

Preventive Effects of Edible Mushroom (*Hypsizigus marmoreus*) on Mouse Type IV Allergy: Fluctuations of Cytokine Levels and Antioxidant Activities in Mouse Sera

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ABSTRACT: We investigated the mechanisms for the antiallergic effects of *Hypsizigus marmoreus* mushrooms on an oxazolone-induced type IV allergy in male ICR mice. Serum levels of interleukin-12 (IL-12) and γ -interferon (γ -IFN) were significantly increased in mice, which showed a severe ear edema induced by the type IV allergy in comparison with those of normal mice. The oral administration of an ethanol extract of *H. marmoreus* for 3 d at a dose of 250 mg/kg body weight prevented the increase of serum IL-12 levels but not γ -IFN levels. Lowering of serum levels of interleukin-2 (IL-2), spleen natural killer (NK) cell activity, and serum antioxidant activity in mice with allergic symptoms were also prevented by oral administration of the ethanol extract. These results suggested that one of the antiallergic mechanisms of *H. marmoreus* would be due to maintenance of antioxidant status and inhibition of the fluctuations of cytokines in allergic mice.

Keywords: antioxidant activity, cytokine, *Hypsizigus marmoreus*, mouse, type IV allergy

Introduction

The increase in allergic diseases in the world has become an important medical problem in recent years. Allergies are divided into 4 types based on their mechanisms. The most frequent allergic diseases are type I, such as asthma and pollinosis, and type IV, such as contact hypersensitivity. Type IV is known as delayed type hypersensitivity and involves cell-mediated immunity. The cell-mediated immunity is mediated by cytokines such as interleukin-12 (IL-12), which is secreted by antigen presenting cells (APC) such as macrophage-like cells, and stimulates naive T cells; interleukin-2 (IL-2), which is secreted by type 1 T helper (Th1) cells and mononuclear cells (MNC), and stimulates cytotoxic T lymphocytes (CTL) and natural killer (NK) cells; and γ -interferon (γ -IFN) and tumor necrosis factor- α (TNF- α), which are secreted by Th1 cells and macrophages and stimulate macrophages. γ -IFN and TNF- α are known as proinflammatory cytokines. Severe inflammation is a key process in the development of type IV allergy. Inflammation could be induced with excess active oxygen species such as the superoxide anion radical and nitric oxide produced from phagocytes like macrophages stimulated in an allergic response and some chemical mediators such as leukotrienes and prostaglandins, formed during the enzymatic oxidation of arachidonic acid. Some antioxidants such as methionine (Del Maestro and others 1981), mannitol (Shingu and others 1982), tea catechins (Katiyar and others 1995), and superoxide dismutase (Yoshikawa

and others 1985) could inhibit inflammation. Tea catechins also exhibited antiallergic effects on type IV allergy in mice (Suzuki and others 2000).

Our previous paper demonstrated that an ethanol extract of *Hypsizigus marmoreus* (Peck) Bigel. (bunashimeji), an edible mushroom, inhibited type IV allergy in mice when administered orally and percutaneously (Sano and others 2002). Our other reports also demonstrated that feeding of a diet containing 5% to 10% dried powder of extracts of *H. marmoreus* enhanced antioxidant activities in mouse plasma (Matsuzawa and others 1997, 1998). It is thought that the active components in the ethanol extract prepared from *H. marmoreus* are hydrophilic and have low molecular weights. This mushroom includes several antioxidants and anti-inflammatory components such as L-methionine (Harada and others 2003), mannitol, trehalose (Shingu and others 1982; Yoshida and others 1982; Oku and others 2003), ergosterol, and its peroxides (Yasukawa and others 1994). In this study, we investigated the effects of the ethanol extract of *H. marmoreus* on serum cytokine levels, spleen NK cell activities, and serum antioxidant activities in mice affected by a type IV allergy induced with oxazolone.

Materials and Methods

Preparation of ethanol extract of *H. marmoreus*

An ethanol extract of fruiting bodies of *H. marmoreus* from the Japan Agricultural Cooperatives Zennou Nagano, Japan was prepared. Two hundred grams of *H. marmoreus* were crushed with a food mill and extracted with 600 mL ethanol at 40 °C for 3 h using heating extractor with a reflux condenser. The extract was filtered and obtained filtrate was lyophilized.

Mouse model of type IV allergy

Oxazolone-induced edema of the ear in mice was used to model type IV allergy (Nakano 1977; Nakamura and others 1980).

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Oxazolone, a sensitizer, was purchased from Wako Pure Chem. Ind., Osaka, Japan. Male ICR mice, 4 wk old, were purchased from Japan SLC Inc., Shizuoka, Japan. The hair of the abdominal region was carefully cut away, and 0.1 mL of a 0.5% oxazolone solution in ethanol was applied to the skin (sensitization). Five days after the sensitization, 20 μ L of a 0.5% oxazolone solution in acetone were applied to both sides of each animal's right ear (challenge). Twenty-four hours after the challenge, the mice were sacrificed, under ether anesthesia, and circular samples (5.0 mm in diameter) of both ears were removed using a punching apparatus. Mouse blood was taken and centrifuged at $1500 \times g$ for 10 min, and the supernatant was used as the serum. The weights of the right and left ears were measured and the ear swelling ratio was calculated. The ethanol extract of *H. marmoreus* was orally administered to mice 1 h before the challenge at a dose of 250 mg/kg body weight once a day for 3 d. Control mice were not administered any agents, though they were subjected to the other procedures. Normal mice were not subjected to any of the procedure. The number of mice in each group was five. Throughout the experiment, the animals were handled in accordance with "The Guide for Animal Experiments in the Numazu Natl. College of Technology."

Determination of cytokines in mouse serum

The levels of IL-12, γ -IFN, and IL-2 in mouse serum were determined by enzyme-linked immunosorbent assay (ELISA) with a Murine IL-12 ELISA Development Kit (Pepro Tech EC, Ltd., London, U.K.), ChemikineTM Mouse IFN γ Sandwich ELISA Kit (Chemicon Intl. Inc., Temecula, Calif., U.S.A.), and TiterZyme EIA (mouse Interleukin-2 assay) kit (Assay Designs Inc., Ann Arbor, Mich., U.S.A.), respectively.

Determination of natural killer cell activities in mouse spleen cells

The cytolytic function of NK cells in mouse spleen was determined by a Cr⁵¹ release assay with YAC-1 target cells, murine lymphoma cells, labeled with Cr⁵¹. The assay was performed at Mitubishi Kagaku Bio-Clinical Laboratories Inc., Tokyo, Japan.

Determination of antioxidant activities in mouse serum

Male ddY mice, 6 wk old, were purchased from Japan SLC Inc. The supernatant of a 5% mouse brain homogenate in 0.04 M phosphate buffer (pH 7.4) was prepared and 0.25 mL of it and 0.5 mL of phosphate buffer were mixed. The mixture with or without 3 μ L of serum, which was obtained from mice used in the allergy experiment, was autooxidized at 37 °C for 60 min (Stocks and others 1974). The level of lipid peroxide in the reaction mixture was determined by the thiobarbituric acid method (Yagi 1976) and the inhibitory activities of mouse serum against the lipid peroxidation were evaluated.

Statistical analysis

Statistical analyses were performed with the nonparametric Mann-Whitney U test to determine the significance of differences between the appropriate experimental groups. A *P* value less than or equal to 0.05 was considered statistically significant.

Results and Discussion

Effects of ethanol extract from *H. marmoreus* on serum cytokine in mice with type IV allergy

The yield of the ethanol extract prepared from *H. marmoreus* was 20.2% in dry weight terms. When administered orally at a

dose of 250 mg/kg body weight, the extract exhibited significant antiallergic activities as in our previous report (Sano and others 2002). The rate of inhibition of type IV allergy was 40.5% in this study.

Effects of the oral administration of the ethanol extract on the serum levels of IL-12, γ -IFN, and IL-2 in mice affected by the type IV allergy induced with oxazolone are shown in Figure 1. These cytokines are important for the allergic process. Serum IL-12 levels were significantly higher in control mice having intense allergic symptoms than in normal mice. This increase was significantly prevented by the oral administration of the extract at 250 mg/kg. IL-12 is a cytokine secreted by APC such as macrophages, and induces the differentiation of native T cells into Th1 cells. Serum γ -IFN levels were also significantly higher in allergic mice (control mice) than in normal mice. However, this increase was not significantly prevented by the oral administration of the ethanol extract at 250 mg/kg. γ -IFN and TNF- α , proinflammatory cytokines, have multiple actions such as the stimulation of macrophages, Th1 cells, and CTL and the suppression of type 2 T helper cells. Serum IL-2 levels were significantly lower in allergic mice (control mice) than normal mice. This decrease was significantly prevented by the oral administration of the extract at 250 mg/kg. IL-2 produced by Th1 cells and MNC could stimulate CTL and NK cell activities. These results suggest that the ethanol extract prepared from *H. marmoreus* has some modulatory effect on the immune responses in mice affected by the type IV allergy induced with oxazolone. In particular, the production and/or release of IL-12 from APC were efficiently inhibited by the extract. In order to clarify the more detailed mechanism of these phenomena, it is necessary to investigate the influences of the ethanol extract of *H. marmoreus* on the gene expression systems of some proinflammatory cytokines in cultured T cells or macrophages, including the activation of transcription factors such as activator protein-1 and nuclear factor kappa B.

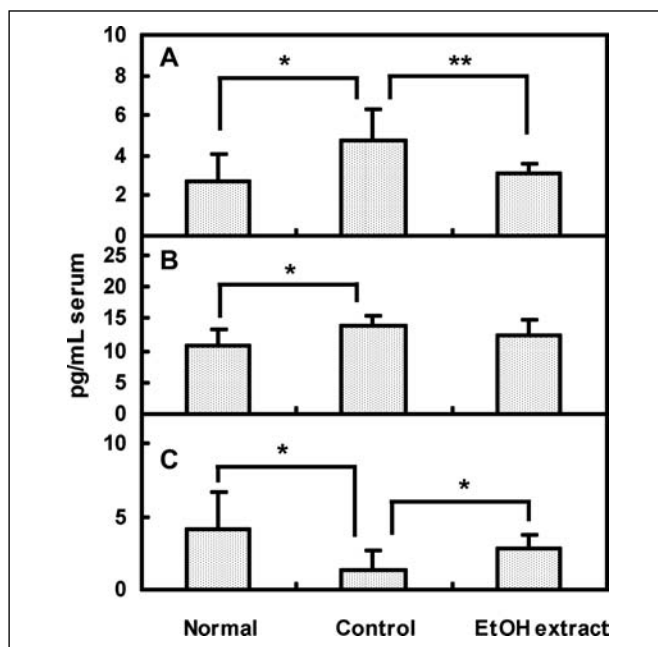


Figure 1 – Effects of the oral administration of an ethanol extract prepared from *Hypsizigus marmoreus* on levels of several cytokines in the sera of 5-wk-old male ICR mice affected by a type IV allergy induced with oxazolone. A, IL-12; B, γ -IFN; C, IL-2. Mean $\pm$ SD. *N* = 5. Dose; 250 mg/kg body weight. Significant differences from control value: **P* < 0.05, ***P* < 0.01.

Effects of ethanol extract from *H. marmoreus* on spleen NK cell activities in mice with type IV allergy

Spleen NK cell activities in mice affected by the type IV allergy induced with oxazolone are shown in Figure 2. The activities were significantly lower in allergic mice (control mice) than in normal mice. The lowering of spleen NK cell activities was significantly prevented by the oral administration of the extract at 250 mg/kg. IL-2 and NK cell activities are generally thought to play an important role in cell-mediated immunity. Since NK cell activities could be stimulated by IL-2, the changes of NK cell activities associated with those of the serum IL-2 levels are reasonable. It was reported that changes in NK cell activities little affected the degree of delayed type hypersensitivity such as mouse arthritis (Barthold and de Souza 1955; Nilsson and others 1999) and the lowering of NK cell activities did not affect the severity of inflammation in oxazolone-induced ear edema (Kunzendorf and others 1996). These data suggest the complexity of type IV allergic responses such as oxazolone-induced ear edema.

Effects of ethanol extract from *H. marmoreus* on serum antioxidant activities in mice with type IV allergy

The antioxidant activities in the serum of mice affected by the type IV allergy induced with oxazolone are shown in Figure 3. The activities were significantly lower in allergic mice (control mice) than in normal mice. The lowering of serum antioxidant activities was significantly prevented by the oral administration of the extract at 250 mg/kg. The excess production of superoxide anion radical and nitric oxide by macrophages and neutrophils is thought to provoke inflammation in the neighbor-

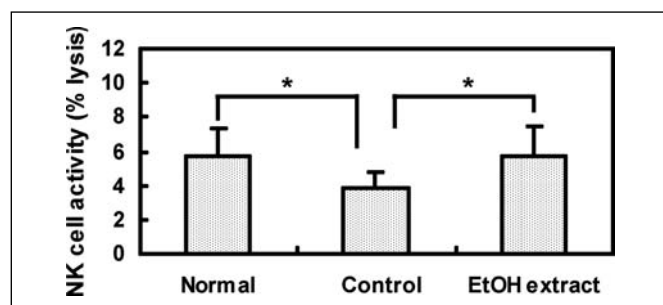


Figure 2—Effects of the oral administration of the ethanol extract of *Hypsizigus marmoreus* on NK cell activities in the spleen cells of 5-wk-old male ICR mice affected by a type IV allergy induced with oxazolone. Mean $\pm$ SD. $N = 5$. Dose; 250 mg/kg body weight. Significant differences from control value: * $P < 0.05$.

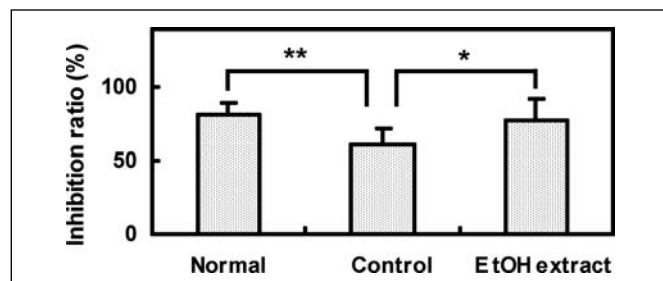


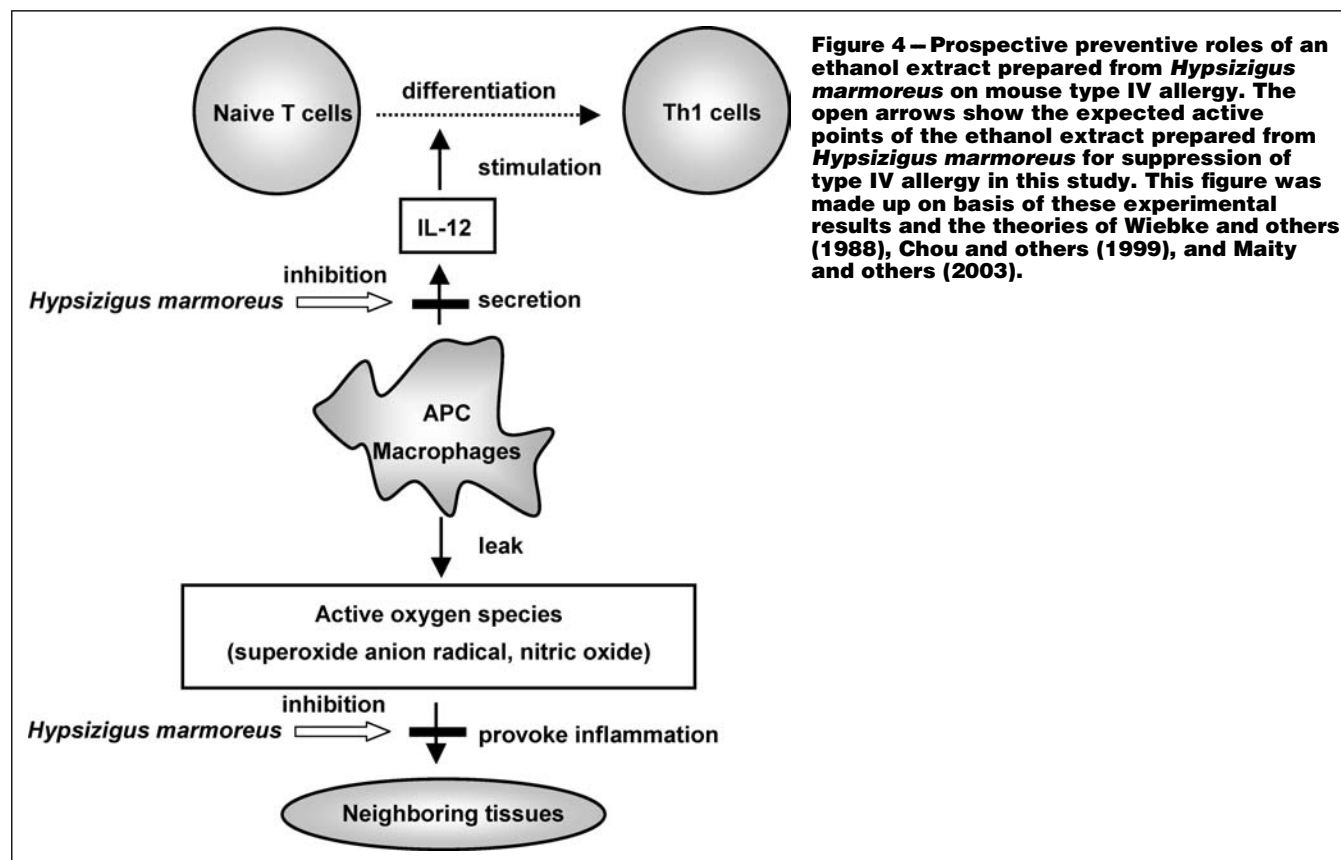
Figure 3—Effects of the oral administration of the ethanol extract of *Hypsizigus marmoreus* on antioxidant activities in the sera of 5-wk-old male ICR mice affected by a type IV allergy induced with oxazolone. Mean $\pm$ SD. $N = 5$. Dose; 250 mg/kg body weight. Significant differences from control value: * $P < 0.05$, ** $P < 0.01$.

ing tissues (Shingu and others 1982; Oyanagui 1983). The enhancement of serum antioxidant activities in mice administered the ethanol extract may contribute to the antiallergic effects. In this experiment, the suppressive activities of the ethanol extract prepared from *H. marmoreus* against the serum levels of γ -IFN, which was one of the proinflammatory cytokines stimulating macrophages, were not so strong. Then the elevation of serum antioxidative activities in the allergic mice was thought to be important for the prevention of the inflammation caused by some active oxygen species produced by macrophages. It was reported that the inflammation caused by oxazolone-sensitization could be mediated by the metabolites of arachidonic acid obtained with 5-lipoxygenase and cyclo-oxygenase (Nakamura and others 1988). An inhibitory effect of *Albatrellus dispansus* (Lloyd) Canf. et Gilbn. on 5-lipoxygenase was reported and the active component was identified as analogues of grifolin (Nozoe and Takahashi 1989). Though the same effects of *H. marmoreus* have not been reported, its positive effect on serum antioxidative activities in mice may help to inhibit the enzymatic oxidation of arachidonic acid.

We previously reported that the administration of the extracts prepared from *H. marmoreus* enhanced antioxidant activities in mouse plasma (Matsuzawa and others 1997, 1998). *H. marmoreus* contains antitumor β -glucans (Ikekawa and others 1992; Motoi and others 2003). Polysaccharides such as β -glucans and glycoproteins in some edible mushrooms have antioxidant activities (Liu and others 1997), anti-inflammatory activities (Ukai and others 1983), and modulatory effects on immune responses (Wang and others 1995). However, these high-polymeric substances are not expected in the ethanol extract. On the other hand, ergosterol (7.9 mg/g extract), mannitol (108 mg/g extract), and trehalose (248 mg/g extract) were found in the ethanol extract. These low-molecular-weight substances have also antioxidant and anti-inflammatory activities. These substances were thought to enhance the antioxidant activities in the serum. The enhancement of serum antioxidant activities was observed 25 h after the oral administration of the ethanol extract in this study. We previously reported that the administration of the ethanol extract prepared from *H. marmoreus* enhanced antioxidant activities in mouse plasma (Matsuzawa and others 1997, 1998).

These effects of *H. marmoreus* against type IV allergy have also been observed in our preliminary experiment though the number of mice used was 3 to 4 in each group. Briefly, oral administration of the ethanol extract at same dose level into mice with type IV allergy inhibited the decreases of serum IL-2 levels, spleen NK cell activities, and serum antioxidant activities by 67.8%, 42.3%, and 75.8%, respectively. Inversely, the administration of the ethanol extract inhibited the increase of serum IL-12 levels by 81.0%, while not affecting serum γ -IFN levels in mice with type IV allergy. Then, the ethanol extract of *H. marmoreus* was thought to have active constituents against type IV allergy but further investigations are needed to clarify the mechanisms for the curative properties.

In summary, the effects of the ethanol extract of *H. marmoreus* on serum cytokine levels, spleen NK cell activities, and serum antioxidant activities in mice with type IV allergy were investigated. The following processes are important in the type IV allergy and inflammation: (1) stimulation of naive T cells by IL-12 secreted from APC; (2) stimulation of macrophages by proinflammatory cytokines such as γ -IFN and TNF- α , secreted from Th1 cells and macrophages; (3) stimulation of NK cells and CTL by IL-2 secreted from Th1 cells and MNC; and (4) release of active oxygen species from macrophages. Oral administration of an ethanol extract of *H.*



marmoreus prevented the increase of serum IL-12 levels and the decreases of serum IL-2 levels, spleen NK cell activities, and serum antioxidant activities in mice with type IV allergy. However, it did not prevent the increase of serum γ -IFN levels.

Conclusions

Figure 4 shows a scheme of conceivable preventive effects of *H. marmoreus* on type IV allergic mice. The results obtained in this study suggest that an ethanol extract of the edible mushroom *H. marmoreus* plays a prominent role in the antiallergic action mainly via the 2 inhibitory actions on APC and macrophages, inhibition of the production and/or release of IL-12, and suppression of oxidative stress induced by active oxygen species.

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