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Note

Effects of Hanabiratake (*Sparassis crispa*) on Allergic Rhinitis in OVA-Sensitized Mice

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The anti-rhinitis properties of *Sparassis crispa* were investigated in mice. To examine the immunomodulative activity of oral administration of *S. crispa*, splenocytes obtained from ovalbumin-sensitized BALB/c mice fed *S. crispa* were restimulated *in vitro* with the same antigen. Oral administration of *S. crispa* induced IFN-γ, but inhibited IL-4 and IL-5 secretion, and suppressed ovalbumin-specific IgE secretion by ovalbumin-stimulated splenocytes. The effects of *S. crispa* were further investigated by using the allergic rhinitis model in BALB/c mice. Nasal symptoms, sneezing and nasal rubbing induced by ovalbumin challenges were inhibited by oral administration of *S. crispa* in a dose-dependent manner. Furthermore, ovalbumin-specific serum IgE levels were diminished in this model. These results demonstrated that *S. crispa* may be effective in suppressing symptoms of allergic rhinitis through its immunomodulating activities.

Keywords: allergic rhinitis, cytokines, hanabiratake, ovalbumin-specific IgE, *Sparassis crispa*

Introduction

Type I allergies, including pollinosis, allergic rhinitis, atopic dermatitis and asthma, are characterized by an elevated production of IgE and mast cell degranulation that result in release of histamine as well as other chemical mediators of allergy (Platts-Mills, 2001). The production of IgE from B cells is regulated by T-helper (Th) cells, which have been classified into Th1 and Th2 subtype (Mosmann *et al.*, 1986). Th2 cells synthesize IL-4, which enhances the IgE production by B cells through inducing IgE isotype class switching (Coffman *et al.*, 1986) and the proliferation of Th2 cells (Le *et al.*, 1990; Swain *et al.*, 1990). Th2 cells also synthesize IL-5 which enhances the IL-4-dependent IgE production (Pene *et al.*, 1988). Therefore, Th2 cells and IL-4 are considered to be critical for IgE production. Conversely, IFN-γ produced by Th1 cells suppresses IgE production both by interfering with the IL-4-derived isotype class switching (Thyphronitis *et al.*, 1989) and by inhibiting the proliferation of Th2 cells (Gajewski and Fitch, 1988). IL-12 produced by antigen-presenting cells, such as dendritic cells and macrophages, is known to stimulate both NK and Th1 cells to make them produce IFN-γ. These two Th1-type cytokines, IL-12 and IFN-γ, enhance the proliferation of Th1 cells (Belosevic *et al.*, 1989; Hsieh *et al.*, 1993). It is generally accepted that enhancement of Th2-mediated immunity causes IgE-dependent allergic diseases. Therefore, properly regulating the balance between Th1- and Th2-type immune responses to heterogeneous antigens is considered to be an important mechanism in the prevention and therapy of the diseases mentioned earlier. In fact, it has been indicated that oral administration of some *lactobacillus* can modulate the host Th1/Th2 balance and decrease serum IgE levels (Fujiwara *et al.*, 2004; Kimoto *et al.*, 2004). Furthermore, oral administration of the extract from Hatakeshimeji (*Lyophyllum decastes*) mushroom can modulate the cytokine production from splenocytes and decrease serum IgE levels in NC/Nga mice (Ukawa *et al.*, 2007).

Sparassis crispa, known as hanabiratake in Japanese, is a tasty edible, medicinal fungus. *S. crispa* has recently become cultivable in Japan. Previously, we reported that both serum IgE levels and the scratching amount of NC/Nga mice that were induced dermatitis by a continuous application of hapten are reduced by oral administration of *S. crispa* (Hasegawa *et al.*, 2004). Furthermore, a branched beta-glucan from *S. crispa* can induce IFN-γ in DBA/2 mice (Harada *et al.*, 2002). However, the effects of oral administration of *S. crispa* on Th1/Th2 balance and allergic rhinitis have not yet been clarified.

In the present study, we examined the effects of oral

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administration of *S. crispa* on allergen-induced IgE and cytokines production by murine splenocytes, which were obtained from ovalbumin (OVA)-sensitized BALB/c mice fed either with or without *S. crispa*. In addition, we examined the effects of *S. crispa* on allergen-specific serum IgE levels and symptoms by the murine allergic rhinitis model.

Materials and Methods

S. crispa sample preparation Fruiting bodies of *S. crispa* were cultivated in the Central Research Laboratories, Unitika (Kyoto, Japan). The *S. crispa* was freeze-dried and ground into ultrafine powder by a mill. The average diameter of the powder was 8 μm. The endotoxin content of the powder was below the detection limit of the Endospecy ES-50M kit (Seikagaku, Tokyo, Japan).

Animals Female BALB/c mice (6 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The animals were housed in an air-conditioned room maintained at 23 ± 2°C and a relative humidity of 55 ± 15% with a 12 h light/dark cycle (8:00 - 20:00). They were given a CRF-1 diet (Oriental Yeast, Tokyo, Japan) and water *ad libitum* for at least 1 week before the experiments. Experiments were performed according to the Guidelines for the Care and Use of Experimental Animals of the Japanese Association for Laboratory Animals.

Reagents The reagents used in the experiments include OVA (Sigma, St. Louis, MO, USA), alum (LSL Co., Tokyo, Japan) and *B. pertussis* (Sigma).

Measurement of ovalbumin-induced cytokines and ovalbumin-specific IgE The effects of oral administration of *S. crispa* on the antigen-induced production of cytokines and the antigen-specific IgE were investigated using a culture system with OVA-stimulated splenocytes derived from mice fed *S. crispa* as described by Fujiwara *et al.* (2004) with some modifications. BALB/c mice (n = 8 per group) were intraperitoneally sensitized with 20 μg of OVA absorbed onto 2 mg of alum in 0.1 ml sterile saline. Intraperitoneal injections were given twice on day 0 and day 14. They were fed a diet containing 0.25% *S. crispa* for 21 days after the first sensitization. The mean food intake was about 3 g/day per mouse, indicating that each mouse was fed approximately 7.2 mg/day of *S. crispa*. The control group was fed a normal diet without *S. crispa*. Mice were sacrificed on day 21 to obtain splenocytes. After depletion of erythrocytes, the cells (2.5×10^6/ml) were cultured with OVA (100 μg/ml) in 0.2 ml of RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with heat-inactivated fetal bovine serum (100 ml/l, Invitrogen, CA, USA), penicillin (50 U/ml), and streptomycin (50 μg/ml) in a 96-well culture plate. Supernatants were collected on day 28 for the cytokine assay and stored at –80°C for further analysis. To monitor OVA-specific IgE production, we slightly modified the culture system (Shida *et al.*, 1998). Splenocytes were cultured in 1 ml culture medium with OVA for 3 days in a 48-well plate. The cultured cells were harvested, washed to remove OVA, and cultured without OVA for a further 7 days. Supernatants were collected for the OVA-specific IgE assay and stored at –80°C for further analysis.

ELISA for OVA-specific IgE and cytokines Measurement of OVA-specific IgE was performed by sandwich ELISA as described by Ishida *et al.* (2003) with some modifications. For measurement of OVA-specific IgE in mice sera and in culture supernatants, a purified goat anti-mouse IgE antibody affinity (Bethy Laboratories, Inc., Montgomery, TX, USA) was diluted with coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) to 10 μg/ml. Then, 0.1 ml of diluted antibody was soaked into each well of a 96-well Maxisorp immunoplate (Nunc, Roskilde, Denmark), and was incubated for 60 min. The microplate was washed 3 times with the washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and blocked with blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 30 min. After the microplate was washed 3 times with the washing solution, 0.1 ml of diluted samples and standard (mouse anti-OVA-specific IgE, MCA2259, Serotec, Oxford, UK) were added to each well, and incubated for 120 min. Sera samples were diluted with sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05 % Tween 20, pH 8.0) and supernatants of the culture were diluted with complete RPMI 1640 medium without OVA. After 3 washes with the washing solution, biotinylated OVA was diluted with sample diluent (20 μg/ml) and 0.1 ml was added to each well. Biotinylated OVA was prepared by ECL protein biotinylation module (Amersham, Buckinghamshire, UK) and the plate was incubated for 60 min. After 3 washes with the washing solution, 0.1 ml of streptavidin-peroxidase adjusted to 0.5 μg/ml with sample diluent was added to each well and incubated for 60 min. After 5 washes with the washing solution, 0.1 ml of TMB reagent (R&D systems, MN, USA) was added to the each well, followed by incubation for 30 min in the dark. Then, 0.1 ml of 2 M H_2SO_4 was added to each well and absorbance at 450 and 550 nm was measured using a microplate reader. The value of (OD_{450} - OD_{550}) was used for the specific absorbance from TMB.

IFN-γ, IL-4 and IL-5 were measured using the IFN-γ ELISA kit, IL-4 ELISA kit, and IL-5 ELISA kit (Pierce Biotechnology, Rockford, USA), respectively.

Sneezing and nasal rubbing behavior induced by antigen in sensitized mice The effects of oral administration of *S. crispa* were investigated using the allergic rhinitis model in mice as described by Kayasuga *et al.* (2002) with some modifications. Mice were given an intraperitoneal injection

consisting of OVA (100 μg), alum (1 mg) and pertussis toxin (300 ng) in 0.1 ml sterile saline on day 0. On day 5, they were boosted by a subcutaneous injection of 1 ml of saline containing OVA (50 μg) in the back. After general sensitization, local sensitization was performed once a day from day 18 to day 53 by the instillation of OVA in saline (5 mg/ml, 10 μl/nostril) into the bilateral nasal cavities using a micropipette. They were fed either with or without *S. crispa* daily from day 25 to day 53. The number of sneezing and nasal rubbing after nasal instillation of OVA solution into the bilateral nasal cavities were counted for 30 min every week from day 25. Sera were collected on day 46 and day 53 for OVA-specific IgE quantification and stored at –80°C for further analysis.

Statistical analysis Data were summarized with descriptive statistics, such as the mean and standard error of the mean. Statistical comparisons were analyzed by using either a Student's *t*-test or a Dunnett's test. A p value of less than 0.05 was considered significantly different.

Results

Effect of Sparassis crispa on immunomodulative activity in sensitized mice To examine the immunomodulative activity of oral administration of *S. crispa*, splenocytes obtained from ovalbumin-sensitized BALB/c mice fed *S. crispa* were restimulated *in vitro* with the same antigen. Cytokines and OVA-IgE levels in the splenocyte culture supernatants were measured by using ELISA. Splenocytes from the *S. crispa* group showed significantly higher IFN-γ production ($p<0.01$; Fig. 1A) and significantly lower IL-4 production ($p<0.01$; Fig. 1B) compared to the control group. Splenocytes from the *S. crispa* group produced less IL-5 and OVA-specific IgE than the control group (Fig. 1C and D), but showed no significant difference. The inhibition of Th2-

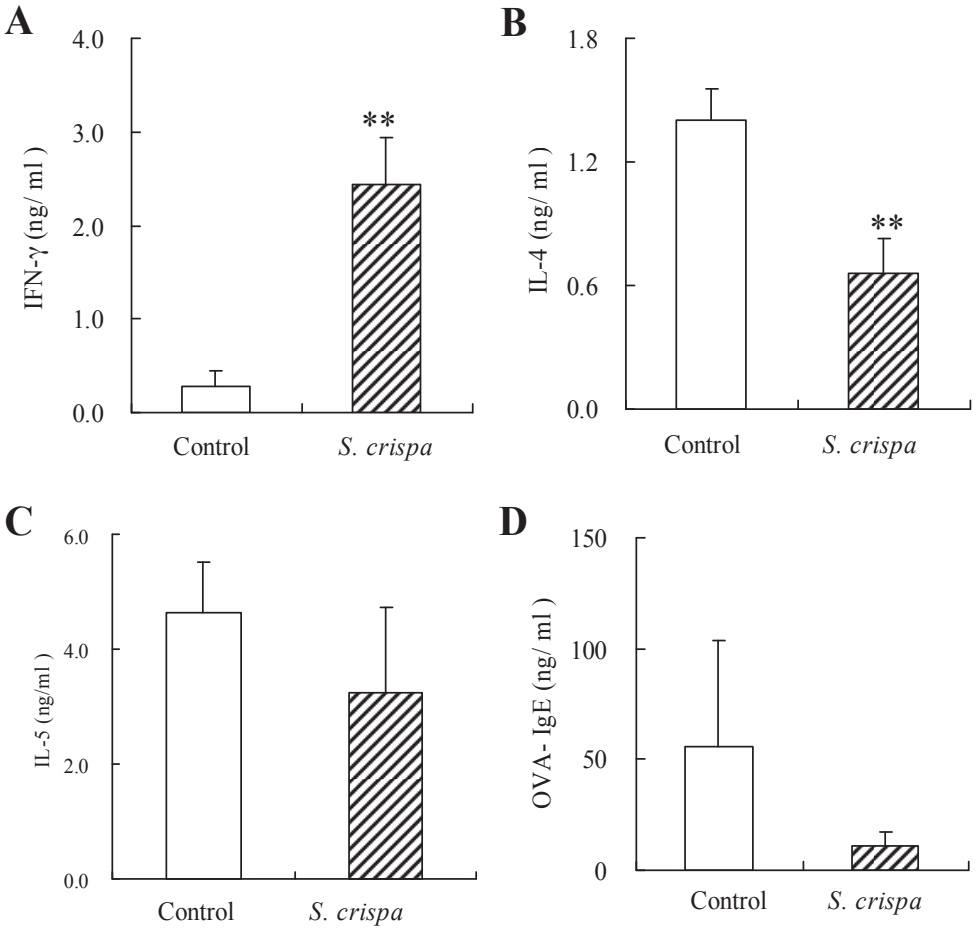

Fig. 1. Effects of oral administration of *Sparassis crispa* on IFN-γ, IL-4, IL-5, and OVA-specific IgE secreted by splenocytes derived from OVA-sensitized BALB/c mice.
IFN-γ (A), IL-4 (B), IL-5 (C), and OVA-specific IgE (D). The averages and error bars representing the standard error were obtained from the data of 8 mice, and Student's *t*-test was used for statistical analysis. Control group (open columns); administered group (hatched columns). There was a significant difference of $^{**}p<0.01$ compared to the control group.

type cytokine and OVA-specific IgE production seemed to be associated with the oral administration of the *S. crispa* that induced IFN-γ.

Effect of Sparassis crispa on nasal rubbing and sneezing induced by antigen Figure 2 shows the effects of repeated oral administration of *S. crispa* on nasal symptoms induced by a local application of the antigen in BALB/c mice. The *S. crispa* at doses of 36 or 120mg/kg was administered orally every day from day 25 to day 53. Both sneezing and nasal rubbing movements were observed immediately after topical antigen challenge and lasted for more than 30 min. The number of sneezing and nasal rubbing movements was increased progressively by daily intranasal sensitization. The oral administration of *S. crispa* caused a dose-dependent inhibition of the nasal rubbing movements from day 32 to day 53, and a significant effect was observed at a dose of 120 mg/kg on day 53 (Fig. 2A). The number of sneezing was also inhibited significantly by oral administration of *S. crispa* at a dose of 120 mg/kg on day 46 and day 53 (Fig. 2B). The effects of *S. crispa* on OVA-specific serum IgE levels in the allergic rhinitis model are shown in Fig. 3. The oral administration of *S. crispa* reduced the level of OVA-specific IgE in sera on day 46 and day 53, but showed no significant difference.

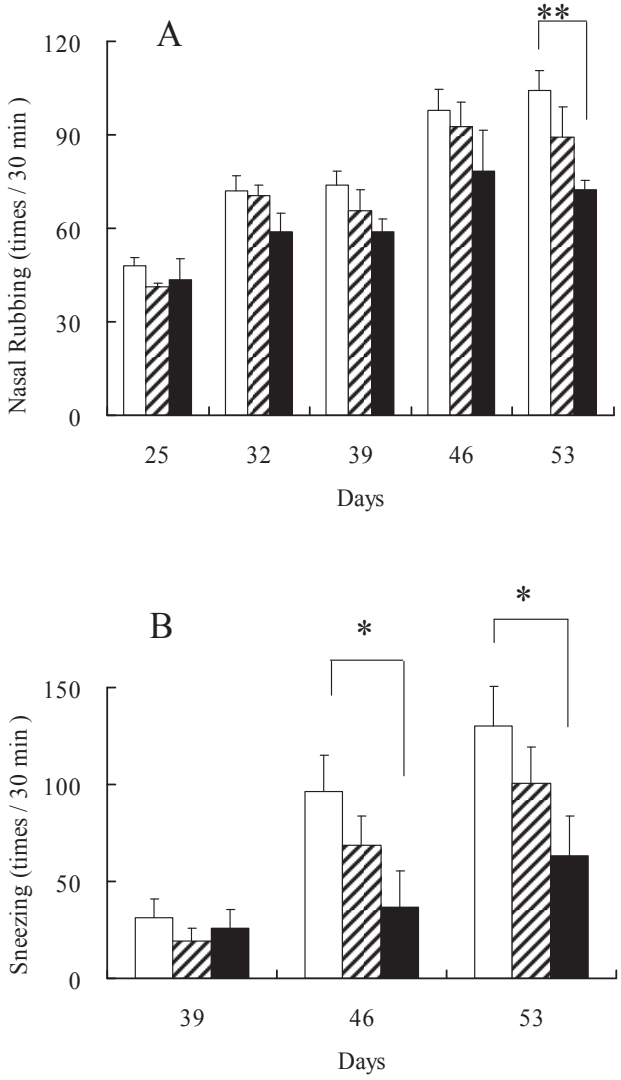

Fig. 2. Effects of oral administration of *Sparassis crispa* on symptoms in the allergic rhinitis model.

Mice were repeatedly injected OVA solution (50 μg/10 μl) into the bilateral nostrils every day from day 18 to day 53 after the first immunization and were fed either with or without *S.* daily from day 25. The number of nasal rubbing (A) and sneezing (B) was counted for 30 min after topical application of antigen. The averages and error bars representing the standard error were obtained from the data of 10 mice, and Dunnett's test was used for statistical analysis. Control group (open columns); 36 mg/kg (hatched columns); 120 mg/kg (solid columns). There was a significant difference of $*p<0.05$ and $**p<0.01$ compared to the control group.

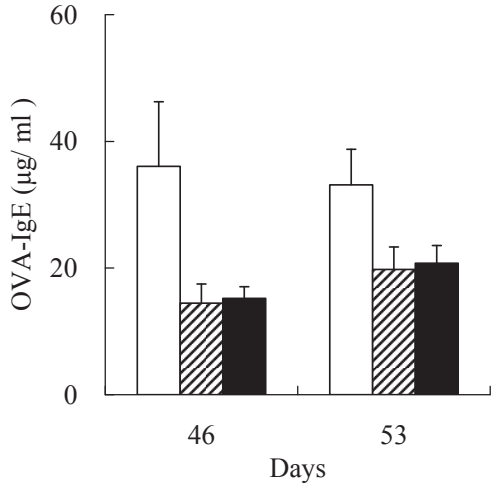

Fig. 3. Effect of oral administration of *Sparassis crispa* on OVA-specific serum IgE levels in the allergic rhinitis model.
The averages and error bars representing the standard error were obtained from the data of 10 mice. Control group (open columns); 36 mg/kg (hatched columns); 120 mg/kg (solid columns).

Discussion

It was indicated that oral administration of *S. crispa* could reduce both blood IgE level and scratching number of NC/Nga mice that were induced dermatitis (Hasegawa *et al.*, 2002). Moreover, a branched β-glucan from *S. crispa* can induce IFN-γ in DBA/2 mice *in vitro* (Harada *et al.*, 2002). Therefore, we investigated whether oral administration of *S. crispa* inhibits IgE production through promoting the Th1-type immune response.

In the present study, oral administration of *S. crispa* inhibited OVA-specific IgE production by OVA-stimulated murine splenocytes through promoting a dominant Th1-type cytokine profile with enhanced IFN-γ and diminished IL-4 and IL-5 production. These results demonstrated that oral administration of *S. crispa* caused inhibition of antigen-specific IgE production through both promoting the Th1-type immune response and inhibiting the Th2-type immune response.

The reduction of the number of sneezing and nasal rubbing movements in the murine allergic rhinitis model seemed to be associated with the reduction in antigen-specific serum IgE levels. In addition, diminished production of IL-4 may be associated with suppression of the symptoms because IL-4 can promote the proliferation of mast cells, which play an important role in allergic reactions as well as allergen-specific IgE antibody production (Powrie *et al.*, 1993).

In conclusion, *S. crispa* showed inhibitory effects on immediate allergic reactions, and its mechanism of action is generated mainly by suppression of Th2-type immune response.

In this report, the integrant for immunomodulating activities in *S. crispa* is unknown as the powder of *S. crispa* was used for our experiments. However, there have been some reports that suggest the active ingredient may be β-glucan, which makes up 43.6% of *S. crispa* as measured by the enzyme method (Anonymous, 2000). Thus, *S. crispa* is a good source material for preparing β-glucan in high yields (Harada *et al.*, 2004). Lentinan (Chihara *et al.*, 1969), schizophyllan (Okamura *et al.*, 1986), and krestin (Mizutani *et al.*, 1992), all of which contain β-glucan, have been reported to have antitumor activities, and lentinan has been reported to have anti-allergic effects (Hamuro, 2005), indicating that β-glucan enhances cellular immunity. Therefore, β-glucan of *S. crispa* may be responsible for anti-rhinitis properties in OVA-sensitized mice.

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