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Reduction of Development of Late Allergic Eosinophilic Rhinitis by *Kurozu Moromi* Powder in BALB/c Mice

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The preventive effect of the development of the late allergic eosinophilic rhinitis (LAER), a T helper (Th)2 type immune response, in BALB/c mice by *kurozu moromi* powder (kmp) was examined. Mice were pre-treated with or without (control) kmp intragastrically for 30 days (50 mg/kg/day) and then LAER was induced by ovalbumin. Compared to the control group, kmp treatment resulted in reduction of the number of eosinophils, but not lymphocytes, in the blood. The kmp treatment significantly decreased concentration of interleukin (IL)-4, the Th2 cytokine, compared to the control in nasal cavity fluid (NF). However, no difference or increase in concentration of interferon-γ, a Th1 cytokine, in NF was observed in the two groups. The control group showed slight to moderate rhinitis, such as hyperplasia of respiratory epithelium and goblet cells with prominent mucous secretion and infiltration of eosinophils and lymphocytes in nasal mucosa. These changes were significantly reduced in mice treated with the kmp. The inhibitory effect of the kmp on the development of LAER may be due to decreased local Th2 cell activity promoting eosinophil infiltration, which is separate from the mutual Th1/Th2 inhibitory effect. In addition, the present study suggests that the kmp may be a useful food for prevention of the development of LAER in humans.

Keywords: *kurozu,* rhinitis, eosinophil, Th2, IL-4

Introduction

The numbers of allergic airway diseases are increasing in industrialized countries. Several factors such as air pollution (D'Amato, 2002), viral infection (Marsland *et al.,* 2004) and lifestyle (Huovinen *et al.,* 2003) may cause increased susceptibility to respiratory allergic diseases. Moreover, declining exposure to bacterial agents may increase the incidence of allergic airway disease, according to hygiene hypothesis (Strachan, 1989; Von Herdzen and Haahtela, 2004).

Allergic rhinitis is characterized by upper airway hyperresponsiveness (Salib *et al.,* 2003). There is a general agreement that T-helper (Th) 2 cytokines, such as IL-4 (Coyle *et al.,* 1995), IL-5 (Kaneko *et al.,* 1991) and IL-13 (Zhu *et al.,* 1999) mediate the allergic response, because they correlate with disease severity. It has been proposed that allergic airway inflammation may consist of an immediate phase mediated by IgE and mast cells (Corry *et al.,* 1996; Galli, 1997) and a late phase mediated mainly by $CD4^+$ Th2 cells and eosinophils (O'Byrne *et al.,* 1987; Lei *et al.,* 1989; Corrigan *et al.,* 1995; Kosgren *et al.,* 1997; Takeda *et al.,* 1997).

For more than 200 years, *kurozu* (rice black vinegar) has been produced in the Fukuyama district of Kagoshima Prefecture in Japan. Among *kurozu* manufactures, *kurozu moromi* powder (kmp) is a byproduct obtained during processing. It has been suggested that the kmp may have a lot of advantageous pharmacological effects on lipid metabolism and haematological functions (Fujino *et al.,* 1990, 1993; Nagano *et al.,* 2001a). Moreover, it has been reported that the kmp has preventive effects on an allergic dermatitis mouse model (Nagano *et al.,* 2001) and also delayed eosinophilic allergic asthma model in mice (Hayashi *et al.,* 2006). These suggest that the kmp may be useful as a type of health food to prevent the development of allergic diseases, although multiple drugs, including antihistamines and steroids, are still being used to treat allergic diseases (Hussain and Kline, 2003).

In the present study, the preventive effects of kmp on the development of the late allergic eosinophilic rhinitis (LAER) induced by ovalbumin in BALB/c mice were examined.

Materials and Methods

Animals A total of 23 specific pathogen-free, 8-week-old female BALB/c mice (Japan Charles River Co., Yokohama, Japan) were used (kmp group, $n=15$; control group, $n=8$). In each set of experiments, the numbers of mice used were stated as below. They were kept in 25 ± 2

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℃ room with 55±10% humidity and a 12-h light/dark cycle (light, 08: 00–20: 00). The animal experiments were approved by Research Ethics Board of the Faculty of Agriculture, Yamaguchi University.

Diets Kurozu can be fermented from brown rice in ceramic pots. Generally the kmp, an insoluble material, was formed during kurozu fermentation (Fig. 1). The powder was prepared by Sakamoto Brewing Co., Ltd. (Kagoshima, Japan).

Experimental design Mice were treated daily for 30 days with kmp (0.1 ml, 50.0 mg/kg) dissolved in distilled water using a cannula for mice (1.5 mm diameter×70 mm length; Natsume Co., Tokyo, Japan) intra-gastrically. As described previously, this treatment was effective for the suppression of the development of allergic dermatitis (Nagano *et al.,* 2001b) and late allergic asthma (Hayashi *et al.,* 2006) in mice. For the control group, the same treatment was carried out using distilled water.

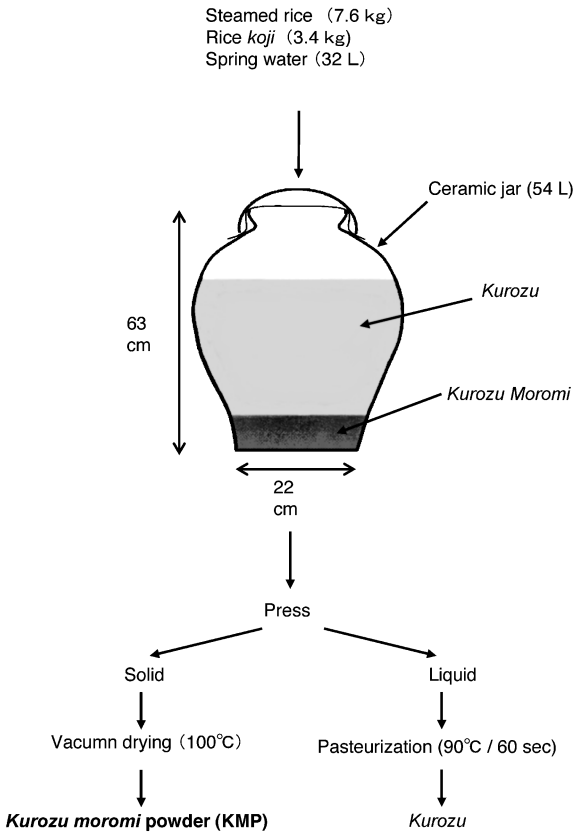

Fig. 1. The procedure of production of *kurozu moromi* powder (kmp).

The sensitization and challenge procedures were carried out by the methods described previously (Hayashi *et al.,* 2002). In brief, as shown in Fig. 2, animals were sensitized on the next day after the final treatment of kmp intraperitoneally (i.p.) with 10 μg of ovalbumin (OVA: Grade V; Sigma Chem. Co., MO, USA) in 0.1 mL phosphate-buffered saline (PBS; pH 7.4) of aluminium hydroxide (Alum. 1.2 mg; Serva, Heidelberg, Germany) on day 0 and day 10. Fourteen days after the first sensitization, each mouse was challenged intranasally with 200 μg of OVA in 50 μl of PBS under anaesthesia i.p. with ketamine (45 mg/kg body weight; BW: Sankyo Co., Tokyo, Japan) and xylazine (8 mg/kg BW; Bayer Co., Tokyo, Japan).

Mice were anesthetized i.p. as described above and a whole blood was obtained from the heart puncture using a heparinized syringe 4 days after the challenge. The percentage of lymphocytes and eosinophils ($n=5$, control; $n=8$, kmp) were counted from blood smear stained with Giemsa solution. The total number was determined by multiplying the percentage of each cell type. The heads ($n=8$, control; $n=15$, kmp) were fixed in 10% neutral buffered formalin (pH 7.0) for 2 days, and decalcificated in 20% EDTA in 1 mL Tris-HCL (pH 7.4) for 2 weeks and the nasal region was sliced at the anterior margin of orbit transversely (Fig. 3A), since it has been reported that allergic rhinitis was mainly localized in respiratory epithelium of this area (Fig. 3B: bold line) in a murine AR model (Hussain *et al.,* 2001; Farraj *et al.,* 2004). Paraffin sections (5-μm thickness) were stained with haematoxylin and eosin (HE), alcian blue or Periodic acid Schiff (PAS).

Figure 3C shows the right side of transverse section. Rectangles indicate lower portion of the nasal septum (S), lower (L) and upper (H) lateral walls of respiratory epithelium and the number of eosinophils and lymphocytes infiltrated was examined. Total number of each inflammatory cell type (eosinophils or lymphocytes/100 μm^2) of nasal mucosa at 3 different parts; S, L and H, as described above) was counted by an eyepiece with a grid and calculated by two observers who were blinded to the study design.

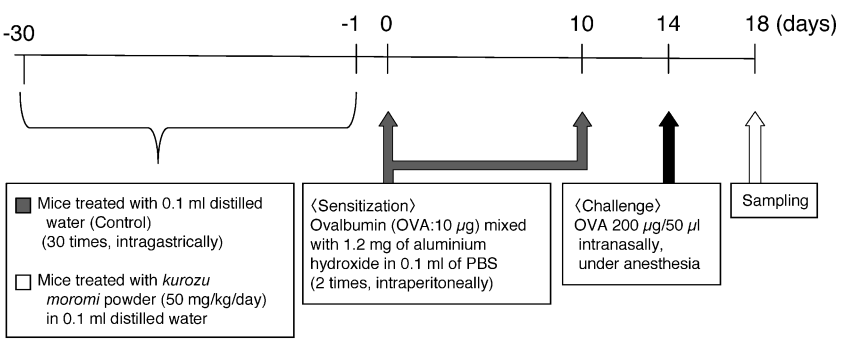

Fig. 2. Experimental protocol. After pre-treatment with kmp, BALB/c mice were sensitized twice i.p. with 10 μg OVA with Alum on day 0 and day 10, and thereafter challenged intranasally with 200 μg OVA in PBS 4 days after second sensitization. Samples were collected 4 days after challenge and used for assays in each experiment.

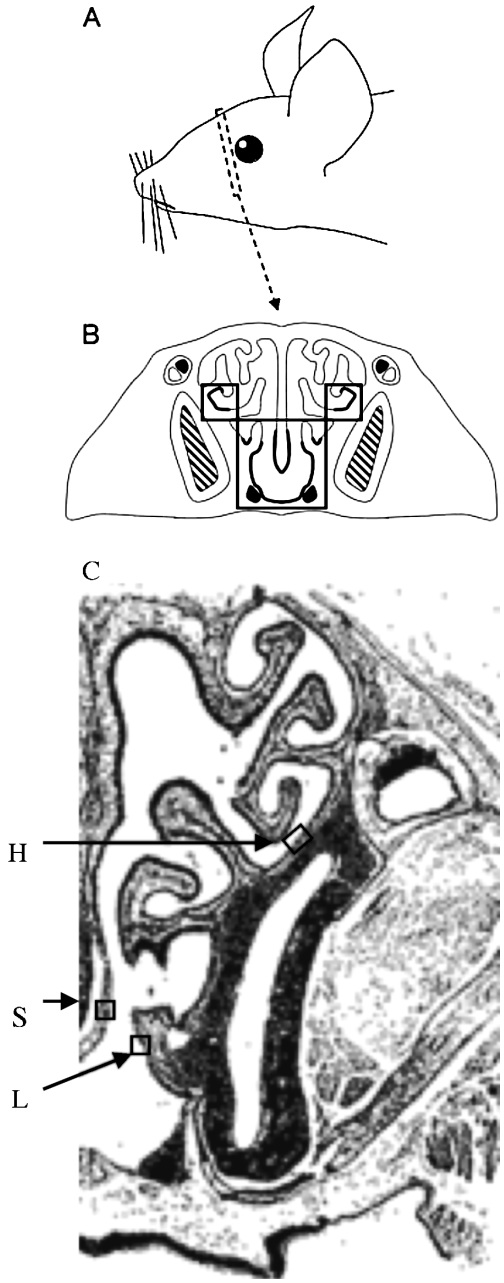

Fig. 3. Site of nasal tissue examined (A), schematic figure of transverse section of nasal region at anterior margin of orbit (bold lines indicate respiratory epithelium) and number of eosinophils counted at three different parts (S, L and H: arrows).

The total number of leukocytes (except for erythrocytic series and megakaryocytes) and eosinophils in bone marrow (BM), which is located at nasal septum areas of tissue sections, was counted, and the percentage of eosinophils was determined in each mouse ($n=5$; control, $n=8$; kmp).

Collection of nasal cavity fluid (NF) On day 18, NF ($n=4$ in each group) was collected by the method described by Rhee *et al.* (2004). In brief, the mouse trachea at the upper level was ligated and then a catheter was guided into the nasopharynx. The nasal passages were then gently perfused with 1 ml of cold PBS. NF was then collected in a petri dish and centrifuged at 800 *g* for 10 min at 4℃

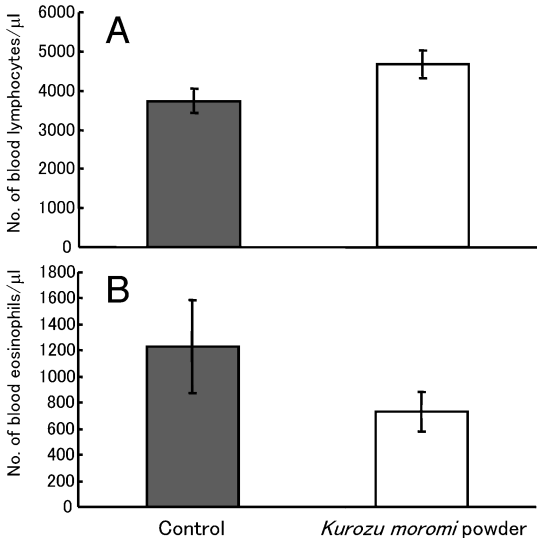

Fig. 4. Total number of blood lymphocytes (A) and eosinophils (B) in both groups.

to separate cells and supernatants.

Concentration of IL-4 and IFN-γ in NF by ELISA IL-4 and IFN-γ mouse ELISA kits were purchased (Techne Co., Minneapolis, MN, USA). The minimal detectable concentration was 2 pg/ml for IL-4 and IFN-γ ($n=4$ in each group).

Statistical analysis The data are expressed as the mean of samples examined±standard error (±SE). Unpaired Student's *t*-test was used to evaluate the significance of differences; a *P*-value less than 0.05 were considered significant.

Results

Effects of kmp on the number of blood eosinophils and lymphocytes were examined. The number of blood eosinophils (control *vs.* kmp=1229±359 *vs.*732±154/μL) but not lymphocytes (control *vs.* kmp=3728±313 *vs.* 4665 ±358/μL), was slightly decreased in kmp-treated mice compared to control mice (Fig. 4), although there was no statistical difference between the two groups. No significant difference was observed in number of monocytes and neutrophils between the two groups (data not shown). Next, the percentage of eosinophils in the bone marrow after kmp treatment was examined; again, no significant differences in percentage of eosinophils between the two groups were observed (Fig. 5; control *vs.* kmp=22.6±3.7 *vs.* 23.6±2.7%).

Effects of the kmp-treatment on concentrations of IL-4, which is responsible for eosinophil infiltration (Coyle *et al.*, 1995), and IFN-γ in NF were examined. Compared to the control mice, the kmp treatment reduced IL-4 concentrations (Fig. 6A; $P<0.003$, control and kmp=4.39±1.05 vs. 0.46±0.30 pg/mL), whereas there was no significant increase in IFN-γ concentrations in either group (Fig. 6B). In addition, no IFN-γ production in the kmp group was observed (Fig.6B; the control *vs.* the kmp: 0.56±0.28 *vs.* 0.00±0.00 pg/mL).

The reduction in the development of nasal lesion by the kmp-treatment was confirmed by histopathology. In gen-

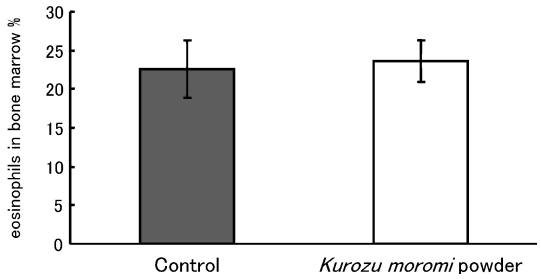

Fig. 5. Percentage of eosinophils of bone marrow in both groups.

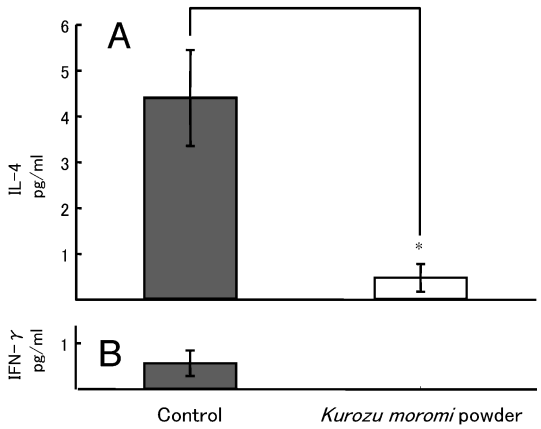

Fig. 6. Production of IL-4 and IFN-γ in nasal fluid in both groups. Data shown are mean±SE. $^{*}P<0.003$ between two groups.

eral, as shown in Fig. 7A, there were many goblet cell metaplasia and hyperplasia of nasal swelled respiratory epithelial cells with elongated cilia and hyaline droplets within cells. In some cases, desquamated epithelial cells mixed with some eosinophils, lymphocytes, neutrophils and macrophages within lumens were observed. A moderate infiltration of lymphocytes and eosinophils with some neutrophils, macrophages and plasma cells with oedema and hyperaemia in mucosal walls were observed. No thickening of the nasal epithelial basement membranes and hyperplasia of nasal gland was observed. While a few mast cells were detected in the nasal mucosa, there was no increase in the number in both groups. Histological changes of nasal mucosa in the mice treated with kmp were prominently reduced compared to those in the control group (Fig.7B).

In nasal mucosa, the number of infiltrated eosinophils (the control *vs.* the kmp; 22.5±5.3 *vs.* 4.5±0.8, Fig. 8A; $P<0.012$) and lymphocytes(the control *vs.* the kmp; 27.6±2.4 *vs.* 8.3±1.5, Fig. 8B; $P<0.01$) in the kmp-treated group was significantly reduced in comparison with that in the control group.

Discussion

The present study demonstrated that the kmp-treatment reduced the development of late allergic eosinophilic rhinitis in BALB/c mice, which show genetically Th2-like responses to exogenous antigens (Reiner and Locksley,

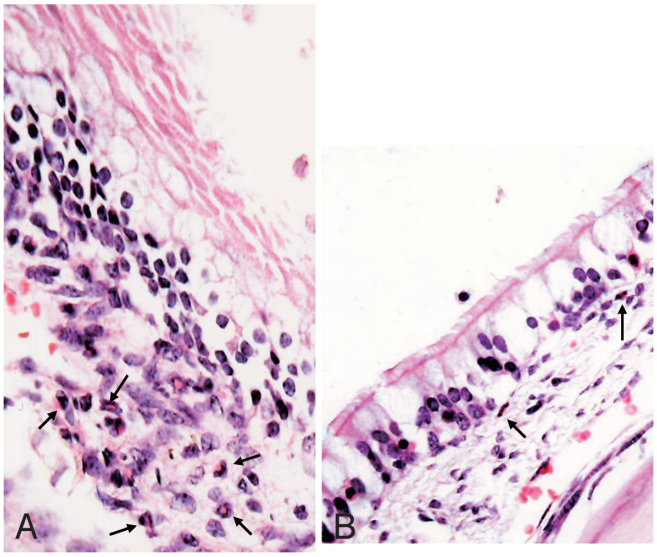

Fig. 7. Representative histology from a control (A) and a mouse treated with the kmp (B). Compared to a treated mouse, the nasal mucosa from a control mouse demonstrated a prominent goblet-cell metaplasia and/or hyperplasia with a remarkable infiltration of lymphocytes and eosinophils (A; arrows). However, those changes were less severe in the kmp mouse (arrows indicate eosinophils).×100. HE.

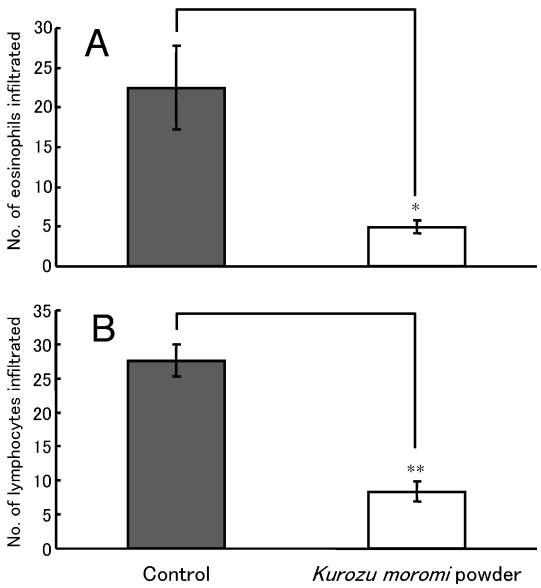

Fig. 8. Number of infiltrated eosinophils in lower portion of the nasal septum, the lower and upper lateral walls of respiratory nasal epithelium in the right side were examined histologically. Compared to the number of eosinophils (A) and lymphocytes (B) in the control group, that of eosinophils (A) and lymphocytes (B) was prominently reduced. $^{*}P<0.012$ and $^{**}P<0.01$ between the two groups.

1995; Hayashi *et al.*, 2003). The kmp treatment caused reduced concentrations of IL-4 in nasal fluids, which are produced by Th2 cells (Mosmann and Coffman, 1989). Additionally, IL-4 is required for the induction of lower respiratory allergic Th2 immunity (Coyle *et al.*, 1995). Taken together, the reduced LAER in the mice treated

with kmp may be due to locally reduced infiltration of lymphocytes and eosinophils.

Reduced local IL-4 production may not be mediated by mutual Th1/Th2 inhibitory effect (Mosmann and Coffman, 1989), since there was no increase in local IFN-γ (typical Th1 cytokine) production in both groups. Moreover, there were no differences in eosinophil percentage of bone marrow and the number of blood eosinophils and lymphocytes between the two groups, suggesting that local, but not systemic, IL-4 production may be suppressed by the kmp treatment. This may be responsible for the reduced infiltration of these cells. However, at present, the inhibitory mechanisms of kmp, consisting of complex mixtures of organic materials including bacteria metabolites, are unknown. Further studies are needed to clarify these points, including the determination of the active component(s) of kmp.

IgE may also contribute to eosinophil migration by interacting with mast cells (Shakoory *et al.,* 2004). Although we have reported previously that blood total IgE values were reduced by the treatment of the kmp (Hayashi *et al.,* 2006), this may not contribute to decreased eosinophil infiltration, since no difference in infiltration and an increase in the number of mast cells at the nasal mucosa were observed in both groups.

All mice treated with the kmp showed temporary erythematous swelling of the skin (e.g., mouth, hand and foot) shortly after OVA challenge (within 30 min-2 h in sensitized mice), although these changes disappeared soon afterward (data not shown). Human subjects treated with nearly same dose of the kmp used here did not show any such changes (Fujino *et al.,* 1990, 1992). Additionally, the preventive experiments of the kmp on allergic dermatitis in BALB/c mice (Nagano *et al.,* 2001b) did not show such changes. Thus, these changes may possibly, at least in part, be due to the effects of the artificially-induced experimental methods used here and/or species difference between humans and rodents.

There is general agreement that reduced early-life exposure to strong Th1 stimuli in industrialized countries has skewed the Th1/Th2 balance towards Th2 responses. Thus, the reduced development of LAER by the treatment of kmp suggests that this food may have the potential to prevent the development of LAER in humans, and to act as an ideal food.

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