

## Amelioration of late asthmatic bronchial-bronchiolar lesions in BALB/c mice by kurozu moromi powder

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### Abstract

The preventive effect of the development of bronchial-bronchiolar lesions of the late asthmatic response (LAR), a T helper (Th)2 type immune response, in BALB/c mice kurozu moromi powder (kmp) was examined. Mice were pre-treated with or without (control) kmp intra-gastrically for 30 days (50 mg/kg/day) and LAR was induced by ovalbumin (OVA). Compared to the control group, it was recognizable that kmp resulted in reduction of the IgE values and the number of eosinophils, but not lymphocytes, in the blood. Also, the kmp treatment increased significantly in a production of OVA-specific interferon (IFN)- $\gamma$  from splenic cells. On the other hand, there was no difference in the production of interleukin (IL)-4 and IL-5 between two groups. The control group showed severe bronchial-bronchiolar inflammation, such as goblet cell hyperplasia with prominent mucous secretion, infiltration of eosinophils and lymphocytes, and hypertrophy of smooth muscle in bronchial-bronchiolar walls. These changes were less severe in the mice treated with the kmp. Compared between the two groups, the beneficial effects of kmp were clearly demonstrated and the development of LAR was reduced. This may be due to the activated OVA-specific Th1 cell responses induced by the effects of kmp which leads to the suppression of the development of LAR mediated by Th2 responses.

**Keywords:** *Asthma, late asthma, bronchitis, eosinophil, Th2, kurozu moromi powder, amelioration.*

### Introduction

The numbers of allergic diseases like asthma are increasing in Japan and other developed countries. According to hygiene hypothesis, declining exposure to infectious agents may increase the risks of suffering from allergic asthma (Strachan 1989, Von Hertzen & Haahtela 2004). The use of multiple drugs including

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antihistamines and steroids to treat allergic diseases dose not significantly suppress the diseases, especially allergic asthma (Hussain & Kline 2003).

Allergic asthma is characterized by airway hyperresponsiveness, eosinophil recruitment into peribronchial walls and mucus overproductions in bronchial- and bronchiolar epithelial cells (Seminario & Gleich 1994; Galli 1997). 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 eosinophil infiltration and disease severity. It has been proposed that allergic asthma 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<sup>+</sup> 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 (black rice vinegar) has been produced in the Fukuyama district of the Kagoshima prefecture in Japan. Kurozu moromi powder (kmp) is a by-product obtained during processing. It has been suggested that kmp may have a lot of pharmacological effects on lipid metabolism and haematological functions (Fujino et al. 1990, 1992). Moreover, it has been reported that kmp has preventive effects on an allergic dermatitis mouse model (Nagano et al. 2001b). This suggests that kmp may be useful as part of healthy food to ameliorate allergic diseases.

In the present study, the preventive effects of kmp on the development of the late allergic bronchial-bronchiolar inflammation induced by ovalbumin in BALB/c mice were examined.

## Materials and methods

### *Animals*

A total of 30 specific pathogen-free, 8-week-old female BALB/c mice (Japan Charles River Co., Yokohama, Japan) were used (the kmp group,  $n = 16$ ; the control group,  $n = 14$ ). In each set of experiments, the numbers of mice used were stated as below. They were kept in  $25 \pm 2^\circ\text{C}$  room temperature with  $55 \pm 10\%$  humidity and a 12-h light/dark cycle (lighting time 08:00–20:00). The animal experiments were approved by the Research Ethics Board of the Faculty of Agriculture, Yamaguchi University.

### *Diets*

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

### *Experimental design*

The mice were divided into the following two groups (Figure 2). The mice were treated daily for 30 days with the kmp (0.1 ml; 50.0 mg/kg) dissolved in distilled water using cannula for mouse (1.5 mm diameter  $\times$  70 mm length; Natsume Co., Tokyo, Japan) intra-gastrically as described previously, since this treatment was effective for the suppression of the development of allergic dermatitis (Nagano et al. 2001b). As a control, the same treatment was carried out using distilled water. Body weight

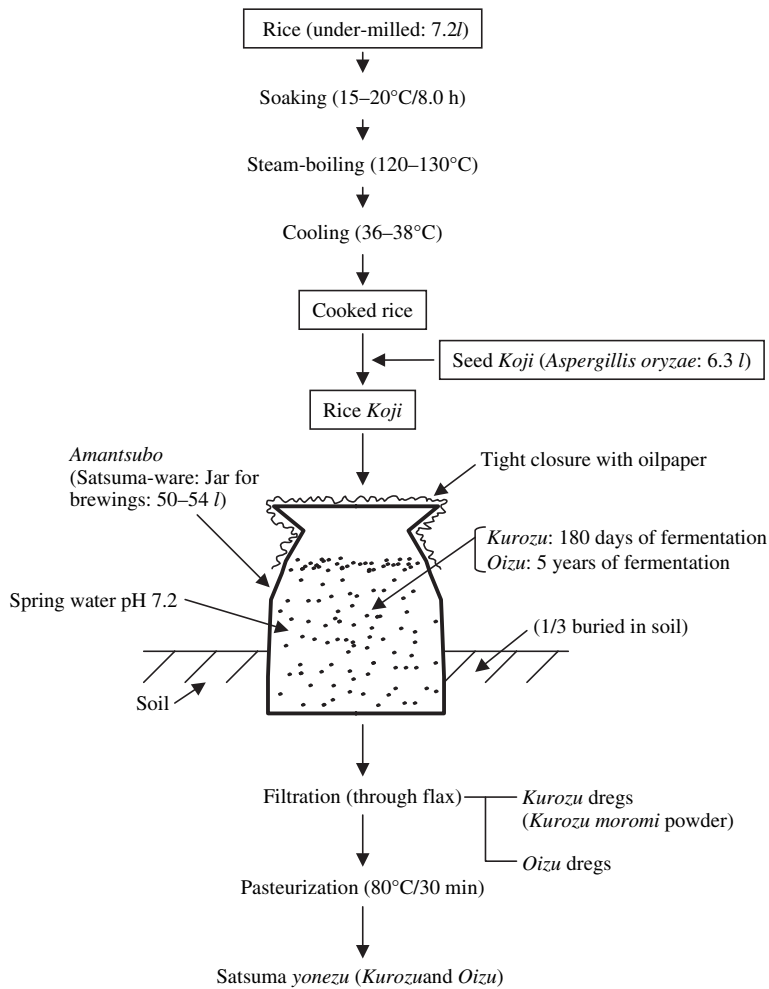


Figure 1. The procedure of production of kurozu moromi powder (kmp).

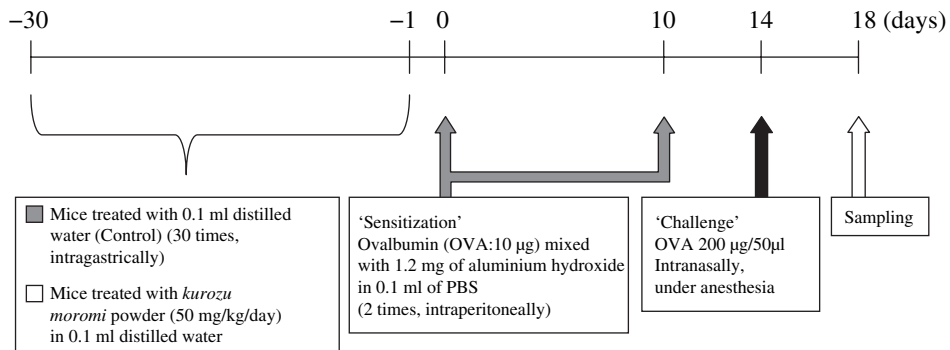


Figure 2. Experimental protocol. After pre-treatment of the kmp, BALB/c mice were sensitized twice i.p. with 10 µg OVA with Alum on day 0 and day 10, and thereafter challenged intranasally with 200 µg OVA in PBS 4 days after second sensitization. Samples were collected 4 days after challenge and used for assays (Figures 4, 6, 7 and 8).

was examined ( $n = 14$ , control;  $n = 16$ , kmp) every day before the first sensitization. Also, plasma was obtained ( $n = 10$  in each group) for IgE assay from the orbital venous plexus with heparinized micropipettes (Terumo, Tokyo, Japan) under anaesthesia with ketamine (45 mg/kg BW; Sankyo Co., Tokyo, Japan) and xylazine (8 mg/kg body weight; Bayer Co., Tokyo, Japan) intraperitoneally (i.p.).

The sensitization and challenge procedures were carried out by the methods described previously (Hayashi et al. 2002). In brief as shown in Figure 2, animals were sensitized on the next day after the final treatment of the kmp intraperitoneally (i.p.) with 10  $\mu$ g 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 200  $\mu$ g of OVA in 50  $\mu$ l of PBS under anaesthesia i.p. with ketamine (45 mg/kg 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 ( $n = 6$ , control;  $n = 8$ , kmp) were counted after blood smear stained with Giemsa solution. The total number was determined by multiplying the percentage of each cell type. The whole left lung ( $n = 13$ , control;  $n = 16$ , kmp) was fixed in 10% neutral buffered formalin (pH 7.4) and embedded in paraffin blocks. Five  $\mu$ m thickness sections prepared from embedded tissue were stained with haematoxylin and eosin (HE), toluidine blue or Periodic acid Schiff (PAS). The severity of lesions in each transversely sectioned bronchus-bronchiole was evaluated by light microscopy and scored (Hayashi et al. 2001) as follows: no lesions, 0; mucus or desquamated epithelial cells in the lumen, 1; a few goblet cells in the epithelial layer, 1; many such cells, 2; thickened basement membrane, 1; mild infiltration of eosinophils and lymphocytes in the mucosa, 1; severe infiltration in the mucosa, 2; hypertrophy of smooth muscle cells in mucosa, 1. In each mouse the score index (SI) was calculated by dividing the total score by the number of bronchi-bronchiole examined. The SI average was then determined for each group.

#### *Isolation of splenic cells*

Spleens from mice were aseptically removed and placed in phosphate buffered-balanced salt solution (PBBS; pH 7.4). Single-cell suspension was made by teasing spleens apart with scissors and filtering through a 250- $\mu$ m disposable syringe (Terumo Co., Tokyo, Japan). Cell suspensions were collected in sterile conical tubes (Assist Trading Co., Tokyo, Japan) after red blood cells were haemolysed by Tris-buffered  $\text{NH}_4\text{Cl}$  solution and washed in PBBS medium containing 0.1% heat-inactivated foetal calf serum (FCS; Gibco, Grand Island, NY, USA) followed by centrifugation at 800 *g* for 10 min at 4°C. Cells were counted using a haemocytometer and diluted in Dulbecco's modified Eagles's medium (DMEM; KC biological, Lenexa, Kans, USA) to a density of  $5 \times 10^6$  cells/ml. Viability of cells was more than 95% by trypan blue dye exclusion test. Splenocytes ( $1 \times 10^6$  cells in 200  $\mu$ l DMEM with FCS) mixed with OVA (20 $\mu$ g) were cultured for 48 h at 37°C in a 5% atmosphere and supernatants were obtained.

*Concentration of IFN- $\gamma$ , IL-4 and IL-5 in supernatants from cultured spleen cell fluid and plasma IgE by ELISA*

IL-4, IL-5, IFN- $\gamma$  (Techne Co., Minneapolis, MN, USA) and IgE (Endogen Inc., Woburn, MA, USA) mouse ELISA kits were purchased. The minimal detectable concentration was 2 pg/ml for IFN- $\gamma$ , IL-4 and IL-5, and 47 pg/ml for IgE. Supernatants from cultured splenic cells ( $n = 4$  in each group) at the time of sampling were assayed for those cytokines. The increased serum IgE ratio was expressed as follows. Increased ratio = IgE values at the time of final treatment of the kmp, or at the time of sampling/IgE values just before kmp treatment.

*Statistical analysis*

The data are expressed as the mean of samples examined  $\pm$  standard error ( $\pm$ SE). Unpaired Student's  $t$ -test was used to evaluate the significance of differences; a  $p$ -value less than 0.05 was considered significant.

## Results

The kmp treatment did not result in any growth retardation (Figure 3) from  $-31$  to  $-1$  days. The number of blood eosinophils, but not lymphocytes, was decreased in the kmp-treated mice compared to the control mice ( $p < 0.05$ ; Figure 4). On the other hand, no significant differences were observed in a number of monocytes and neutrophils between two groups (data not shown).

Compared to the control mice, the kmp treatment inhibited the increase in IgE, and this effect has been continued at the time of the sampling ( $p < 0.01$ ; Figure 5). There was no significant difference in IL-4 and IL-5 productions, whereas the values of IFN- $\gamma$  from splenic cells increased significantly in the mice treated with kmp compared to those in the control mice ( $p < 0.01$ ; Figure 6).

The severity of bronchial-bronchiolar lesions (Score index: Figure 7) in the mice treated with the kmp (SI: 2.4) was significantly reduced compared to that in the control mice (SI: 5.0,  $p < 0.01$ ).

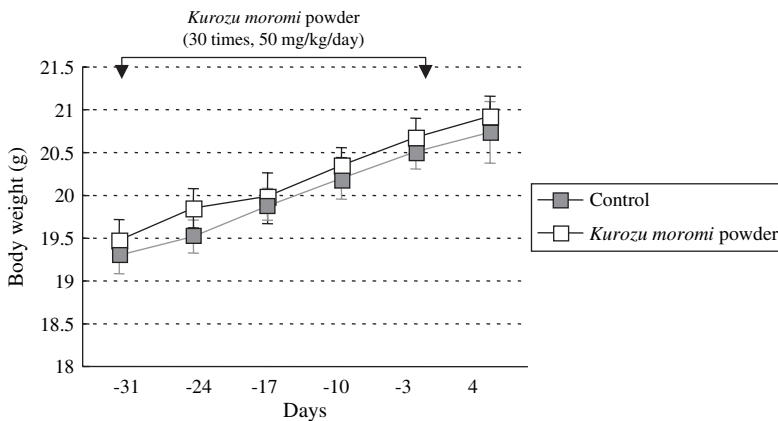


Figure 3. Body weight changes in mice treated with kmp or without kmp (the control group) until starting the induction of LAR. Data shown are mean  $\pm$  SE.

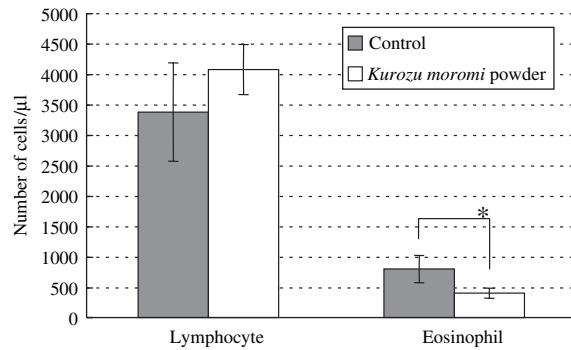


Figure 4. Total number of blood lymphocytes and eosinophils in both groups. Data shown are mean  $\pm$  SE. \* $p < 0.05$  between two groups.

In general, the lesions in bronchi were more severe than those in bronchiole. As shown in Figure 8A, there was many goblet cell metaplasia and hyperplasia of bronchial-bronchiolar swelled epithelial cells with prominent mucous exudate in bronchial-bronchiolar lumens in the control mice. In some cases, desquamated epithelial cells mixed with some eosinophils, lymphocytes and macrophages within lumens were observed. A severe peri-bronchial and -bronchiolar infiltration of lymphocytes and eosinophils with oedema and hyperaemia in bronchial-bronchiolar walls and hypertrophy and/or hyperplasia of the bronchial smooth muscles were observed. There was a thickening of the bronchial epithelial basement membranes in some cases. In addition, a large number of eosinophils and lymphocytes infiltrated in small arteries with swelled endothelial cells and degeneration of walls. A few mast cells were detected in the bronchial-bronchiolar mucosa in both groups. The changes in the mice treated with the kmp were much less severe than those in the control group (Figure 8B).

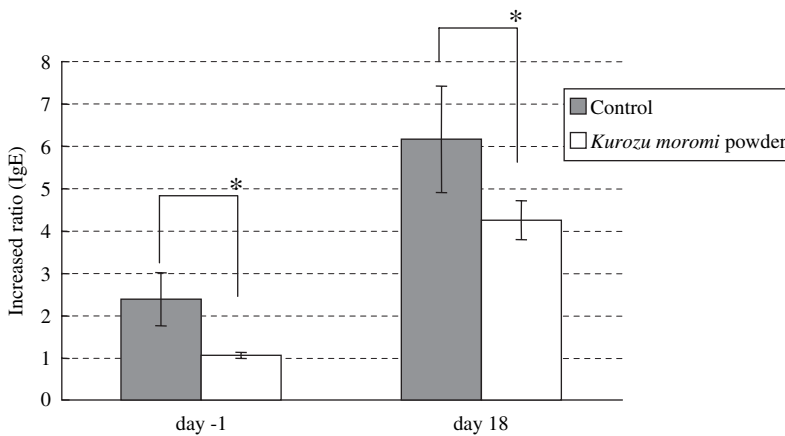


Figure 5. Increased IgE ratio in blood at the final time of the kmp-treatment in both groups. IgE ratio: IgE values at day 0/those at day  $-30$ . Data shown are mean  $\pm$  SE. \* $p < 0.01$  between two groups.

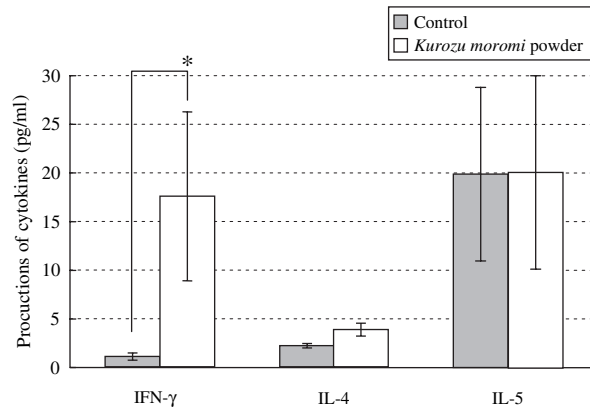


Figure 6. Production of IL-4, IL-5 or IFN- $\gamma$  by splenic cells in both groups. Data shown are mean  $\pm$  SE. \* $p < 0.01$  between two groups.

## Discussion

We demonstrated that BALB/c mice, which show genetically Th2-like responses to exogenous antigens (Reiner & Locksley 1995), pre-treated with kmp showed less severe late allergic bronchitis and bronchiolitis, which are characterized by infiltration of eosinophils and lymphocytes with an increased mucus production, compared to the control mice. The kmp treatment caused reduction of values in blood IgE, which produce Th2 cells (Mosmann & Coffman 1989), in BALB/c mice. Moreover, the increased systemic OVA-specific IFN- $\gamma$  (representative Th1 cytokine) production was paralleled with the decreased number of blood eosinophils. In addition, the kmp did not affect the bodyweight gain. Taken together, these data suggest that the reduced LAR in the mice treated with kmp may be due to the systemic enhancement of Th1 cell activity induced by the kmp. Furthermore, this treatment may, at least in part, shift the imbalance toward the Th1 type immunity from Th2 type reaction. This may not be mediated by mutual Th1/Th2 inhibitory effect (Mosmann & Coffman 1989) in LAR, since the production of Th2 cytokines (IL-4 and IL-5) was not suppressed.

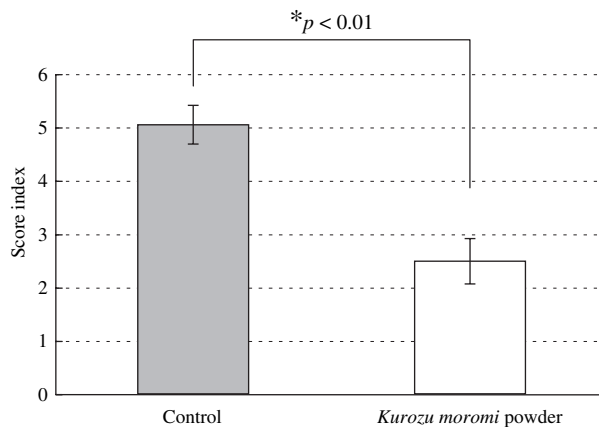


Figure 7. Score index (severity of bronchi-bronchial lesions) in both groups. Data shown are mean  $\pm$  SE. \* $p < 0.01$  between two groups.

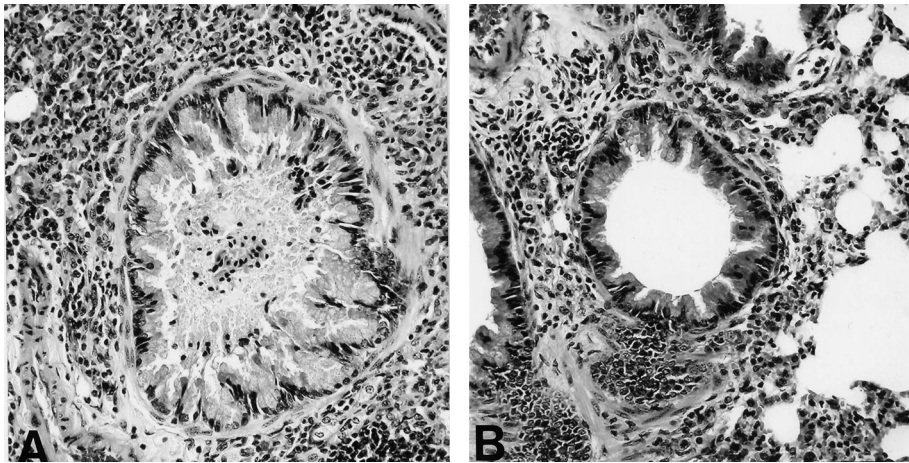


Figure 8. Representative histology from a control (A) and a mouse treated with kmp (B). Compared to a treated mouse, the lung tissues from a control mouse demonstrated a prominent goblet-cell metaplasia and hyperplasia with mucus and bronchiole epithelial cell shedding in the bronchial lumen, alterations in the integrity of the hypertrophic smooth muscle cell and a remarkable peribronchial infiltration of lymphocytes and eosinophils in bronchiole mucosa.  $\times 100$  HE.

Thus kmp may have an ability to induce Th1 cytokines. In addition, systemic Th1 responses induced by kmp may also operate locally as Th1 responses at bronchi-bronchiole areas as demonstrated previously (Hayashi et al. 2003).

The exact mechanisms of reduced LAR and the increased Th1 response by the treatment of kmp remain unclear. We suppose that Th1-reactions by kmp may be the result of the presence of arginine indirectly and/or directly as follows. It has been suggested that arginine enhances induction of Th1 and Th2 cytokine synthesis by Peyer's patch alpha/beta T cells, when mice are fed on an arginine-supplemented liquid diet (Kobayashi et al. 1998). Also dietary methionine and cysteine influence immune response *in vitro* (Takahashi et al. 1997). These suggest that administrated kmp containing amino acids may affect digestive immune systems leading to the development Th1 response. It seems likely, since kmp contains several kinds of amino acids and peptides (Fujino et al. 1990, 1992; Nagano et al. 2001a, 2001b). In addition, mice treated with kmp showed enlarged caecum containing foods with swelled lymphoid tissues compared to the control group (data not shown), suggesting another possibility that the administrated kmp may effect intestinal lymphoid tissues via the affecting intestinal bacterial flora (Kobayashi et al. 1998).

All mice ( $n = 16$ ) treated with kmp showed temporally erythaematous swelling of skins (e.g. mouth, hand and foot) shortly after OVA challenge (within 30 min to 2 h) in sensitized mice and thereafter those changes disappeared soon, whereas one out of 14 mice in the control group showed such changes (data not shown). Human subjects treated with nearly the same dose of kmp used here did not show any such change (Fujino et al. 1990, 1993). Also, the preventive experiments of kmp on allergic dermatitis in BALB/c mice (Nagano et al. 2001b) did not show such changes. Thus, the possibilities may, at least in part, be due to the effects of the artificially induced experimental methods used here, since one of the control mice showed such changes and/or species differences between human and mice.

There are general agreements that reduced early-life exposure to strong Th1 stimuli in industrialized country has skewed the Th1/Th2 balance towards Th2 responses. Thus, the reduced LAR by the treatment of kmp suggests that this food may have a potential to reduce LAR in human as an ideal food.

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