Original paper

Effect of Oral Administration of the IgE-suppressive Wild Yeast Strain *Saccharomyces paradoxus* P01 on the Development of Atopic Dermatitis-like Symptoms in NC/Nga Mice

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The oral intake of probiotic microorganisms has been investigated as a potential approach to prevent IgE-associated allergic diseases, including atopic dermatitis (AD). However, the effects of potentially edible wild yeast strains of the genus *Saccharomyces* on allergic symptoms remain poorly understood. In this study, we investigated the effects in the NC/Nga AD mouse model of *Saccharomyces paradoxus* P01 (P01), a wild strain selected based on its suppressive effect on IgE production in vitro. Compared to animals on a P01-free diet, mice fed a P01-supplemented diet had a markedly reduced incidence of skin lesions and picryl chloride-induced ear thickening and inflammation. Serum IgE levels and IgE production by spleen cells in mice fed the P01-supplemented diet were significantly lower than those of control mice. These results suggest that oral intake of specific IgE-suppressive wild strains of *Saccharomyces* yeast may be effective in suppressing IgE production and alleviating IgE-associated allergic symptoms.

Keywords: *Saccharomyces*, probiotics, atopic dermatitis, anti-allergic, IgE, IL-12

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with intense pruritus and a series of exacerbations and remissions (Williams et al., 2000). The prevalence of AD has risen dramatically in recent years in both developed and developing countries (Pawankar et al., 2013). AD is mainly characterized by IgE-associated immunologic reactions in response to allergen exposure (Liu et al., 2011). In addition, many studies have suggested that T-cell-mediated delayed hypersensitivity was also involved in the pathological state of AD (Werfel and Wittmann, 2008). Therefore, AD is recognized as a type I and partially type IV allergy as per the Coombs and Gell classification. Human AD is thought to be caused by the complex interaction among defects in skin barrier function, immune abnormalities, and environmental and infectious agents (Barnes, 2010). Although studies have been conducted to develop new treatments for individuals suffering from AD, its pathophysiology remains incompletely elucidated, and patients rely primarily on supportive care involving long-term use of topical corticosteroids (Simpson, 2010). Given that such treatments are generally very expensive and sometimes have adverse side effects, the development of therapeutic options, such as anti-allergic food ingredients, is desirable.

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Various dietary supplements have been investigated for the treatment of AD. Among them, the use of probiotics is a unique approach. Probiotics are broadly defined as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host (Salminen et al., 1999). Probiotic microorganisms prevent allergic symptoms by improving the intestinal microbial environment and/or directly modulating the host immune system (Kuitunen and Boyle, 2012). The principal requirement for anti-allergic probiotics is the absence of side effects, and most microorganisms used as probiotics are derived from normal gut flora, such as lactic acid bacteria and bifidobacteria. Accordingly, the usage of other microorganisms as probiotics remains limited (Kim and Ji, 2012).

We previously reported that specific non-industrial Saccharomyces cerevisiae and Saccharomyces paradoxus yeast strains obtained from fruits and soil suppressed IgE production from mouse splenic B cells (Kawahara et al., 2013). In contrast to the above-mentioned probiotics, Saccharomyces yeasts are not typically present in the normal gut flora. Industrial strains of Saccharomyces, particularly S. cerevisiae, are important microorganisms in the manufacturing of foods for humans because of their capacity to metabolize sugar into ethanol and CO₂, and thus they are frequently used to produce various foods and alcoholic beverages (Sicard and Legras, 2011). S. paradoxus, a close relative of S. cerevisiae, is also commonly used to introduce aromatic compounds during the production of food and alcoholic beverages (Majdak et al., 2002; Orlic et al., 2007). Among the Saccharomyces yeast strains, those isolated from natural sources are frequently referred to as “wild” yeasts in contrast to “domesticated” industrial yeasts. The physiological effects of Saccharomyces yeast strains on human health are poorly understood, except for those of S. cerevisiae var. boulardii (S. boulardii), the only probiotic strain reported to modulate both innate and adaptive host immunity by enhancing secretory IgA production (Qamar et al., 2001; Rodrigues et al., 2000; Moslehi-Jenabian et al., 2010).

The Saccharomyces yeast cell wall component zymosan is one of the pathogen-associated molecular patterns (PAMPs). Zymosan consists mainly of β1,3-glucan with β1,6-linked branches, and is recognized by both Toll-like receptor (TLR)2/6 and CD14 in cooperation with dendritic cell-associated lectin-1 (Dectin-1) (di Carlo and Fiore, 1958; Ganttner et al., 2003; Ozinsky et al., 2000). Although β1,3-glucans and β1,6-glucans are typically present in the inner layer of the fungal cell wall, they are exposed at the cell surface in budding scars (Wheeler and Fink, 2006). The heat killing process exposes the β-glucan-containing structures on the intact yeast body and increases the immune-enhancing activity of the yeast (Gow et al., 2007). Therefore, oral intake of heat-killed wild Saccharomyces strains may affect the human immune system, and we considered that heat-killed IgE-suppressive Saccharomyces yeast strains may have potential as probiotics to prevent allergic symptoms in vivo.

In the present study, we investigated whether oral administration of Saccharomyces paradoxus P01, the most IgE-suppressive strain in our previous study, improves IgE-associated allergic symptoms in picryl chloride (PiCl)-treated NC/Nga mice, a model of AD. There is a close relationship between plasma total IgE levels and the development of skin lesions in NC/Nga mice (Matsumoto et al., 1999). IgE activates key effector cell types involved in allergic inflammation and plays a general role in the allergic response (Zheng et al., 2011). In addition, T-cell-dependent skin lesions, such as type IV allergy induced by haptens (e.g., PiCl) are known to be exacerbated by the antigen-independent effects of IgE (Bryce et al., 2004). Therefore, serum IgE levels were measured to investigate the effect of the P01 strain on IgE production.

Materials and Methods

Microorganisms The Saccharomyces paradoxus P01 strain, isolated in our previous study (Kawahara et al., 2013), was propagated in yeast-peptone-sucrose medium (1% yeast extract, 2% peptone, and 2% sucrose; pH 5.8) at 29°C under aerobic conditions. The MM-3 diet supplemented with 0.05% (wt/wt) heat (90°C)-killed P01 strain (P01 strain-supplemented diet) was produced by the Funabashi Farm (Chiba, Japan). A standard MM-3 diet without the P01 strain, prepared using the same process, was employed as the control diet.

Mice Specific pathogen-free male NC/Nga mice aged 5 weeks were purchased from Oriental Yeast (Tokyo, Japan) and housed at 23 ± 3°C under a 12-h light/dark cycle. All of the animal protocols used in this study were approved by the Committee for Animal Experiments of Shinshu University.

Feeding program Sixteen NC/Nga mice were acclimatized for 7 days and given free access to the MM-3 diet and water. They were then assigned to two groups consisting of 8 mice each on the basis of their body weights, and then fed the control diet or the P01 strain-supplemented diet ad libitum for 138 days.

Induction of AD-like skin lesions AD-like skin lesions were induced by repeated topical application of picryl chloride (PiCl; Tokyo Kasei, Tokyo, Japan) according to the method described by Segawa et al. (2008), with the following modifications. Briefly, 150 μL of 5% PiCl dissolved in an ethanol and acetone mixture (4:1) was applied to the abdomen of the mice on day 21 to establish sensitization. At 5 days after sensitization, 15 μL of 1% PiCl (P01 strain-supplemented diet) was produced by the Funabashi Farm (Chiba, Japan). A standard MM-3 diet without the P01 strain, prepared using the same process, was employed as the control diet.

Scoring of dermatitis Throughout the experimental period, clinical skin severity scores, body weight, and ear thickness were recorded weekly. The total clinical severity of the five symptoms (erythema/hemorrhage, edema, acoma/excoriation, dryness, and antherma) was calculated from the sum of the individual scores graded as 0 (no symptoms), 1 (mild), 2 (moderate), and 3 (severe) for each symptom. Ear thickness was measured for the right ear of...
Fig. 1. Experimental schedule for the induction of atopic dermatitis (AD)-like dermatitis in NC/Nga mice.

the mice using a digital caliper.

**Sampling of blood and organs** Blood samples were collected from the caudal vein of the mice at day 0 and at 14-day intervals from day 26 to day 138. The collected blood samples were stored overnight at 4°C to allow clotting and then separated by centrifugation at 500 × g for 30 min to obtain serum. The serum was stored at –80°C until use.

After the end of the experiment, the mice were euthanized using the vertebral dislocation method. The spleen was aseptically collected from the mice to evaluate the IgE productivity of the immune cells. The ears of the mice were collected for histopathological study. The livers and kidneys of the mice were harvested to evaluate the change in weight resulting from harmful side effects.

**Histopathological studies** The collected ears were immediately fixed in 10% neutral buffered formalin and left in the formalin for 48 h. The fixed ear tissues were sliced and embedded in paraffin, sectioned, deparaffinized and rehydrated using standard techniques by the Biopathology Institute (Oita, Japan). The sections were subjected to hematoxylin and eosin staining to evaluate edema. Histopathological changes were examined using light microscopy. An arbitrary score was assigned to each microscopic field viewed at a magnification of 200×.

**Preparation and cultivation of immune cells** Single-cell suspensions from the spleen were prepared by gently mincing the tissue in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan). To remove red blood cells, spleen cells were treated with hemolytic buffer (17 mM Tris-HCl buffer containing 0.144 M ammonium chloride; pH 7.2) for 5 min at room temperature. Cells were then washed with RPMI 1640 medium followed by centrifugation to remove the hemolytic buffer. Pelleted cells were designated as splenic cells and resuspended in fresh RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and a mixture of 100 IU/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA, USA).

Splenic cells were seeded at a density of 5 × 10⁶ viable cells/mL in Falcon 96-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ, USA) and cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air. The supernatants were collected on day 3 and day 7 for measurement of IL-12 and total IgE, respectively, according to the method described by Ohata *et al.* (2011), with the following modifications. Culture supernatants were collected and stored at –80°C until use.

**Enzyme-linked immunosorbent assay (ELISA)** Total IgE and IL-12 levels were measured using sandwich ELISA. In total, 50 μL of primary antibody dissolved in 0.1 M carbonate buffer (pH 10.0) was added to each well of a 96-well Nunc Immuno Plate MaxiSorp (Thermo Fisher Scientific, Roskilde, Denmark) and incubated at 37°C for 90 min. The concentrations of the primary antibodies for IgE and IL-12 measurement were 10 μg/mL goat anti-mouse IgE (Bethyl Laboratories, Montgomery, AL, USA) and 10 μg/mL rat anti-mouse IL-12 p40/70 (BD Pharmingen, San Diego, CA, USA). Each well was washed 3 times with phosphate-buffered saline (PBS; pH 7.2) containing 0.05% Tween 20 (PBST) and then post-coated with 300 μL of Block Ace (DS Pharma Biomedical, Osaka, Japan) in 0.1 M sodium carbonate buffer (pH 10.0) at 4°C overnight. After the plates were washed 3 times with PBST, 50 μL of the culture supernatant or a standard solution diluted optimally with PBS was added to each well and incubated at 37°C for 60 min. For measurement of IgE and IL-12, mouse IgE (Sigma Aldrich, St. Louis, MO, USA) or recombinant murine IL-12 (PeproTech, Rocky Hill, NJ, USA) was used as the standard, respectively. The plates were then washed 5 times with PBST. Next, 100 μL of the second antibody dissolved in PBS was added to each well and incubated at 25°C for 60 min. The concentrations of the secondary antibodies were 2 μg/mL horseradish peroxidase-conjugated goat anti-mouse IgE (clone LO-ME-3; GeneTex, Irvine, CA, USA) and 2 μg/mL biotin-conjugated rat anti-mouse IL-12 p40/70 (BD Pharmingen). For IL-12, after 5 washes with PBST, the wells were filled with 100 μL of 2 μg/mL horseradish peroxidase-conjugated streptavidin (BD Pharmingen) and incubated at 25°C for 60 min. The wells were washed 5 times with...
PBST, after which 100 μL of TMB Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was added to each well, and the plate was incubated at 25°C for 15 min. The reaction was stopped by adding 100 μL of 1 M phosphoric acid. The absorbance at 450 nm was measured on an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis Data were statistically analyzed using a two-sided Mann-Whitney’s U test or a two-sided Student’s t-test. P-values less than 0.05 were considered statistically significant.

Results
Effect of oral administration of the P01 strain on AD-like skin lesions in NC/Nga mice NC/Nga mice exhibited progressive AD-like skin lesions from day 54 of the experiment onwards. Acumia appeared first, followed by mild dryness, erythema, hemorrhage and edema. Relative to the control diet group, the clinical severity score in the P01-supplemented group was significantly lower on and after day 68 of the experiment (Fig. 2).

Effect of oral administration of the P01 strain on the development of PiCl-induced inflammation in the ears of NC/Nga mice Repeated application of PiCl in mice is known to induce an immediate hypersensitivity response followed by a late reaction (Kitagaki et al., 1995). In response to repeated PiCl application, the ears of NC/Nga mice exhibited progressive thickening beginning after the first application on day 35. Relative to the control diet group, ear thickening was significantly reduced in the P01 strain-supplemented diet group on or after day 68 (Fig. 3A and 3B).

Next, histological analysis was performed to determine the effects of P01 diet supplementation on PiCl-induced allergic symptoms (Fig. 3C). Relative to the control diet group, outer skin thickening and hyperplasia of collagen fibers and cartilage were reduced in the ears of mice fed the P01-supplemented diet.

Effect of oral administration of the P01 strain on serum total IgE levels in NC/Nga mice Total IgE levels were increased after day 40 of the experiment. IgE levels tended to be lower in mice fed the P01-supplemented diet than in the control group on or after day 82 (Fig. 4). On day 124 and day 138, the IgE scores of the P01 strain-supplemented diet group were 686.8 ± 75.8 ng/mL and 537.7 ± 53.8 ng/mL, respectively, which were significantly lower (P < 0.001) than those of the control group (1,057.1 ± 79.6 ng/mL and 1,054.2 ± 158.6 ng/mL, respectively).

Effect of oral administration of the P01 strain on IgE and IL-12 production by splenic cells In mice fed the P01 strain-supplemented diet, IgE production by splenic cells (4.96 ± 1.0 ng/mL) was significantly decreased relative to that in control mice (7.9 ± 1.7 ng/mL; Fig. 5A).

Next, we investigated the effect of P01 supplementation on IL-12 production by splenic cells. IL-12 production by splenic cells from mice fed the P01-supplemented diet and the control diet was 476.4 ± 6.1 pg/mL and 440.0 ± 2.8 pg/mL, respectively (Fig. 5B).

Discussion
In this study, we investigated the effect of dietary supplementation with the heat-killed IgE-suppressive P01 strain on PiCl-treated NC/Nga mice. The NC/Nga mouse was established as an inbred strain that spontaneously suffers from dermatitis under conventional housing conditions (Matsuda et al., 1997). Repeated application of PiCl develops AD-like skin lesions in NC/Nga mice (Okada et al., 2000).

Oral administration of the P01 strain reduced both the dermatitis score (Fig. 2) and PiCl-induced ear thickening (Fig. 3A).
Serum IgE levels in mice fed the P01-supplemented diet were significantly reduced (Fig. 4). Given the role of IgE in the immediate phase allergic reaction and as an enhancer of hapten-induced antigen-independent effects, the attenuation of allergic symptoms in NC/Nga mice by oral administration of the P01 strain...
can be attributed to the suppression of IgE production. In fact, IgE production by splenic cells, which reflects the systemic immune response, was significantly decreased in mice fed the P01-supplemented diet (Fig. 5A). We also evaluated the effect of the P01 strain on IgA production from mouse splenic cells. As a result, IgA production was not enhanced by the P01 strain (Data not shown), suggesting that the suppressive effects of the P01 strain on AD did not occur through IgA enhancement and were different from the known IgA-enhancing effects of *S. boulardii*.

Our previous study indicated that the yeast cell wall component zymosan has IgE-suppressive and IL-12 enhancing effects similar to those of the P01 strain (Kawahara et al., 2013). A recent study demonstrated the protective effects of agents containing PAMPs in AD and other allergic conditions (Heederik and von Mutius, 2012). In particular, ligands for TLR2 or Dectin-1 have been reported to modulate Th1 and Th2 responses, and may serve as therapeutic agents against allergic diseases (Lin et al., 2013; Patel et al., 2005). Oral ingestion of a TLR2/6 agonist has been reported to alleviate inflammatory symptoms by rebalancing Th2-skewed immune responses (Fuchs et al., 2010; Knothe et al., 2011). Therefore, to determine whether the IL-12-enhancing effects of the P01 strain were effective in vivo, the IL-12 production of splenic cells was
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Although the yeast strains, such as P01, are

Beta-glucan by

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safety of oral administration of these wild isolates on

therapeutic probiotics to alleviate IgE-associated allergic potential candidates as anti-allergic food components and novel suppressive wild strains will need to be carefully evaluated in several models.

vivo risk of infection can be reduced by using heat-killed yeast, the immune-deficient patients (de Llanos et al., 2006). A comparison of clinical and food *Saccharomyces cerevisiae* isolates on the basis of potential virulence factors. *Antonie Van Leeuwenhoek*, 90, 221-231.


