cells are deeply involved in the mechanism of IgE production. Th cells are classified into several subtypes, including Th0, Th1, Th2, Th3, Th17, etc., which produce various cytokines (Masuda *et al.*, 2010; Romagnani, 1999; Saito *et al.*, 2010). Th cells mutually regulate and maintain an optimal balance within the immune system. When allergens enter the body, antigen-presenting cells (APCs), macrophages (Mφ) and dendritic cells (DC) engulf these allergens and present them to T (particularly Th2) cells. Activated Th2 cells then differentiate, expand and produce cytokines. Th2 cells produce interleukin (IL)-4 and induce IgE production from B cells. When antigens crosslink IgE binding to the surfaces of mast cells, the mast cells secrete chemical mediators such as histamine, which cause allergic symptoms. Type-1 allergies develop as a result of the disruption of Th1/Th2 balance represented by interferon (IFN)-γ/IL-4 value through the predominance of Th2 cells and excess IgE production (Masuda *et al.*, 2010; Segawa *et al.*, 2008a; Yoshida *et al.*, 2010).

It has been reported that some probiotics, such as lactic acid bacteria (LAB) and *Bifidobacterium*, suppress IgE production by improving the Th1/Th2 imbalance through production of Th1-type cytokines (IL-12 and IFN-γ) to ameliorate development of dermatitis (Masuda *et al.*, 2010; Yoshida *et al.*, 2010). On the other hand, it has also been reported that some probiotics decrease IgE production by suppressing excess production of Th2-type cytokine rather than by inducing production of Th1-type cytokines to ameliorate development of type-1 allergies (Iwabuchi *et al.*, 2007; Iwabuchi *et al.*, 2009; Kanzato *et al.*, 2008; Niers *et al.*, 2005; Takahashi *et al.*, 2006).

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Recent studies have indicated that Treg cells are likely to be involved in controlling the expression of allergic diseases (Francis et al., 2003; Karlsson et al., 2004; Ling et al., 2004; Niers et al., 2005). Treg cells, which have an immune suppressive function, are able to inhibit the excessive response of Th cells (Th1, Th2 and Th17 cells) through the production of IL-10 and transforming growth factor (TGF)-β (Shi and Qin, 2005; Tanabe et al., 2008). It has been reported that oral administration of one strain of probiotics alleviates the clinical symptoms of AD by enhancing IL-10 and TGF-β production (Segawa et al., 2008c). IL-17, produced by Th17 cells and other non-T cells, acts as a potent inflammatory cytokine and is related to various types of inflammation, including allergic inflammation. It has been reported that one strain of probiotics suppresses IL-17 production and may be useful in the treatment of Th17-mediated disease (Tanabe et al., 2008).

NC/Nga mice, an inbred strain established from Japanese fancy mice, develop AD-like skin lesions under conventional care or upon repeated challenge with 2,4,6-trinitrochlorobenzene (picryl chloride, PiCl; Tokyo Kasei Kogyo, Tokyo, Japan) under specific pathogen free (SPF) conditions (Kato et al., 2005; Matsuda et al., 1997; Sasakawa et al., 2001; Segawa et al., 2008c). Induced dermatitis is accompanied by elevated serum IgE levels, increased expression of Th2-type cytokines, eosinophil accumulation in the lesions and frequent scratching behavior, which are characteristic features of human AD (Segawa et al., 2008b; Segawa et al., 2008c).

We have previously shown that MN45 isolated from the process of miso fermentation significantly suppressed IgE production in peyer’s patch (PP) cells and splenocytes of ovalbumin (OVA)-induced allergic diarrhea model mice (Ohata et al., 2011). In this study, we investigated the inhibitory effects of oral administration of MN45 on IgE elevation and the development of AD induced by topical application of PiCl under SPF conditions using AD model NC/Nga mice. The inhibitory mechanisms of the development of dermatitis and IgE elevation by oral administration of MN45 were investigated by measuring Th1-type (IL-12 and IFN-γ), Th2-type (IL-4), regulatory (IL-10) and inflammatory (IL-17) cytokine production in splenocytes.

Materials and Methods

Animals Five-week-old male NC/Nga mice were purchased from Charles River Japan (Kanagawa, Japan). Mice were housed in a filter-laminar flow enclosure in a bioclean room. They were given standard laboratory rodent chow MM-3 (Funabashi Farm, Chiba, Japan) and water ad libitum, and were acclimated for 1 week before experiments. All animal experiments in the present study were conducted in accordance with the guidelines for animal experiments of Shinshu University.

Microorganisms Tetragenococcus halophilus MN45 strain (MN45) isolated from miso was used in this study. This strain was incubated in de Man, Rogosa and Sharpe (MRS) broth containing 15% NaCl at 30°C for 2 days, harvested by centrifugation at 2,000 rpm for 15 min, washed three times with phosphate buffered saline (PBS), and then killed at 100°C for 10 min. These microorganisms were then freeze dried.

In vivo experiments AD-like skin lesions were induced by repeated topical application with PiCl. Eight-week-old male NC/Nga mice were sensitized with 150 μL 5% PiCl dissolved in 99.5% ethanol and acetone mixture (4:1) by topical application onto foot pads and shaved abdomen. Four days after the first sensitization, 15 μL of 1% PiCl dissolved in olive oil was applied to each side of the ears once per week for a total of nine times. Mice (n = 7 per group) were fed an MM-3 diet containing 0%, 0.05% or 0.5% MN45 from 2 weeks before the first sensitization (Day 0) to the end of the study (Day 82) (Fig. 1). Clinical skin severity scores, ear thickness, and total IgE concentration in serum were periodically examined throughout the study period. Dermatitis symptoms were evaluated according to the scoring method described by Matsuda et al. (Matsuda et al., 1997). Edema was evaluated by measuring ear thickness and 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) or 3 (severe).
(severe) for each of the five symptoms (redness/hemorrhage, edema, acedia/excoriation, dryness and anthema). Ear thickness was measured by using a micrometer (Mitsutoyo Corp., Kanagawa, Japan). IgE in serum collected from the tail at various time points was measured by ELISA. Cytokine (IL-4, IL-10, IL-12, IL-17 and IFN-γ) and IgE production by splenocytes from the mice were also measured. Splenocytes were harvested on day 3 to measure cytokine (IL-4, IL-10, IL-12, IL-17 and IFN-γ) production and on day 14 to measure IgE production. Concentrations of cytokines and IgE in the supernatants were determined by sandwich ELISA.

**ELISA** Measurement of IL-4, IL-10, IL-12, IL-17, IFN-γ and IgE in the culture supernatants and measurement of total IgE in serum were performed by sandwich ELISA. Monoclonal antibody mouse IL-4 (clone 11B11; Mabtec, Nacka Strand, Sweden), purified anti-mouse IL-10 (Biologend, San Diego, CA, USA), purified rat anti-mouse IL-12 (p40/p70) monoclonal antibody (cloneC15.6; BD Biosciences, San Diego, CA, USA), rabbit (polyclonal) anti-mouse/rat IFN-γ (Biosource, Camarillo, CA, USA) or anti-IgE (LO-ME-2 purified; EIU, Belgium) was coated on the wells of a 96-well ELISA plate (Nunc, Roskilde, Denmark) using carbonate buffer, and this was incubated overnight at 4°C. After blocking the unoccupied sites on the plate with bovine serum albumin (BSA), test sample and standard recombinant mouse recombinant murine IL-4 (Strathmann Biotec, Hanover, Germany), recombinant mouse IL-10 (Biologend), recombinant mouse IL-12 (Techne Corporation, MN, USA), recombinant murine IFN-γ (Biosource) or purified mouse IgEκ monoclonal (BD Biosciences) was added. Subsequently, for the determination of cytokines, biotin-conjugated antibody mouse monoclonal IL-4 (clone BVD6-24G2; Mabtec), biotinylated anti-mouse IL-10 (Biolegend), biotinylated rat anti-mouse IL-12 (p40/p70) monoclonal antibody (clone C15.8; BD Biosciences), mouse (monoclonal) anti-rat/mouse IFN-γ biotin conjugate (clone DB-1; BD Biosciences) or biotinylated anti-IgE (LO-ME-2 biotin; Techopharm Biotechnology, Frankfurt, Germany) was used. Streptavidin-horseradish peroxidase conjugate was added to each well. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added to the wells and then the reaction was stopped by 1M sulfuric acid. The absorbance of the contents of the wells was read at 450 nm on a Microplate Reader (Bio-Rad Laborato-

The concentration of IL-17 was measured using mouse IL-17 kit (R&D Systems, USA).

**Histological analysis** Mouse ears were removed on the final day of the experiment (Day 82). Each ear was fixed in a 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque, Kyoto, Japan), embedded in paraffin, cut into 3-mm sections, and stained with hematoxylin and eosin.

**Statistical analysis** All values are expressed as means ± standard deviation (SD). Statistical evaluation of the differences in clinical score was performed by Steel’s test. Statistical evaluations of the difference in ear thickness, total IgE in serum and various cytokines and IgE production by splenocytes were performed by Dunnett’s test. Probability values of less than 0.05 were considered to be statistically significant.

**Results**

**Effects of oral administration of MN45 on development of dermatitis** Figure 2 shows the changes in clinical skin severity score and ear thickness in NC/Nga mice with ag-

**Fig. 2.** Effects of oral administration of MN45 on clinical skin score and ear thickness in NC/Nga mice induced by topical application of PiCl. Clinical skin score (A) (full score, 15) and ear thickness (B) was assessed once per week. Each value represents the mean ± SD, n = 7. Asterisks indicate significant differences vs. controls (Cont). * p < 0.05.
slight degree of acomia were observed (Fig. 3B). Histological analysis of ear skin was performed, and typical photographs are shown in Fig. 4. In comparison with the control group, the MN45 fed group showed less ear thickening. This clearly demonstrates that oral administration of MN45 is effective in preventing the development of dermatitis induced by topical application of PiCl in a dose-dependent manner.

Effects of oral administration of MN45 on total IgE levels in serum

It has been reported that the clinical severity of type-1-allergies is associated with increased serum IgE levels in mice (Masuda et al., 2010; Matsuda et al., 1997). Therefore, IgE concentration in serum was measured. As shown in Fig. 5, total IgE levels in the serum in the control group increased from Day 61 after topical application of PiCl, and

Fig. 3. Symptoms of dermatitis in NC/Nga mice. Clinical features of AD-like skin lesions on the face and ears on Day 82. Representative mice from the control group (A), the 0.05% MN45-fed group (B), the 0.5% MN45-fed group (C) are shown.

Fig. 4. Histological features of skin lesions in NC/Nga mice. Ear sections from the control group (A), the 0.05% MN45-fed group (B) and the 0.5% MN45-fed group (C) were stained with hematoxylin and eosin (Day 82).
IgE levels in serum and prevented the development of AD-like skin lesions, clinical skin severity and ear swelling in NC/Nga mice induced by periodical application of PiCl under SPF conditions (Figs. 2, 3, 4 and 5). Th1 type cytokines (IL-12 and IFN-γ) did not increase, but decreased significantly in splenocytes of NC/Nga mice fed MN45 (Fig. 6A, B). Production of IL-4 was suppressed significantly in splenocytes of NC/Nga mice fed 0.5% MN45 (Fig. 6C). Although continued to increase until Day 82. On the other hand, in the 0.5% and 0.05% MN45-fed groups, the increase in total IgE levels in serum was suppressed significantly. These results indicate a relationship between total IgE levels in serum and the progress of dermatitis (Figs. 2, 3, 4 and 5).

**Effects of MN45 ingestion on cytokines and IgE production from splenocytes of NC/Nga mice**  
In order to analyze the suppressive mechanism of MN45 on the development of dermatitis in NC/Nga mice, cytokine and IgE production from splenocytes in NC/Nga mice were measured. At Day 82, splenocytes prepared from each group were cultured for 3 days to measure IL-4, IL-10, IL-12, IL-17 and IFN-γ production, and for 14 days to measure IgE production. As shown in Fig. 6A, IL-12 (Th1-type cytokine) production decreased significantly in the 0.05% and 0.5% MN45-fed groups. IFN-γ (Th1-type cytokine) production decreased significantly in the 0.5% MN45-fed group (Fig. 6B). IL-4 (Th2-type cytokine) production decreased significantly in the 0.5% MN45-fed group (Fig. 6C) and IgE production decreased significantly in the 0.05% and 0.5% MN45-fed groups (Fig. 6D). As shown in Fig. 7, production of IL-10, which is an anti-inflammatory cytokine increased significantly in the 0.05% MN45-fed group and tended to increased in the 0.5% MN45-fed group. Inflammatory cytokine (IL-17) production decreased significantly in the 0.05% and 0.5% MN45-fed groups (Fig. 8).

**Discussion**  
In this study, the oral administration of MN45 decreased

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**Fig. 5.** Effects of oral administration of MN45 on serum IgE levels of NC/Nga mice.  
Serum was collected once every two weeks from NC/Nga mice after first challenge with PiCl. Concentrations of serum IgE were determined by ELISA. Each value represents the mean ± SD, n = 7. Asterisks indicate significant differences vs. controls (cont). * p < 0.05.

**Fig. 6.** Effects of oral administration of MN45 on various cytokines and total-IgE production from splenocytes prepared from NC/Nga mice.  
Eighty-two days after the start of the experiment, splenocytes prepared from NC/Nga mice were cultured at 37°C under 5% CO₂ for 3 days to measure cytokine (IL-4, IL-12 and IFN-γ) production and for 14 days to measure IgE production. Cytokines and IgE concentrations in the supernatant were determined by ELISA. Each value represents the mean ± SD, n = 7. Asterisks indicate significant differences vs. controls (Cont). * p < 0.05.
MN45 (Fig. 7). IL-10 is an anti-inflammatory cytokine and has immunosuppressive properties. It has been reported that IL-10 suppresses ε transcript expression and IgE production induced by IL-4 (Jeannin et al., 1998; Segawa et al., 2008c). It has also been reported that subcutaneous injection of TGF-β induced by IL-10 suppresses the development of AD-like skin lesions in NC/Nga mice and is accompanied by reductions in IgE levels in serum (Segawa et al., 2008c; Sumiyoshi et al., 2002). Accordingly, the increase in IL-10 production by oral administration of MN45 may induce TGF-β and be involved in the reduction of IgE synthesis and the amelioration of AD-like skin lesions in NC/Nga mice.

In this study, the oral administration of MN45 did not increase IFN-γ/IL-4 production, production of IgE was suppressed significantly (Fig. 6D). Therefore, we believe that the suppression of IgE production do not depend on the improvement of Th1/Th2 imbalance.

IL-4 is an important factor for immunoglobulin class-switching to IgE. IgE production induced by IL-4 is strongly blocked by Th1-type cytokine IFN-γ (Del et al., 1988; Pène et al., 1998; Segawa et al., 2008c). It has been reported that some probiotics inhibit IgE production through improvement of Th1/Th2 imbalance by inducing production of Th1-type cytokines and suppressing production of Th2-type cytokine (IL-4) (Masuda et al., 2010; Yoshida et al., 2010). On the other hand, it has also been reported that some probiotics suppress the excess production of IgE and the development of allergic inflammation through the suppression of excessive Th2 cell response rather than by inducing production of Th1-type cytokines (Iwabuchi et al., 2007; Iwabuchi et al., 2009; Kanzato et al., 2008; Niers et al., 2005; Takahashi et al., 2006). It has been reported that one strain of Bifidobacterium suppresses the production of Th2 cell-attracting chemokines and that one strain of LAB induces Th2 cell apoptosis (Iwabuchi et al., 2009; Kanzato et al., 2008). Oral administration of MN45 may suppress the production of Th2-type cytokines (IL-4) from splenocytes of NC/Nga mice through these mechanisms.

In this study, an increase in IL-10 production from splenocytes was observed following oral administration of MN45 (Fig. 7). IL-10 is an anti-inflammatory cytokine and has immunosuppressive properties. It has been reported that IL-10 suppresses ε transcript expression and IgE production induced by IL-4 (Jeannin et al., 1998; Segawa et al., 2008c). It has also been reported that subcutaneous injection of TGF-β induced by IL-10 suppresses the development of AD-like skin lesions in NC/Nga mice and is accompanied by reductions in IgE levels in serum (Segawa et al., 2008c; Sumiyoshi et al., 2002). Accordingly, the increase in IL-10 production by oral administration of MN45 may induce TGF-β and be involved in the reduction of IgE synthesis and the amelioration of AD-like skin lesions in NC/Nga mice.

In this study, the oral administration of MN45 suppressed IL-17 production (Fig. 8). IL-17, an inflammatory cytokine, is produced by Th17 cells and causes tissue inflammation by inducing the expression of proinflammatory cytokines and chemokines. IL-17 is also involved in the proliferation, maturation and chemotaxis of neutrophils (Tanabe et al., 2008). Tanabe et al. reported that B. infantis suppresses IL-17 production by inducing the immunoregulatory cytokine IL-10 (Tanabe et al., 2008). In this study, suppression of IL-17 production by splenocytes after oral administration of MN45 may be attributed to the increase in IL-10 production, which may ameliorate AD.

It has recently been reported that Treg cells play a key role in regulating immune systems, and that the proliferation of Treg cells may be an important mechanism involved in the suppression of excessive Th cell response (Francis et al.,
References


