



Dietary Pulverized Konjac Glucomannan Prevents the Development of Allergic Rhinitis-Like Symptoms and IgE Response in Mice

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Konjac is a traditional Japanese food with a peculiar texture, and it has been suggested that its main ingredient, konjac glucomannan (KGM), ameliorates metabolic disorders such as diabetes and hypercholesterolemia. We have found that feeding with pulverized KGM (PKGM) prevents skin inflammation in a murine model of atopic dermatitis. Here, we show that dietary PKGM suppresses allergic rhinitis-like symptoms in mice upon immunization and nasal sensitization with ovalbumin (OVA). The PKGM-fed mice showed a much lower frequency of sneezing than in control animals. We also found that PKGM supplementation exclusively suppressed OVA-specific IgE response without affecting IgG1/IgG2a responses as well as systemic Th1/Th2 cytokine production. These results suggest that PKGM can be a beneficial foodstuff in preventing nasal allergy such as seasonal pollinosis.

Key words: allergic rhinitis; IgE; pulverized konjac glucomannan

Konjac, long consumed as a Japanese food, is manufactured using a konjac powder, which is obtained from the tubers of *Amorphophallus konjac*. The main ingredient of konjac powder is konjac glucomannan (KGM), a highly viscous polysaccharide composed of glucose and mannose residues at a molar ratio of 2:3 with β 1-4 linkages. Several lines of evidence suggest that dietary KGM has beneficial effects on health: oral intake of KGM prevents hyperglycemia and hyperlipidemia, and its potency is correlated with high viscosity.¹⁾ Lim *et al.*²⁾ reported that dietary KGM enhances IgA and IgG

secretion by lymphocytes from the rat mesenteric lymph node, suggesting that KGM can also modulate the immune system. However, the mode of action of dietary KGM in regulating the metabolic and/or immune pathway is largely unknown.

We have found a novel, beneficial immunomodulatory function of processed KGM: feeding with pulverized low-viscous KGM (PKGM) prevented the development of eczematous skin inflammation and hyper-IgE production in NC/Nga mice,^{3,4)} a well-known animal model of atopic dermatitis (AD).⁵⁾ More recently, we found that AD-like skin lesions (dermal thickening, eosinophilia, and mastocytosis), as well as local overproduction of substance P and proinflammatory cytokines, were all impaired in PKGM-fed NC/Nga mice,⁶⁾ suggesting that dietary PKGM suppresses a wide array of skin inflammatory immune responses. We have also reported that oral intake of PKGM inhibited the increase in serum IgE and IgG levels induced by continuous injection of syngeneic keratinocyte extracts in BALB/c mice.⁷⁾ Taken together, these results suggest that dietary PKGM can be beneficial in preventing allergy, although the precise mechanism underlying the anti-inflammatory action of PKGM and its effect on allergen-induced inflammatory disorders remain to be investigated.

In the present study, we found that dietary PKGM prevents the development of allergic rhinitis-like inflammation in mice upon nasal challenge with OVA. We also found that PKGM exclusively suppresses OVA-specific IgE antibody response without affecting systemic Th1/Th2 cytokine profile.

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Abbreviations: AD, atopic dermatitis; ELISA, enzyme-linked immunosorbent assay; i.p., intraperitoneal; KGM, konjac glucomannan; OVA, ovalbumin; PBS, phosphate-buffered saline; PKGM, pulverized konjac glucomannan

Materials and Methods

Konjac glucomannan. Two kinds of konjac glucomannan (KGM) powder, high viscous KGM (PROPOL[®] A, PA) and pulverized KGM (S-P), were prepared by Shimizu Chemical Corporation (Hiroshima, Japan), and their physicochemical properties were analyzed as previously described.^{3,4)} The GM contents of PA and S-P powder were 98.1% and 97.0% respectively, and the viscosity-average molecular weight of both samples were estimated to be over 1,000 kDa (as assessed using an Ubbelohde viscometer). The mean particle size of these KGM powders was estimated to be 300 μ m for PA and 75–100 μ m for S-P. The peak viscosity of 1% solution was more than 100,000 mPa·s for PA and approximately 35,000 mPa·s for S-P.

Animals and diets. Four-week-old female BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). They were maintained under specific pathogen-free conditions for 8 weeks on a control diet (MF diet, Oriental Yeast, Tokyo) or a KGM diet (an MF diet containing 5% w/w KGM powder) *ad libitum*. All animals were housed in an animal facility kept at $22 \pm 2^\circ\text{C}$ under a 12-h light/12-h dark cycle. All animal studies were carried out using protocols reviewed and approved by the Committee on Animal Experimentation of Hiroshima University.

Immunization, intranasal sensitization, and evaluation of nasal symptoms. Immunization and nasal challenge with ovalbumin (OVA) were performed by previously described protocols,⁸⁾ with some modifications. Briefly, at 7 and 9 weeks of age (*i.e.*, 3 and 5 weeks after the start of feeding with KGM), the mice were immunized intraperitoneally (*i.p.*) with either phosphate-buffered saline (PBS) or 20 μ g of OVA (Sigma, St. Louis, MO) emulsified in 2.25 mg of alum adjuvant (LSL, Tokyo) in 100 μ l of total volume. Two weeks after the second immunization, the mice were intranasally challenged with OVA solution (25 mg/ml solution in PBS, 20 μ l/mouse) by daily instillation without anesthesia (Fig. 1A). To evaluate allergic rhinitis-like symptoms, the mice were placed into an observation cage (one animal/cage) just after intranasal instillation with OVA, and the number of sneezes was counted for 5 min under blinded conditions.

Analysis of plasma immunoglobulins. Twelve hours after the last (eleventh) nasal challenge with OVA, blood samples were collected from all the groups of mice. Total plasma IgE, IgG1, and IgG2a levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA), as previously described.³⁾ Plasma OVA-specific IgE, IgG1, and IgG2a titers were also analyzed by ELISA, as described previously.⁷⁾ Briefly, 96-well microtiter plates were coated with OVA solution (100 μ g/ml) at 4°C overnight, and then incubated with

diluted plasma samples ($\times 10$ for IgE, and $\times 500$ for IgG1 and IgG2a). After reaction with secondary biotinylated rat anti-mouse IgE, IgG1, or IgG2a antibody (BD Bioscience, San Jose, CA, 1:250 dilution) and subsequent incubation with streptavidin-conjugated alkaline phosphatase (Zymed Laboratories, San Francisco, CA, 1:1,000 dilution), enzyme reaction was performed using the AttoPhos[®] substrate system (Promega Corporation, Madison, MI). Fluorescence intensity of each well was then analyzed with a Wallac 1420 ARVO Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA).

Cell culture and cytokine analysis. Spleens were removed from the mice, and total mononuclear cell suspension was prepared by treatment with lysis buffer (150 mM NH_4Cl , 15 mM NaHCO_3 , 0.1 mM EDTA-2Na, pH 7.3) to lyse red blood cells. After washing 3 times with PBS, splenocytes (4×10^6 cells/ml) were stimulated with OVA (100 μ g/ml) for 96 h in RPMI-1640 medium (Sigma) supplemented with 100 U/ml of penicillin, 100 μ g/ml of streptomycin, 50 μ M of 2-mercaptoethanol, and 10% fetal bovine serum (BioWest, Rue de la Caille, France) at 37°C in 5% CO_2 /95% air. The IL-4, IL-5, and IFN- γ levels in the culture supernatant were determined by sandwich ELISA using reagents and instructions from BD Biosciences. IL-13 assay was carried out using DuoSet mouse IL-13 (R&D Systems, Minneapolis, MN).

Statistical analysis. Statistical analysis was performed by Student's *t*-test. $P < 0.05$ was accepted as the level of significance.

Results

Dietary PKGM prevents the development of allergic rhinitis-like sneezing symptoms in OVA-sensitized mice

To investigate the effect of low-viscous pulverized KGM (PKGM) on allergic nasal inflammation, BALB/c mice were fed a PKGM-containing diet or diets containing non-pulverized KGM powder for 3 weeks, and then immunized twice with OVA/alum at a 2-week interval. Two weeks after the second immunization, they were intranasally challenged with OVA solution to induce nasal hyperreactivity (Fig. 1A). All groups of mice were continuously fed KGM-containing diets or a control MF diet during the entire sensitization period.

First we tested the effect of dietary PKGM on rhinitis-like symptoms of the OVA-sensitized mice by counting their sneezing behavior. Control mice immunized with OVA/alum and intranasally challenged with OVA (OVA/OVA) started marked sneezing at the eighth nasal sensitization, and this symptom was further exaggerated towards the eleventh challenge (shown closed circles in Fig. 1B). In contrast, the development of sneezing was significantly prevented in PKGM-fed OVA/OVA mice (indicated by closed triangles in

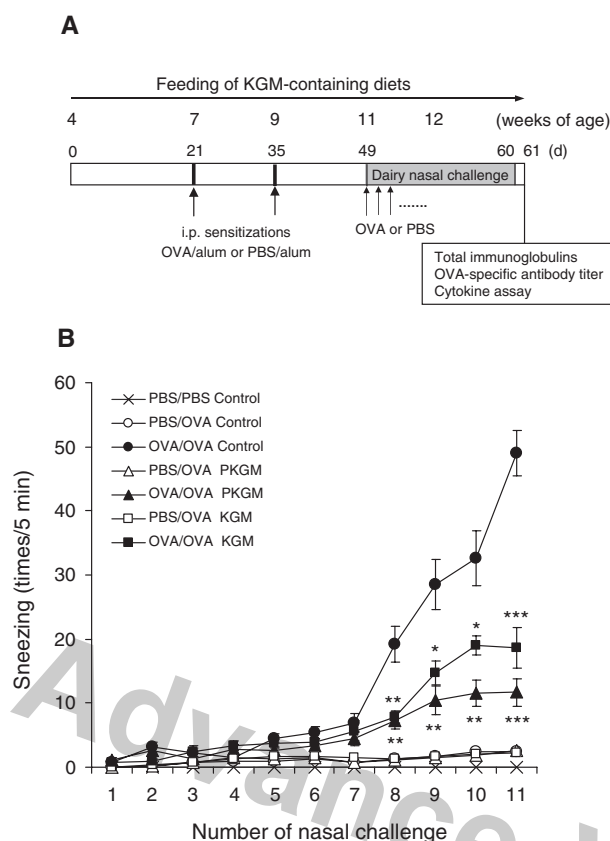


Fig. 1. Dietary KGM Prevents the Development of Nasal Sneezing in BALB/c Mice upon Sensitization with OVA.

A, Experimental scheme. Four-week-old BALB/c mice were continuously fed a PKGM- or non-pulverized KGM-containing diet. Three weeks later, those mice were immunized and intranasally challenged with OVA, as described in "Materials and Methods." B, The numbers of sneezes per 5 min were counted just after nasal challenge with OVA. Values are expressed as means $\pm$ SD with six mice per group. * P < 0.05, ** P < 0.01, *** P < 0.001 (as compared to control OVA/OVA mice).

Fig. 1B). Sham control groups fed a control diet (PBS/PBS or PBS/OVA, shown as x-marks and open squares respectively) or the PKGM diet (PBS/OVA, shown as open triangles) did not show sneezing. These data indicate that oral intake of PKGM prevents rhinitis-like symptoms in mice sensitized with OVA. We observed that feeding the non-pulverized KGM diet also significantly suppressed the development of sneezing (shown as closed squares in Fig. 1B), although the potency was less prominent than that observed in PKGM-fed mice.

Dietary PKGM exclusively suppresses OVA-specific IgE response and total IgE level

To evaluate the effect of dietary PKGM on the immune response, we next examined OVA-specific antibody responses 24 h after the eleventh nasal challenge with OVA. The specific IgE response was evident in control mice immunized and challenged with OVA (OVA/OVA), but the increase in IgE titer was significantly suppressed in PKGM-fed OVA/OVA mice

(Fig. 2A). In contrast to the inhibitory effect on IgE response, the OVA-specific IgG1 and IgG2a responses were indistinguishable as between the PKGM-fed mice and the control mice. We also found that PKGM-fed mice showed a significant decrease in plasma total IgE concentration, while IgG1 and IgG2a levels were unaffected (Fig. 2B). Mice fed non-pulverized KGM also showed antibody responses similar to those seen in PKGM-fed mice; *i.e.*, a significant down-regulation of OVA-specific IgE titer and a trend toward decrease (P = 0.07) in total IgE levels. These results indicate that oral supplementation with KGM-containing diets preferentially down-regulates *in vivo* IgE production.

Oral intake of KGM had no effect on the secretion of Th1/Th2 cytokines from splenocytes stimulated with OVA

Antigen-driven isotype switching of immunoglobulins is tightly regulated by cytokines, which drive germline transcription at the immunoglobulin constant region loci: Th2-dominated cytokine milieu favors the IgE or IgG1 class switch recombination,⁹⁾ whereas isotype switching to the IgG2a subclass is directly modulated by a Th1-associated transcription factor, T-bet.¹⁰⁾ Above exclusive suppression of the OVA-specific IgE response (with no effect on IgG1 or IgG2a responses) in KGM-fed mice implies that down-regulation of IgE production by dietary PKGM or non-pulverized KGM may not be attributable to its effect on the Th1/Th2 cytokine milieu. To test this possibility, we analyzed cytokine production by splenocytes from these mice upon *in vitro* stimulation with OVA. We found that secretion of Th2 cytokines (IL-4, IL-5, and IL-13) by splenocytes from PKGM- or non-pulverized KGM-fed OVA/OVA mice was comparable to those seen in control OVA/OVA mice (Fig. 3). Similarly, splenic IFN- γ production was not affected by feeding of KGM samples. These results indicate that oral intake of KGM has no effect on systemic Th1/Th2 cytokine production by splenocytes.

Discussion

We have reported that dietary PKGM of small particle size suppressed skin inflammation and hyper-IgE production in AD-prone NC/Nga mice.^{3,4)} In the present study, we found that PKGM prevented allergic rhinitis-like symptoms and IgE response in mice upon nasal challenge with OVA, further providing evidence that PKGM is a beneficial foodstuff in preventing allergy and allergen-specific IgE response.

Our previous studies with NC/Nga mice indicated that neither non-pulverized KGM nor re-granulated PKGM suppressed AD-like skin lesions, suggesting that a small particle size of PKGM powder is critical for its eczema-preventive effect.^{3,4)} By contrast, here we found a significant anti-allergic effect of non-pulverized KGM powder, although its potency was less prominent than that of PKGM (Fig. 1B). The differential efficacy of

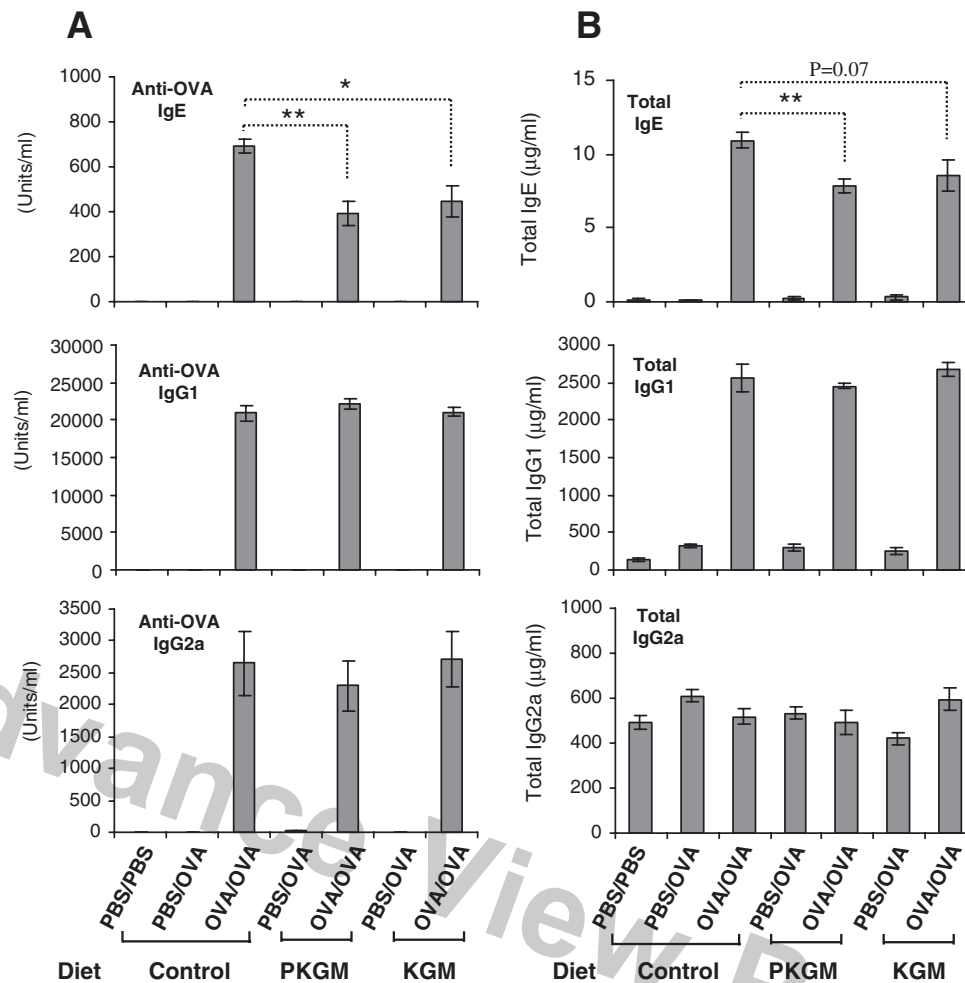


Fig. 2. Dietary PKGM Preferentially Suppresses Allergen-Specific IgE Response and Total IgE Levels upon Sensitization with OVA.

After the eleventh nasal challenge, plasma samples were collected from each group of mice, and antibody responses were analyzed by ELISA. A, OVA-specific IgE, IgG1, and IgG2a responses. B, Total immunoglobulin levels. Values are expressed as means $\pm$ SD with six mice per group. * $P < 0.05$, ** $P < 0.01$ (as compared to control OVA/OVA mice).

non-pulverized KGM on these two atopy models might be due to distinct mechanisms underlying the pathogenesis of OVA-induced rhinitis in BALB/c mice and those of eczema development in NC/Nga mice. Indeed, OVA-induced nasal hyperreactivity requires Fc ϵ RI as well as IL-13,^{8,11} whereas AD-like skin lesions are seen in STAT6-deficient NC/Nga mice that have no Th2 cells or IgE antibodies.¹² Thus the OVA-induced rhinitis model appears to be more dependent on the classical IgE-mast cell axis of the allergic response than the NC/Nga model. Non-pulverized KGM might preferentially down-regulate these type-I allergic pathways, although which are largely dispensable for eczema development in NC/Nga mice.

We found that dietary PKGM exclusively suppressed the OVA-specific IgE response without affecting the IgG1/IgG2a responses and systemic Th1/Th2 cytokine production (Figs. 2A and 3). These data imply that suppression of the IgE response is not attributable to a skewed Th1 response, but rather driven by hitherto unknown mechanisms other than canonical Th1/Th2

dogma. We also found that PKGM feeding specifically suppressed the increase in total IgE levels, but not that of IgG1 (Fig. 2B). This result differs from our previous data, which indicated that dietary PKGM suppressed both total IgE and IgG1 levels in NC/Nga mice^{4,6} and in BALB/c mice upon injection with syngeneic keratinocyte extract.⁷ This discrepancy in the data might be due to differences in experimental settings (*e.g.*, mouse strain, immunization protocols).

How does dietary PKGM preferentially down-regulate the IgE response in our rhinitis model? We have several assumptions about the underlying mechanisms. The first possibility is that dietary PKGM suppresses only the IgE class switch recombination (CSR), but not IgG1 CSR. One such IgE-inhibitory candidate is IL-21, which specifically down-regulates IL-4-driven C ϵ germ-line transcription without abrogating STAT6 activation.¹³ *In vivo*, IL-21-deficient mice show exaggerated IgE production.¹⁴ More recently, natural killer T cell-derived IL-21 has been found to trigger B ϵ cell apoptosis to inhibit IgE production.¹⁵ All these data

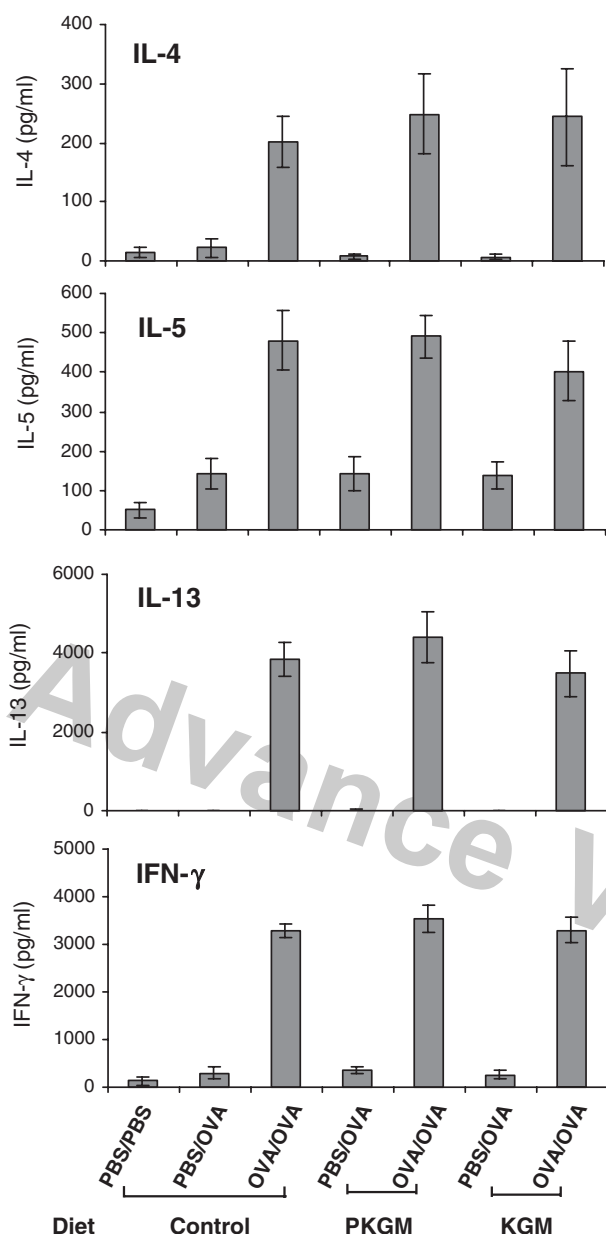


Fig. 3. Dietary KGM Did Not Affect Th1/Th2 Cytokine Production by Splenocytes from OVA-Sensitized Mice.

Total splenocytes from KGM-fed mice after the eleventh nasal challenge were stimulated with OVA for 96 h, and IL-4, IL-5, IL-13, and IFN- γ levels in the culture supernatant were quantified by sandwich ELISA, as described in "Materials and Methods." Values are expressed as means $\pm$ SD with 6 mice per group.

establish IL-21 as a critical negative regulator of IgE synthesis, although further investigation is needed to assess the actual involvement of this cytokine in our system. Second, IgE CSR might be down-regulated by a cell-mediated pathway. Obayashi *et al.*¹⁶⁾ recently demonstrated that dendritic cells (DCs) selectively inhibited IgE CSR, and thus a similar IgE inhibitory loop might also be activated in the mucosal DC-B cell interface of PKGM-fed mice. Finally, dietary PKGM might specifically suppress IgE⁺ plasma cell differentiation *in vivo*. This possibility is based on a recent

report indicating that IgE⁺ plasma cells are generated *via* an exceptional *in vivo* maturation program, in which somatic hypermutation and affinity maturation take place in IgG1⁺ cells, and a post-IgE-switching phase in which IgE⁺ B cells differentiate swiftly into plasma cells.¹⁷⁾ Hence it is also possible that dietary PKGM negatively regulates the latter unique plasma cell differentiation pathway to inhibit IgE production. Whatever the mechanism is, analysis of *in vivo* IgE⁺ and IgG1⁺ B cell/plasma cell subsets in PKGM-fed mice should be the first experiment to address these possibilities.

In addition to the IgE-suppressive mechanisms, the precise mode of action by which dietary PKGM prevents allergic rhinitis is still unknown. Since Fc ϵ RI signaling is essential to OVA-induced nasal hyperreactivity,⁸⁾ suppression of allergen-specific IgE would be the primary target of dietary PKGM in inhibiting rhinitis. However, other anti-inflammatory roles of PKGM might also be involved, because we have found that PKGM feeding abrogated a wide array of skin inflammatory immune responses in NC/Nga mice, including defective skin thickening, decreased local mastocytosis/eosinophilia, and overall impairment in cutaneous overproduction of substance P, proinflammatory cytokines, and a Th2 cell-attractive chemokine, CCL17/TARC.⁶⁾ Our preliminary histopathological analysis indicates that nasal mast cell infiltration is severely impaired in PKGM-fed mice (unpublished data), suggesting that nasal proinflammatory immune responses might be down-modulated upon feeding with PKGM.

Another important issue is how dietary KGM interacts with gut mucosa to fulfill an anti-allergic function. One plausible possibility is that KGM modulates gut microflora, which in turn prevent IgE production and nasal inflammation. Indeed, it has been reported that dietary KGM increases fecal bifidobacteria,¹⁸⁾ an intestinal microflora whose reduction is highly correlated with atopy predisposition.¹⁹⁾ Another noteworthy evidence is that acid-hydrolyzed KGM has a greater prebiotic effect than does KGM in increasing cecal and fecal bifidobacteria.²⁰⁾ This result might provide a mechanistic insight into our observation that PKGM showed a more potent anti-rhinitis effect than did non-pulverized KGM (Fig. 1B). That is, the superior anti-allergic effect of PKGM might be attributable to its better bioavailability to gut microbiota. Practically, PKGM, with its finer particle size, appears to be degraded more easily into oligosaccharides than non-pulverized KGM, because the increase in specific surface area promotes bacterial adherence and/or enzymatic access to the surface area of KGM molecule. In addition to the possible prebiotic action, the direct effect of dietary PKGM on the gut immune system is another intriguing possibility to be addressed.

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