

Allergic Airway Eosinophilia Is Suppressed in Ovalbumin-Sensitized Brown Norway Rats Fed Raffinose and α -Linked Galactooligosaccharide¹

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ABSTRACT We recently found that dietary raffinose suppressed allergic airway eosinophilia in ovalbumin-sensitized Brown Norway rats. Using this model in the present study, we compared the efficacy of other oligosaccharides with that of raffinose. Brown Norway rats were immunized s.c. with ovalbumin on d 0 and exposed to aerosolized ovalbumin on d 20; broncho-alveolar lavage fluid was obtained on d 21. In Expt. 1, rats were fed a control diet or diets supplemented with different oligosaccharides (50 g/kg diet, raffinose, α -linked galactooligosaccharide, fructooligosaccharide, and xylooligosaccharide). The number of eosinophils in the fluid was significantly lower in rats fed raffinose and α -linked galactooligosaccharide diets than in those fed the control diet. Dietary fructooligosaccharide and xylooligosaccharide did not affect airway eosinophilia. In Expt. 2, i.p. administration of raffinose and α -linked galactooligosaccharide, but not fructooligosaccharide and xylooligosaccharide, suppressed airway eosinophilia in rats fed the control diet. In Expt. 3, suppression of airway eosinophilia by dietary α -linked galactooligosaccharide occurred in cecetomized rats administered neomycin. Reduced levels of interleukin (IL)-4 and IL-5 mRNA in lung tissue were associated with the suppression of airway eosinophilia. We propose that indigestible oligosaccharides differ in their suppressive effect on allergic airway eosinophilia in ovalbumin-sensitized Brown Norway rats and that the effect appears not to be mediated by intestinal microflora. *J. Nutr.* 135: 538–543, 2005.

KEY WORDS: • oligosaccharide • ovalbumin • allergy • airway eosinophilia • Brown Norway rats

Prebiotics such as fermentable dietary fibers and indigestible oligosaccharides can influence immune health by modulating the intestinal microflora (1). Because immunological factors contribute to the pathogenesis of atopic diseases (2), improvement of intestinal microflora by consuming prebiotics may potentially play a role in preventing and treating such diseases. There is increasing evidence that consumption of indigestible oligosaccharides increases the proportion of beneficial lactic acid bacteria such as bifidobacteria and lactobacilli in the human colon (3–8). Because administration of lactic acid bacteria (i.e., probiotics) was reported to control the clinical symptoms of atopic diseases in humans (9–12), consumption of indigestible oligosaccharides would theoretically prevent and/or ameliorate such diseases.

However, the idea should be examined more directly by experimental and clinical studies.

Raffinose (RAF)³ is an indigestible oligosaccharide that occurs naturally in many vegetables and fruits (13). Because Benno et al. (14) reported that oral administration of RAF increased bifidobacteria and decreased bacteroides and clostridia in human feces, RAF can be considered to be a prebiotic agent and would be expected to prevent atopic diseases through increasing bifidobacteria in the colon. In fact, it was reported that oral administration of RAF in patients with atopic dermatitis improved symptoms such as eczema and pruritus (15). More recently, we showed that dietary RAF suppressed airway infiltration of eosinophils after antigen exposure in ovalbumin (OVA)-sensitized Brown Norway (BN)

¹ Supported by Grant-in-Aid for Scientific Research (B) from The Ministry of Education, Science, Sports and Culture of Japan and by Technical Developing Program for Making Agribusiness in the Form of Utilizing the Concentrated Know-how from Private Sector from The Ministry of Agriculture, Forestry and Fisheries of Japan.

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³ Abbreviations used: BAL, broncho-alveolar lavage; BALF, broncho-alveolar lavage fluid; BN, Brown Norway; CON, control; DIG, digoxigenin; FOS, fructooligosaccharide; GAPDH, glyceraldehyde triphosphate dehydrogenase; GOS, α -linked galactooligosaccharide; IFN, interferon; IL, interleukin; OVA, ovalbumin; PLSD, protected least significant difference; RAF, raffinose; Th2, T helper type 2; XOS, xylooligosaccharide.

rats (16). Because antigen-induced airway eosinophilia is a condition representing a late-phase inflammation in atopic asthma (17), our results suggest that dietary RAF affects immune health to prevent atopic asthma. Additionally, our study showed that suppression of allergic airway eosinophilia by dietary RAF was still observed in cecectomized rats administered neomycin and that i.p. administration of RAF also suppressed eosinophilia (16). These results suggest little contribution of colonic bacteria to the action of dietary RAF in this asthma model. Thus, mechanism(s) other than prebiotic action may be involved in the prevention of atopic diseases through RAF consumption. The present study was designed to compare the efficacy of different prebiotic oligosaccharides on allergic airway eosinophilia in OVA-sensitized BN rats.

MATERIALS AND METHODS

Animals and diet. Male BN rats (Charles River), 5 wk old at the start of the experiment, were housed in individual cages in a temperature-controlled ($23 \pm 2^\circ\text{C}$) room with a dark period from 1900 to 0500 h. Before the experiment, they had free access to water and a purified diet prepared according to the composition of the AIN-93G diet (18). This diet was used as the control (CON) diet. The oligosaccharide-supplemented diets were prepared by adding (50 g/kg diet) of RAF (raffinose pentahydrate, Nippon Beet Sugar Mfg.), α -linked galactooligosaccharide (GOS) synthesized according to Yamashita et al. (19), fructooligosaccharide (FOS, Fructooligo-95P, Meiji Seika), or xylooligosaccharide (XOS, Xylooligo-95P, Suntory) to the CON diet at the expense of α -cornstarch. Supplementation of these oligosaccharides at this level did not alter the physical characteristics (i.e., texture) of the diet.

This study was approved by the Hokkaido University Animal Use Committee, and rats were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University.

Experimental design. In Expt. 1, after 7 d of consuming the CON diet, 30 rats were actively immunized with OVA on d 0 as described below and fed for a further 7 d. Rats were then divided into 5 groups ($n = 6$) and fed the CON, RAF, GOS, FOS, or XOS diet. All rats were challenged by exposure to aerosolized OVA on d 20, and broncho-alveolar lavage fluid (BALF) and lung tissue were obtained on the next day as described below.

Experiment 2 was carried out similarly to Expt. 1 except that all rats were fed the CON diet and injected i.p. with 0.1 mL saline or 0.5% (wt:v) solution of RAF, GOS, FOS, or XOS daily from d 7 to 20.

In Expt. 3, after 7 d of consuming the CON diet, 24 rats were actively immunized with OVA on d 0 and divided into 2 groups ($n = 12$) of cecectomized and sham-operated rats. The operations were carried out on d 5 as previously described (20). Thereafter, the cecectomized rats were administered neomycin sulfate i.g. (80 mg/kg body weight, Wako Pure Chemical Industries) daily. Rats were fed the CON diet for 7 d after immunization, and the rats in each operation group were then further divided into 2 groups ($n = 6$) of rats fed the CON diet or the GOS diet. All rats were challenged by exposure to aerosolized OVA on d 20, and BALF and lung tissue were obtained on the next day. The colonic contents of all rats were obtained and subjected to enumeration of total anaerobic bacteria.

Immunization and challenge. Each rat was actively immunized by a s.c. injection of 1 mg OVA (Grade V, Sigma Chemical) suspended in 0.5 mL Imject Alum into the back of the neck. At the same time, 0.2 mL *Bordetella pertussis* vaccine (Wako Pure Chemical Industries) containing 6×10^9 heat-inactivated bacilli in saline was administered i.p. as an adjuvant. On d 20 after immunization, the rats were exposed to 1% (wt:v) OVA solution aerosolized by an ultrasonic nebulizer (NE-U12; Omron) for 10 min.

Broncho-alveolar lavage (BAL) and cell count. At 24 h after the OVA challenge, the rats were anesthetized by an i.p. injection of a mixed solution of ketamine hydrochloride (70 mg/kg body weight) and xylazine hydrochloride (8 mg/kg body weight). After a laparot-

omy, the rats were killed by bleeding from the abdominal aorta. BALF was obtained and subjected to a cell count as previously described (21). In brief, BAL was performed with 5×5 mL HBSS (GIBCO-BRL), and the cytospin preparations were subjected to staining with Diff-Quick staining solution (International Reagents). In each sample, at least 500 cells were identified according to standard structures as alveolar macrophages, eosinophils, neutrophils, lymphocytes, or other cells. After BALF collection, the lung tissues were then subjected to RNA isolation.

Isolation and analysis of RNA. Total RNA was isolated from lung tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA samples were treated with DNase RQ1 (Promega) to remove any genomic DNA. The samples were subjected to semiquantitative RT-PCR to amplify interleukin (IL)-4, IL-5, and interferon (IFN)- γ cDNAs as previously described (16). The PCR products, which were separated by 2% agarose gel electrophoresis, were transferred to a nylon membrane (Biodyne Plus), fixed by UV crosslinking, and the blots were hybridized with each inner oligonucleotide probe labeled with digoxigenin (DIG) using a DIG oligonucleotide tailing kit (Roche Diagnostics). The hybridization signals were detected with a DIG luminescence detection kit (Roche Diagnostics), and the bands developed on X-ray film were quantitated using NIH Image. The signal intensity of each band was normalized by a comparison with the intensity of glyceraldehyde triphosphate dehydrogenase (GAPDH). The sequence of PCR primers and hybridization probes for IL-4, IL-5, IFN- γ , and GAPDH were described previously (16).

Enumeration of total anaerobic bacteria in the colonic content. Bacteriological analysis of the large intestinal content of rats was carried out according to the method of Mitsuoka et al. (22). Briefly, the fresh samples were diluted in 10-fold steps with anaerobic phosphate buffer. A sample (0.05 mL) of each dilution was inoculated onto BL agar (Nissui) supplemented with 5% (v:v) defibrinated horse blood for anaerobic bacteria. Anaerobic incubation was carried out at 37°C for 48 h using AnaeroPack kit (Mitsubishi Gas Chemical). The numbers of bacteria were expressed as the logarithm of colony-forming units (cfu).

Statistical analysis. Results were expressed as means $\pm$ SEM. Significance was evaluated by 1-way or 2-way ANOVA followed by post hoc Fisher's PLSD test. Differences were considered significant at $P < 0.05$. The statistical calculations were carried out using StatView 5.0 computer software (SAS Institute). A post hoc power analysis was done using G*Power computer software (freeware by Buchner, A., Faul, F. & Erdfelder, E.; <http://www.psych.uni-duesseldorf.de/aap/projects/gpower>) to ascertain whether the number of rats used had been sufficient to detect significant differences.

RESULTS

Almost all cells in the BALF of normal rats were alveolar macrophages. Our preliminary experiments showed that $>95\%$ of total cells in the BALF of both nonimmunized OVA-challenged rats and OVA-immunized bovine serum albumin (i.e., unrelated protein)-challenged rats were alveolar macrophages (unpublished data). In the OVA-immunized and OVA-challenged rats in the present study, however, ~ 45 – 60% of total cells in BALF were eosinophils (Figs. 1A, 2A and 3A), indicating that this treatment induced OVA-specific allergic airway eosinophilia.

In Expt. 1, total cell, eosinophil, and lymphocyte numbers in BALF were significantly lower in rats fed the RAF and GOS diets than in those fed the CON diet (Fig. 1A). Cell numbers did not differ among rats fed the CON, FOS, and XOS diets. The number of alveolar macrophages did not differ among the groups. Neutrophil numbers tended to be lower in rats fed the RAF and GOS diets than in the other groups ($P = 0.13$). We estimated by power analysis that 19 rats/group were required for a power value of 0.95. IL-4 mRNA levels in the lung tissue of rats fed the RAF and GOS diets tended to be lower than

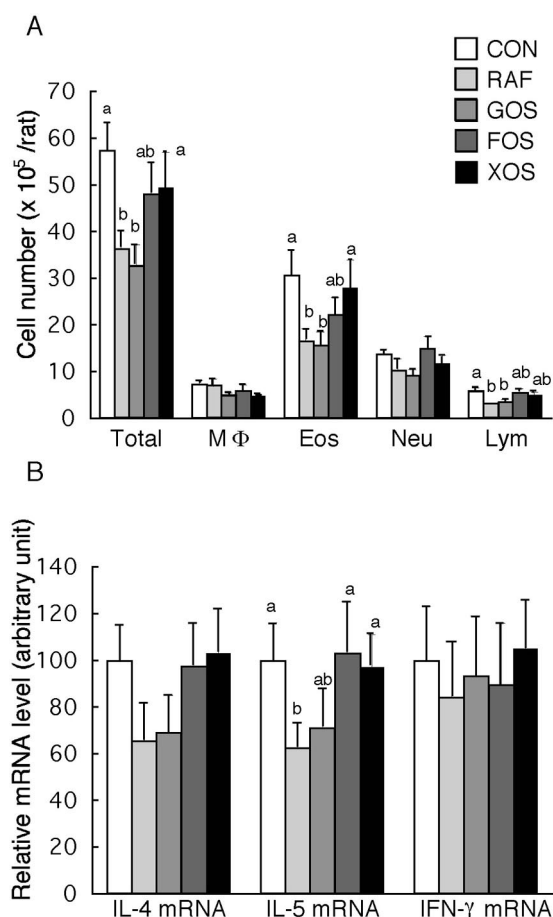


FIGURE 1 Cell profiles in BALF (panel A) and cytokine mRNA levels in lung tissue (panel B) of OVA-sensitized BN rats fed CON, RAF, GOS, FOS, or XOS diets (Expt. 1). BALF and lung tissues were obtained the day after OVA challenge. The mRNA levels were expressed relative to the mean of CON rats, taken as 100. Values are means $\pm$ SEM, $n = 6$. Total, M Φ , Eos, Neu, and Lym represent total cells, macrophages, eosinophils, neutrophils, and lymphocytes, respectively. Means for a variable without a common letter differ, $P < 0.05$.

those of rats fed other diets ($P = 0.27$; Fig. 1B), and we estimated that 21 rats/group would have been needed to detect a significant difference. IL-5 mRNA levels in rats fed the RAF diet were significantly lower than those fed the CON, FOS and XOS diets. IFN- γ mRNA levels were however the same among the groups.

In Expt. 2, an i.p. injection of RAF and GOS significantly reduced total cell and eosinophil numbers in BALF compared with control rats, whereas an i.p. injection of FOS and XOS had no effect (Fig. 2A). The number of alveolar macrophages, neutrophils, and lymphocytes did not differ among the groups. IL-4 and IL-5 mRNA levels in the lung tissue did not differ significantly among the groups ($P = 0.72$ and 0.32 , respectively) but were roughly parallel to total cell and eosinophil numbers in the BALF (Fig. 2B). Using power analysis, we estimated that 86 and 34 rats/group would have been required to detect significant differences in IL-4 and IL-5, respectively. IFN- γ mRNA levels did not differ among the groups.

In Expt. 3, diet, cecectomy, and their interaction affected the total number of anaerobic bacteria in the colonic contents (Table 1). In sham-operated rats, the number of anaerobic bacteria in the colon, including the cecum, was significantly

higher in rats fed the GOS diet than in those fed the CON diet. Given the gross appearance of the bacterial colony, this change appeared to be due to an increase in bifidobacteria. Cecectomy plus neomycin administration significantly reduced the number of anaerobic bacteria in the remaining colon, and there was no difference due to diet in cecectomized rats. Diet did affect the total cell and eosinophil numbers in BALF (Fig. 3A). In both sham-operated and cecectomized rats, these numbers were significantly lower in rats fed the GOS diet than in those fed the CON diet. Cecectomy did not affect the number of total cells and eosinophils. The number of alveolar macrophages, neutrophils, and lymphocytes did not differ among the groups. IL-4 and IL-5, but not IFN- γ mRNA levels in the lung tissue were lower in both cecectomized and sham-operated rats fed the GOS diet than in those fed the CON diet (Fig. 3B).

DISCUSSION

This study used a well-established animal model for investigating allergic asthma (23,24); we examined the effect of

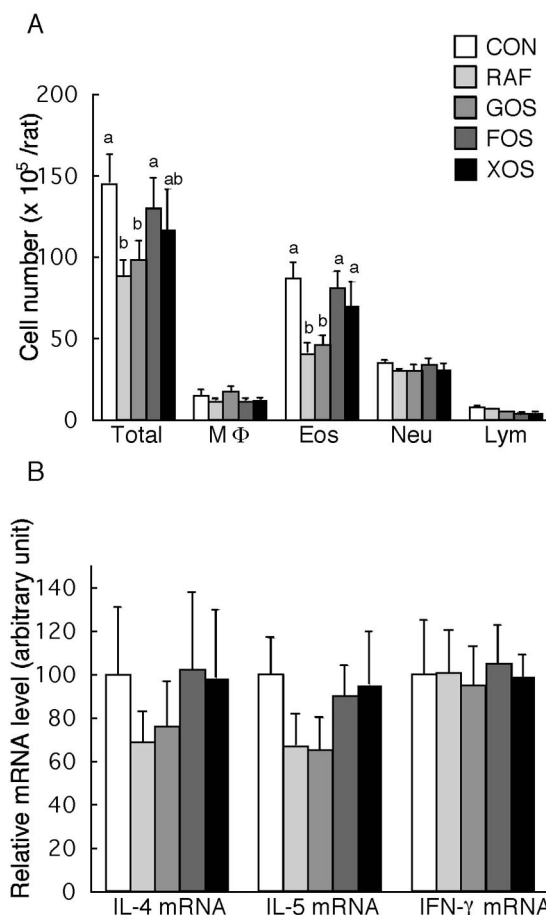


FIGURE 2 Cell profiles in BALF (panel A) and cytokine mRNA levels in lung tissue (panel B) of OVA-sensitized BN rats fed CON and injected i.p. with RAF, GOS, FOS, or XOS solutions (Expt. 2). BALF and lung tissues were obtained the day after OVA challenge. The mRNA levels were expressed relative to the mean of CON rats, taken as 100. Values are means $\pm$ SEM, $n = 6$. Total, M Φ , Eos, Neu, and Lym represent total cells, macrophages, eosinophils, neutrophils, and lymphocytes, respectively. Means for a variable without a common letter differ, $P < 0.05$.

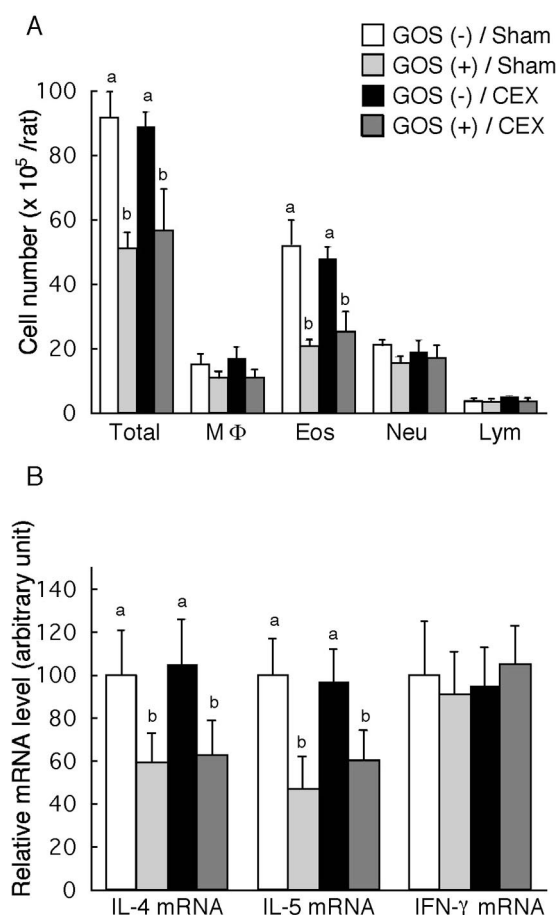


FIGURE 3 Effect of cecectomy (CEX) on cell profiles in BALF (panel A) and cytokine mRNA levels in lung tissue (panel B) of OVA-sensitized BN rats fed CON (no GOS), or a diet supplemented with GOS (Expt. 3). BALF and lung tissues were obtained the day after OVA challenge. The mRNA levels were expressed relative to the mean values of sham-operated rats fed CON, taken as 100. Values are given as means $\pm$ SEM, $n = 6$. The effect of diet was significant ($P < 0.05$) for IL-4 and IL-5mRNA levels. There were no effects of cecectomy and there were no cecectomy $\times$ diet interactions. Means for a variable without a common letter differ, $P < 0.05$. Total, M ϕ , Eos, Neu, and Lym represent total cells, macrophages, eosinophils, neutrophils, and lymphocytes, respectively.

consuming different indigestible oligosaccharides on antigen-induced airway eosinophil infiltration, a condition representing a late-phase inflammation in atopic asthma (17). BN rats were immunized with OVA via the s.c. route using adjuvants, and all rats were successfully sensitized. Rats sensitized in ways other than via the enteral route might not be a suitable model for investigating the effect of prebiotics such as indigestible oligosaccharides on allergic inflammation. However, the intestinal microflora do influence systemic immune responses beyond gut immunity. It was demonstrated that the oral administration of *Lactobacillus casei* strain Shirota modified humoral and cellular immune responses to intradermally injected type II collagen (25). Therefore, it seemed appropriate to use systemically immunized rats to examine whether dietary oligosaccharides modulate allergic inflammation via a prebiotic mechanism.

In the present study, the efficacy of dietary GOS, FOS, and XOS on allergic airway eosinophilia in OVA-sensitized BN rats was compared with that of RAF. GOS was synthesized

enzymatically from galactose by α -galactosidase from *Aspergillus niger* APC-9319, with a shift in the reaction equilibrium from hydrolysis toward synthesis by an increase in substrate concentration (19). GOS contained 58% disaccharide, 28% trisaccharide, and 14% oligosaccharides larger than trisaccharide (19). We reported previously that GOS was not hydrolyzed by human salivary amylase, porcine pancreatic amylase, or glycosidases in rat intestinal brush border membrane and that GOS was utilized by *Bifidobacterium bifidum* and *Clostridium butyricum* (19). FOS and XOS were studied extensively and shown to be indigestible by human enzymes in the small intestine but extensively fermented in the large bowel (8,26,27). FOS and GOS were implicated in increasing the densities of bifidobacteria (27,28) and lactobacilli (29) in the gastrointestinal tract. Thus, these oligosaccharides could be considered to be prebiotics.

Experiment 1 in the present study clearly demonstrated that the dietary RAF and GOS but not XOS and FOS suppressed the airway infiltration of eosinophils after exposure to aerosolized OVA in immunized BN rats. In addition, RAF and GOS were also effective when administered via the i.p. route in Expt. 2, and the data on RAF agree well with our previous study (16). These data suggest that a postabsorptive mechanism is involved in the eosinophilia-suppressing effect of dietary RAF and GOS. However, it could not be ruled out that RAF and GOS administered i.p. influenced the colonic microflora, which contributed to the suppression of airway eosinophilia. In Expt. 3, therefore, rats were cecectomized and administered neomycin sulfate orally to investigate the effect of the elimination of colonic bacteria on the airway eosinophilia-suppressing effect of dietary GOS. In sham-operated rats, the number of total anaerobic bacteria in the colon was higher in rats fed the GOS diet than in those fed the CON diet, which could possibly be due to the increase in bifidobacteria. As expected, cecectomy plus administration of neomycin drastically decreased the number of anaerobic bacteria in the colon, and dietary GOS no longer increased the number of colonic bacteria in the cecectomized rats administered neomycin. Nevertheless, consumption of GOS still suppressed airway eosinophilia in cecectomized rats administered neomycin, which is consistent with an earlier study with RAF (16). These results suggest that colonic bacteria contribute little to the beneficial activity of dietary GOS and RAF on allergic airway eosinophilia. In other words, a reduction in eosino-

TABLE 1

Effect of dietary GOS and cecectomy plus neomycin administration on the total number of anaerobic bacteria in rat colon (Expt. 3)¹

Diet	Operation	Anaerobes
		<i>Log₁₀ cfu/g contents</i>
Control	Sham	9.2 $\pm$ 0.1 ^b
GOS	Sham	10.7 $\pm$ 0.1 ^a
Control	Cecectomy	5.8 $\pm$ 0.2 ^c
GOS	Cecectomy	5.9 $\pm$ 0.2 ^c
ANOVA		
Diet		$P < 0.0001$
Operation		$P < 0.0001$
Diet $\times$ operation		$P = 0.0006$

¹ Values are means $\pm$ SEM, $n = 6$. Means with unlike superscript letters differ, $P < 0.05$.

philic infiltration by dietary GOS and RAF may be associated with a postabsorptive action of GOS and RAF rather than a prebiotic action. In fact, we demonstrated previously that orally administered RAF was absorbed intact in the gastrointestinal tract of rats (16). Nevertheless, because some clinical studies reported the prevention of atopic diseases by consumption of lactic acid bacteria (9–12), the possibility of a prebiotic mechanism for the prevention and treatment of atopic diseases by indigestible oligosaccharides should not be ruled out for clinical applications.

The present study showed that the mRNA expression of IL-4 and IL-5 in lung tissue was associated with airway eosinophilia after exposure to aerosolized OVA in immunized BN rats. Reduced levels of IL-4 and IL-5 mRNA in rats fed the RAF and GOS diets were consistent with our previous study (16). In addition, the present study revealed that the i.p. administration of these oligosaccharides also lowered IL-4 and IL-5 mRNA levels. Thus, RAF and GOS appeared to reduce IL-4 and IL-5 mRNA levels in the lung tissue via a postabsorptive mechanism. A number of cytokines contribute to the accumulation of eosinophils at the site of inflammation (30). Because allergen-specific CD4⁺ T helper type 2 (Th2) lymphocytes, which produce IL-4 and IL-5, migrate primarily to the site of inflammation upon stimulation with antigen (17), expression of IL-4 and IL-5 mRNA in the lung tissue would reflect the generation of these cytokines in Th2 cells. In our preliminary experiment, supplementation of RAF and GOS to the culture media of splenic mononuclear cells isolated from OVA-sensitized BN rats did not suppress the antigen-stimulated production of IL-4 and IL-5 (unpublished data). Therefore, it is conceivable that RAF and GOS would suppress migration of antigen-specific Th2 cells from peripheral blood to the site of inflammation rather than expression of IL-4 and IL-5 in activated Th2 cells at the site of inflammation. A reduction in IL-4 and IL-5 in lung inflammation would possibly contribute to the suppression of eosinophil infiltration in rats administered RAF and GOS because these cytokines were shown to promote eosinophil infiltration (31,32). However, we would have preferred to measure the protein levels rather than the mRNA levels of these cytokines because mRNA levels do not always reflect the protein levels. Further studies will be required to measure the cytokine proteins produced by BALF cells upon stimulation with antigen *ex vivo*.

It remains unclear why consuming FOS and XOS did not suppress allergic airway eosinophilia in OVA-sensitized BN rats. Although dietary FOS and XOS were not absorbed as well as RAF and GOS in the gastrointestinal tract, this is not likely the explanation because FOS and XOS administered i.p. were also ineffective. One possible explanation is that the efficacy may be associated with α -D-galactosidic linkages that exist in RAF and GOS but not in FOS and XOS. Because α -galactosylceramide, a synthetic glycolipid, was reported to be a specific ligand of natural killer T cells, which play a pivotal role in the regulation of diverse immune functions (33), some oligosaccharides that have α -galactosidic linkages may be involved in immune functions by affecting the activation of natural killer T cells after being absorbed in the gastrointestinal tract. Thus, it would be of interest to investigate the structure-activity relation of oligosaccharides as suppressors of allergic airway eosinophilia and also as activators or inhibitors of natural killer T cells.

Taken together, indigestible oligosaccharides differ in their suppressive effect on allergic airway eosinophilia in OVA-sensitized BN rats. RAF and GOS that had α -D-galactosidic linkages were effective in suppressing eosinophil infiltration,

suggesting that α -D-galactosides could be used in the prevention and/or treatment of human allergic diseases. The underlying mechanism seems to be independent of modulation of intestinal microflora and could be associated with reduced migration of activated Th2 cells. However, the possibility of a prebiotic mechanism for the prevention and treatment of atopic diseases by indigestible oligosaccharides should not be ruled out. Nagura et al. (34) reported recently that secretion of IL-12 and IFN- γ from Peyer's patch cells of naïve BALB/c mice was significantly increased by dietary RAF, suggesting suppression of the Th2-type immune response by dietary RAF. Thus, multiple actions of indigestible oligosaccharides may contribute to the prevention and treatment of atopic diseases.

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