

Dietary α -Linked Galacto-Oligosaccharide Suppresses Ovalbumin-Induced Allergic Peritonitis in BALB/c Mice

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To determine whether α -linked galacto-oligosaccharide (α -GOS) prevents allergic peritonitis, BALB/c mice were fed a synthetic diet with and without α -GOS supplementation for 7 d, and were then subcutaneously immunized with ovalbumin on days 0 and 7. The mice were challenged by intraperitoneal injection with ovalbumin on day 14, followed by peritoneal lavage on day 15. The total number of peritoneal exudate cells was significantly lower in the mice fed the α -GOS diet than in those fed the control diet. Peritoneal lavage fluid from mice fed the α -GOS diet not only had less potency to attract peripheral blood leukocytes and peritoneal exudate cells *ex vivo*, but also had lower concentrations of monocyte chemoattractant protein-1 (MCP-1) and eotaxin. Preincubation of the cells with α -GOS failed to affect the migration to peritoneal lavage fluid. We propose that dietary α -GOS reduces cell infiltration in allergic peritonitis by reducing antigen-induced elicitation of MCP-1 and eotaxin in mice.

Key words: α -linked galacto-oligosaccharide; allergic peritonitis; ovalbumin; mice

Indigestible oligosaccharides are representative prebiotics. They are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one of a limited number of bacteria in the colon and thus improve

host health.¹⁾ Because commensal bacteria in the intestinal tract play a major role in the maturation of the immune system and in the maintenance of the homeostasis of the gut-associated immune system,^{2–4)} strategies to manipulate the microbiota are considered to promote immune health. Indeed, administration of *Lactobacillus* and *Bifidobacterium* species prevents and treats rotavirus infections and postantibiotic diarrhea,^{5,6)} allergic diseases,^{7–9)} and the recurrence of inflammatory bowel disease.^{10,11)} Therefore, consumption of indigestible oligosaccharides should theoretically prevent such diseases, but only a limited number of studies have reported the effect of indigestible oligosaccharides on allergic reactions.

α -Linked galacto-oligosaccharide (α -GOS) was synthesized enzymatically from galactose by α -galactosidase from *Aspergillus niger* APC-9319, with a shift of the reaction equilibrium from hydrolysis towards synthesis by increasing the substrate concentration.¹²⁾ α -GOS contains 58% disaccharide, 28% trisaccharide, and 14% oligosaccharides larger than trisaccharides.¹²⁾ We have reported that α -GOS was not hydrolyzed by human salivary amylase, porcine pancreatic amylase, or glycosidases in rat intestinal brush border membrane, and that α -GOS was utilized by *Bifidobacterium bifidum* and *Clostridium butyricum*.¹²⁾ Hence α -GOS was considered to be a prebiotic indigestible oligosaccharide.

We have found that consumption of α -GOS and of

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Abbreviations: α -GOS, α -linked galacto-oligosaccharide; FOS, fructo-oligosaccharide; MCP-1, monocyte chemoattractant protein-1; OVA, ovalbumin; PBLs, peripheral blood leukocytes; PBS, phosphate-buffered saline; PECs, peritoneal exudate cells; PLF, peritoneal lavage fluid; SUP, cell-free supernatant

raffinose (β -D-fructofuranosyl- O - α -D-galactopyranosyl-(1,6)- α -D-glucopyranoside) reduced airway eosinophilia and Th2 cytokines in an ovalbumin (OVA)-induced allergic asthma model of the Brown Norway rat.^{13,14} More recently, Fujitani *et al.* showed that dietary fructo-oligosaccharide (FOS) reduced infiltration of inflammatory cells and edema formation in duodenal mucosa using an OVA-induced food allergy model of NC/jic mice.¹⁵ Vos *et al.* reported that consumption of a mixture of long-chain FOS and short-chain GOS suppressed airway hyperresponsiveness and infiltration of inflammatory cells in an OVA-induced allergic asthma model of BALB/c mice.¹⁶ In addition, we have reported that dietary FOS reduced 2,4-dinitrofluorobenzene-induced contact hypersensitivity in BALB/c mice,¹⁷ but the mechanisms of the anti-allergic effect of dietary indigestible oligosaccharides are not fully understood.

Infiltration of different leukocytes into the site of inflammation is the central feature observed in various allergic diseases.¹⁸ Because indigestible oligosaccharides reportedly reduced the infiltration of inflammatory cells in animal models,^{13–16} investigation of leukocyte mobilization might help in the effort to understand the mechanisms of the anti-allergic effect of dietary indigestible oligosaccharides. The allergic peritonitis model in mice is an easy and reliable tool for the study of inflammatory cell migration.^{19,20} The present study therefore aimed to investigate the effects of α -GOS on OVA-induced allergic peritonitis *in vivo* and on cell migration *ex vivo*. Experiment 1 was designed to test whether dietary α -GOS would prevent peritoneal infiltration of inflammatory cells *in vivo* and the migration of peritoneal exudate cells (PECs) against peritoneal lavage fluid (PLF) *ex vivo*. Because chemokines play important roles in the recruitment of leukocytes from the blood to inflammatory tissues,²¹ we measured the concentration of chemokines in PLF in experiment 2. In addition, the migration of peripheral blood leukocytes (PBLs) against PLF was investigated *ex vivo*. Then, experiment 3 was performed to determine whether α -GOS would directly influence cell migration *in vitro*, because our previous observations suggest that a post-absorptive mechanism is involved in the reducing effect of dietary α -GOS on cell infiltration in allergic airway inflammation in Brown Norway rats.¹³

Materials and Methods

Animals and diets. Female BALB/c mice (aged 4 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and housed in standard plastic cages in a temperature-controlled ($23 \pm 2^\circ\text{C}$) room with a dark period from 20:00 to 8:00 h. They were allowed free access to food and water. The mice were fed either a synthetic diet prepared according to AIN-93G²² or that diet supplemented with α -GOS synthesized following Yamashita *et al.*²³ These diets were referred to as CON and GOS respectively. The GOS diet was prepared by

adding 50 g of α -GOS to each kilogram of the CON diet by substituting α -GOS for α -corn starch.

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

Experimental design. In experiment 1, after 7 d of consuming the CON diet, 24 mice were actively immunized twice (on day 0 and day 7) with OVA, as described below. After the primary immunization, the mice were divided into four groups of six animals. The mice in groups 1 and 2 were fed the CON diet, whereas the mice in group 3 were fed the GOS diet for 14 d. The mice in group 4 were fed the GOS diet for 1 d following 13 d of initial feeding on the CON diet. On day 14, the mice in groups 2, 3, and 4 were intraperitoneally (i.p.) administered OVA to induce allergic peritonitis, as described below. The mice in group 1 were i.p. administered the vehicle. On day 15, the mice were euthanized by decapitation under anesthesia with diethyl ether, and enumeration of PEC in PLF and chemotaxis assay for PEC against the cell-free supernatants (SUP) of PLF were performed as described below.

In experiment 2, after 7 d of consuming the CON diet, 24 mice were actively immunized twice (on day 0 and day 7) with OVA, as described below. After the primary immunization, the mice were divided into two groups of 12 animals and fed either the CON or the GOS diet for 14 d. On day 14, the mice in each diet group were then subdivided into two groups of six animals; one group was left untreated and the other group was i.p. challenged with OVA, as described below. One day later, all the mice were euthanized by decapitation under anesthesia with diethyl ether, and blood samples were collected from the untreated mice while SUPs were prepared from the PLF of the OVA-challenged mice, as described below. PBLs were isolated from blood samples from the untreated mice, and the chemotaxis of PBLs toward SUP was assayed as described below. The concentration of chemokines in SUP of OVA-challenged mice was measured as described below.

In experiment 3, after 7 d of consuming the CON diet, 12 mice were actively immunized twice (on day 0 and day 7) with OVA and fed the CON diet for 14 d after the primary immunization. On day 14, the mice were divided into two groups (six animals per group) of untreated and OVA-challenged mice. Immunization and i.p. challenge with OVA were performed as described below. On the next day, PBLs obtained from untreated mice and PECs and SUPs isolated from OVA-challenged mice were subjected to chemotaxis assay, as described below.

Immunization and intraperitoneal challenge with OVA. The mice were actively immunized with two subcutaneous injections of 100 μg of OVA (grade V, Sigma, St. Louis, MO) suspended in 0.4 ml of Imject

Alum (Pierce, Rockford, IL) on days 0 and 7. One week after the secondary immunization, the mice were i.p. injected with 0.4 ml of phosphate-buffered saline (PBS) containing 10 μ g of OVA. The vehicle-challenged mice were i.p. injected with the same volume of PBS.

Peritoneal lavage and cell count. At 24 h after i.p. antigen challenge, the animals were euthanized by decapitation under anesthesia with diethyl ether. Abdominal skin below the sternum was nicked, and peritoneal lavage was performed by i.p. injection of 7 ml of the complete medium, RPMI-1640 medium (Gibco-BRL, Tokyo) supplemented with 10% fetal calf serum, using a 23 G needle. The abdominal wall was then gently massaged 10 times, and PLF was harvested with a syringe. Approximately 90% of the injected volume was recovered. Samples were discarded when hemorrhages were detected in the cavity. PECs were recovered from PLF by centrifugation (1,000 rpm, 5 min), and SUPs were transferred to another tube. The PECs were resuspended in 1 ml of erythrocyte-lysis buffer (0.15 M NH_4Cl , 10 mM Tris-HCl, pH 7.4) and incubated on ice for 5 min. After centrifugation (1,000 rpm, 5 min), the PECs were washed twice with the complete medium and then resuspended in the medium. The PECs were counted using a Z1 particle counter (Beckman Coulter, Fullerton, CA). The PECs and SUPs were maintained on ice for subsequent chemotaxis experiments.

Isolation of peripheral blood leukocytes. PBLs were isolated from whole blood according to a procedure described elsewhere,^{24,25} with a slight modification. In brief, approximately 1 ml of blood sample from each mouse was collected in a heparin-containing (20 units) tube, and diluted 1:3 with erythrocyte-lysis buffer. The tube was inverted for thorough mixing and incubated for 5 min at room temperature, during which the time it was inverted at least once. After centrifugation ($700 \times g$, 5 min), the supernatant was decanted, leaving behind the cell pellet. The cells were again treated with 1 ml of erythrocyte-lysis buffer, and this step was repeated at least once to clear all contaminated erythrocytes. PBLs were resuspended in the complete medium, counted and stored on ice for later chemotaxis assay.

Chemotaxis assay. In experiments 1 and 2, migration of PECs and PBLs toward SUPs was assayed using Chemotaxicells (5 μ m pore size, Kurabo, Osaka) and 24-well culture plates (Corning Costar, Cambridge, MA). The cells (5×10^5 for PEC or 10^6 for PBL) suspended in 100 μ l of the complete medium were added into Chemotaxicells, the upper compartment. The tested SUPs and the complete medium as a control (600 μ l) were added to the wells of the culture plate, the lower compartment. After 1 h of incubation at 37°C in an atmosphere of 95% air and 5% CO_2 , the fluid in the lower compartment was gently agitated to displace any

adherent cells, and transferred to a FACS tube. The cell suspension was mixed with 15 μ l (equivalent to 15,000 beads) of Flow-CheckTM fluorospheres (10 μ m diameter, Beckman Coulter, Fullerton, CA), and then analyzed on a flow cytometer (Beckman Coulter). Flow cytometric analysis was performed by gating the leukocyte and fluorosphere populations based on differences in relative size (forward scatter) and granularity (side scatter). The total number of migrating cells was calculated by the following equation:

Migrating cell count

= Total number of cells counted

$\times 15,000 / \text{Total number of fluorospheres counted}$

In experiment 3, PECs and PBLs were incubated in the complete medium supplemented with different concentrations of α -GOS (0, 1, and 10 mg/ml) for 30 min at 37°C in an atmosphere of 95% air and 5% CO_2 prior to chemotaxis assay.

ELISA for chemokines. Monocyte chemoattractant protein-1 (MCP-1) and eotaxin concentrations in the SUPs were determined with commercial ELISA kits according to the manufacturers' instructions (Bender MedSystems, Vienna, Austria, and R & D systems, Minneapolis, MN for MCP-1 and eotaxin respectively).

Statistics. Statistical analyses were performed using GraphPad prism version 4.03 for Windows (GraphPad Software, San Diego, CA). Results were expressed as means and the standard errors of the mean. The unpaired *t*-test or Tukey's honestly significantly difference test following one-way analysis of variance was used to compare mean values. *P* values less than 0.05 were considered significant.

Results

In the OVA-immunized mice, i.p. injection of OVA induced peritonitis, as indicated by marked increases in the numbers of PECs in comparison with the vehicle-injected mice (Fig. 1). In addition, our preliminary experiment showed that the number of PECs following i.p. injection of OVA was significantly higher in the OVA-immunized mice than in naïve mice (data not shown). The observed increase in PECs thus appears to have been mediated by an antigen-specific reaction. The number of PECs was significantly lower in the mice fed the GOS diet for 14 d than in the mice fed the CON diet for 14 d (Fig. 1). One-day feeding on the GOS diet failed to reduce the number of PECs.

The SUPs obtained from OVA-challenged mice caused the migration of PECs in a concentration-dependent manner (Fig. 2). The SUPs obtained from the vehicle-challenged mice showed a lower ability to cause the migration of PECs. SUPs from OVA-challenged mice fed the GOS diet yielded significantly a

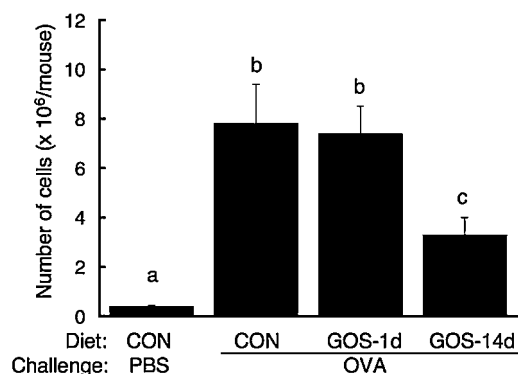


Fig. 1. Effect of Dietary α -Linked Galacto-Oligosaccharide (α -GOS) on Cell Infiltration in an Allergic Peritonitis Model of BALB/c Mice (experiment 1).

All the mice were immunized subcutaneously with ovalbumin (OVA) on days 0 and 7. Thereafter, the mice were intraperitoneally injected with either OVA or the vehicle on day 14. On day 15, all the mice were euthanized, and the number of peritoneal exudate cells recovered from peritoneal lavage fluid was counted. The mice were given a 14-d feeding of the synthetic diet as a control diet (CON), the diet supplemented with α -GOS (GOS), or a 1-d feeding of the GOS diet. Mean values are shown for six mice from each group. The standard errors of the mean are represented by vertical bars. Data not sharing a common letter are significantly different at $P < 0.05$.

lower migration response of PECs than SUPs from the mice fed the CON diet (Fig. 2). This was evident in PECs from the mice fed both the GOS and the CON diet. The migration response of PECs from the mice fed the GOS diet did not differ from that of the mice fed the CON diet.

Likewise, the SUPs obtained from OVA-challenged mice mobilized PBLs in a concentration-dependent manner (Fig. 3). The SUPs obtained from the vehicle-challenged mice showed lower ability to cause migration of PBLs. The migration of PBLs was lower in response to SUPs from mice fed the GOS diet than from those fed the CON diet, although a significant difference was seen in the PBLs from mice fed the CON diet but not the GOS diet (Fig. 3).

Figure 4 shows the chemoattractant concentrations in the SUPs of the OVA-challenged mice. The MCP-1 concentrations in the SUPs were significantly lower in the mice fed the GOS diet than in those fed the CON diet. Similarly, the eotaxin concentrations in the SUPs tended to be lower in the mice fed the GOS diet than in those fed the CON diet.

Incubation of PECs isolated from OVA-challenged mice fed the CON diet with α -GOS prior to chemotaxis assay failed to affect cell migration (Fig. 4). Similarly, preincubation with α -GOS failed to affect the migration of PBLs isolated from the vehicle-challenged mice fed the CON diet (Fig. 4).

Discussion

This study revealed infiltration of peritoneal cells at 24 h after i.p. antigen-challenge in BALB/c mice

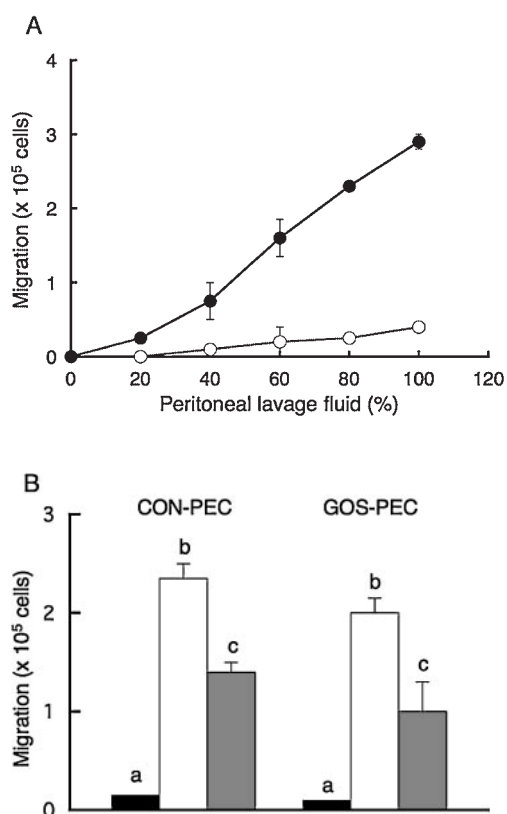


Fig. 2. Effect of Dietary α -Linked Galacto-Oligosaccharide (α -GOS) on the Migration of Peritoneal Exudate Cells (PECs) *ex Vivo* (experiment 1).

A, migration of PEC from ovalbumin (OVA)-challenged mice toward various concentrations of cell-free supernatant (SUP) of peritoneal lavage fluid is shown. Filled and open circles indicate the numbers of cells migrating toward SUP from the OVA- and vehicle-challenged mice respectively. B, migration of PECs from OVA-challenged mice fed the synthetic diet as a control diet (CON-PEC) and the diet supplemented with α -GOS (GOS-PEC) toward SUP is shown. Filled, open, and gray columns indicate the numbers of cells migrating toward the medium, SUP from OVA-challenged mice fed the CON diet and the GOS diet respectively. Mean values are shown for five determinations, and the standard errors of the mean are represented by vertical bars. Data not sharing a common letter are significantly different at $P < 0.05$.

immunized with OVA. Differential cell staining of cytopsin preparations showed that the predominant cell populations in the peritoneal exudates were macrophages and eosinophils (data not shown). Eosinophilia in allergic peritonitis is well established in actively immunized mice.^{19,20} The usefulness of this model depends on the possibility of precise quantification of inflammation-related leukocytes and chemoattractants (*e.g.*, chemokines and cytokines) in peritoneal exudates.²⁶

The present data clearly indicate that dietary α -GOS reduced antigen-induced infiltration of inflammatory cells in allergic peritonitis in mice. This finding is in keeping with our previous observation that dietary α -GOS prevented cell infiltration in an allergic airway inflammation model of Brown Norway rats.¹³ Our preliminary experiments showed that dietary α -GOS

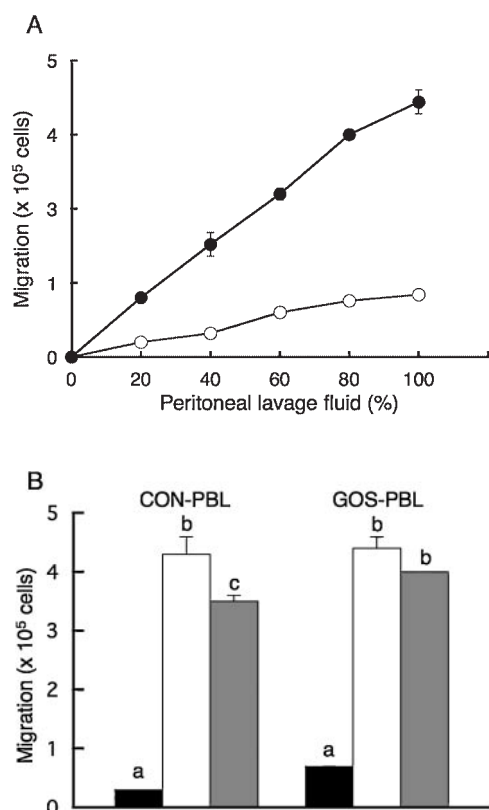


Fig. 3. Effect of Dietary α -Linked Galacto-Oligosaccharide (α -GOS) on the Migration of Peripheral Blood Leukocytes (PBLs) *ex Vivo* (experiment 2).

A, migration of PBL from unchallenged mice toward various concentrations of cell-free supernatant (SUP) of peritoneal lavage fluid is shown. Filled and open circles indicate the numbers of cells migrating toward SUP from the OVA- and vehicle-challenged mice respectively. B, migration of PBLs from OVA-challenged mice fed the synthetic diet as a control diet (CON-PBL) and the diet supplemented with α -GOS (GOS-PBL) toward SUP is shown. Filled, open, and gray columns indicate the numbers of cells migrating toward the medium, SUP from OVA-challenged mice fed the CON diet and GOS diet respectively. Mean values are shown for five determinations, and the standard errors of the mean are represented by vertical bars. Data not sharing a common letter are significantly different at $P < 0.05$.

failed to affect cell infiltration in thioglycolate-induced peritonitis, a well-known acute inflammation model, in BALB/c mice (unpublished data). Hence it appears that dietary α -GOS has an anti-allergic action rather than an anti-inflammatory action.

Chemokines produced in the local microenvironment are factors capable of attracting circulating leukocytes to inflammatory loci.²¹⁾ In the present study, chemotaxis experiments showed that SUPs obtained from antigen-challenged mice stimulated the migration of PECs *ex vivo*, suggesting that peritoneal exudates in antigen-challenged mice contain soluble chemoattractants such as chemokines. When compared to SUPs obtained from mice fed the CON diet, the SUPs from mice fed the GOS diet had less ability to cause mobility of PECs. Perhaps the concentration of chemoattractants in SUPs was

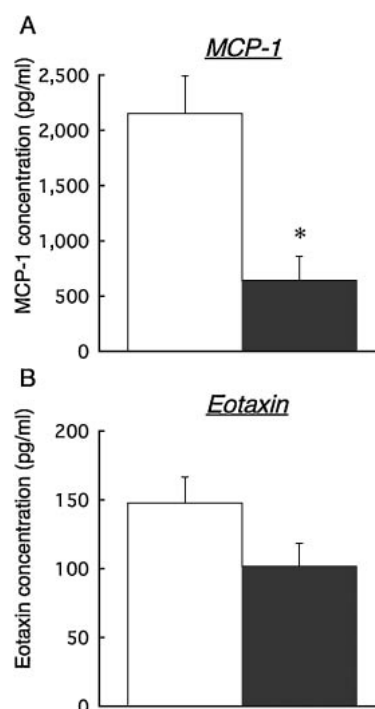


Fig. 4. Effect of Dietary α -Linked Galacto-Oligosaccharide (α -GOS) on the Concentration of Chemokines in Cell-Free Supernatant (SUP) of Peritoneal Lavage Fluid in an Allergic Peritonitis Model of BALB/c Mice (experiment 2).

The concentrations of monocyte chemoattractant protein-1 (MCP-1) and eotaxin in SUP are shown in charts A and B respectively. Open and gray columns indicate mice fed the CON diet and the GOS diet respectively. Mean values are shown for six mice from each group, and the standard errors of the mean are represented by vertical bars. * $P < 0.05$ vs. the CON diet.

lower in the mice fed the GOS diet than in those fed the CON diet. Indeed, concentrations of MCP-1 and eotaxin (chemoattractants for monocytes and eosinophils respectively) in SUPs were lower in the mice fed the GOS diet than those fed the CON diet, suggesting that dietary α -GOS reduced antigen-induced elicitation of these chemokines. Hence the reduced infiltration of PECs, mainly macrophages and eosinophils, in the mice fed the GOS diet might have been associated with reduced elicitation of MCP-1 and eotaxin.

In addition to their role in cell migration, chemokines stimulate degranulation and promote inflammatory mediator release in leukocytes.²⁷⁾ Hence already migrated PECs in the peritoneal cavity are activated and thus show a different migrating response than circulating leukocytes. Hence we tested the migration of PBLs, nonstimulated circulating leukocytes, isolated from immunized mice. Similarly to PECs, the migration of PBLs was lower toward SUPs from mice fed the GOS diet. These data suggest that dietary α -GOS reduces the antigen-induced elicitation of chemoattractants in the peritoneal cavity and does not alter the responsiveness of migrating cells. In addition, 1-d feeding with α -GOS showed no reduction in the infiltration of PECs in antigen-challenged mice. Hence chronic feeding

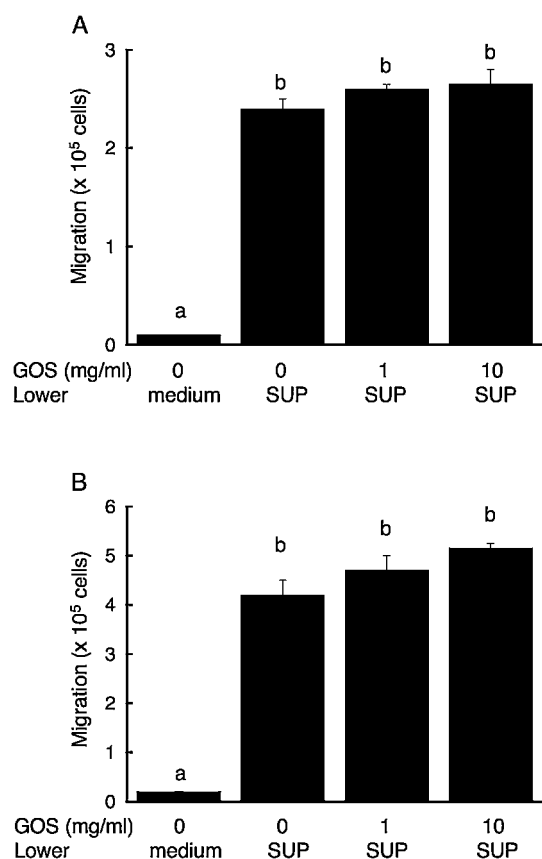


Fig. 5. Effect of *in Vitro* Treatment with α -Linked Galacto-Oligosaccharide (α -GOS) on Leukocyte Migration (experiment 3).

BALB/c mice were fed the synthetic diet and subcutaneously immunized with ovalbumin (OVA) on days 0 and 7. On day 14, the mice were divided into two groups (six animals per group) of untreated and OVA-challenged mice. On the next day, all the mice were euthanized, and peripheral blood leukocytes (PBLs) were isolated from blood samples of untreated mice, while cell-free supernatants (SUP) were prepared from the peritoneal lavage fluid of OVA-challenged mice. PECs (A) and PBLs (B) were incubated with various concentrations of α -GOS (0, 1, and 10 mg/ml) for 30 min, and then subjected to chemotaxis assay. Mean values are shown for five determinations, and the standard errors of the mean are represented by vertical bars. Data not sharing a common letter are significantly different at $P < 0.05$.

with α -GOS may be required to alter responsiveness in eliciting chemoattractants.

Because α -GOS is not hydrolyzed by human salivary amylase, porcine pancreatic amylase, or glycosidases in the rat intestinal brush border membrane,¹²⁾ it is possible to claim that the effect of dietary α -GOS on the infiltration of PECs is mediated by modification of intestinal microbiota. However, our previous study showed that reduction of cell infiltration by dietary α -GOS in allergic airway inflammation in Brown Norway rats was still observed in cecectomized and neomycin-administered animals, and that i.p. administered α -GOS also reduced cell infiltration.¹³⁾ These observations suggest that a post-absorptive mechanism, rather than prebiotic action, is involved in the reducing effect of dietary α -GOS on cell infiltration in allergic airway

inflammation. Hence we speculate that the responsiveness of migrating cells to chemoattractants in inflammatory loci is directly influenced by α -GOS absorbed intact in the intestine. In the present study, however, incubation of PECs and PBLs with α -GOS prior to chemotaxis experiments failed to affect cell migration toward antigen-challenged SUPs. It is thus likely that reduction of peritoneal cell infiltration by dietary α -GOS is not mediated by the direct action of α -GOS on migrating cells. One possible explanation is that α -GOS directly influence the production and/or elicitation of chemoattractants in the inflammatory loci. Additionally, it remains to be determined whether modification of intestinal microbiota is involved in the suppression of allergic peritonitis by dietary α -GOS.

In conclusion, dietary α -GOS reduced cell infiltration in allergic peritonitis in BALB/c mice. This action might be associated with a reduction in antigen-induced elicitation of chemoattractants, including MCP-1 and eotaxin, in the peritoneal cavity rather than with the modification of the responsiveness of migrating cells.

The present study thus provides additional information as to the anti-allergic effect of dietary indigestible oligosaccharides. Moro *et al.* reported that a mixture of long-chain FOS and short-chain GOS reduced the incidence of atopic dermatitis in formula-fed high-risk infants,²⁸⁾ which was the first well-controlled clinical trial showing allergy prevention by indigestible oligosaccharides. However, Osborne and Sinn mentioned in a recent review that there is insufficient evidence to determine the role of prebiotic supplementation of infant formula in the prevention of allergic disease and food hypersensitivity.²⁹⁾ Further study is needed to determine whether indigestible oligosaccharides are beneficial in the prevention of allergic diseases.

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References

- 1) Gibson, G. R., and Roberfroid, M. B., Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.*, **125**, 1401–1412 (1995).
- 2) Cebra, J. J., Influences of microbiota on intestinal immune system development. *Am. J. Clin. Nutr.*, **69**, 1046S–1051S (1999).
- 3) Lanning, D., Sethupathi, P., Rhee, K. J., Zhai, S. K., and Knight, K. L., Intestinal microflora and diversification of the rabbit antibody repertoire. *J. Immunol.*, **165**, 2012–2019 (2000).
- 4) Holt, P. G., and Jones, C. A., The development of the immune system during pregnancy and early life. *Allergy*, **55**, 688–697 (2000).

- 5) Shornikova, A. V., Casas, I. A., Isolauri, E., Mykkanen, H., and Vesikari, T., *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. *J. Pediatr. Gastroenterol. Nutr.*, **24**, 399–404 (1997).
- 6) Cruchet, S., Obregon, M. C., Salazar, G., Diaz, E., and Gotteland, M., Effect of the ingestion of a dietary product containing *Lactobacillus johnsonii* La1 on *Helicobacter pylori* colonization in children. *Nutrition*, **19**, 716–721 (2003).
- 7) Kalliomaki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., and Isolauri, E., Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial. *Lancet*, **357**, 1076–1079 (2001).
- 8) Rosenfeldt, V., Benfeldt, E., Nielsen, S. D., Michaelsen, K. F., Jeppesen, D. L., Valerius, N. H., and Paerregaard, A., Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *J. Allergy Clin. Immunol.*, **111**, 389–395 (2003).
- 9) Viljanen, M., Savilahti, E., Haahtela, T., Juntunen-Backman, K., Korpela, R., Poussa, T., Tuure, T., and Kuitunen, M., Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. *Allergy*, **60**, 494–500 (2005).
- 10) Rembacken, B. J., Snelling, A. M., Hawkey, P. M., Chalmers, D. M., and Axon, A. T., Non-pathogenic *Escherichia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. *Lancet*, **354**, 635–639 (1999).
- 11) Guslandi, M., Mezzi, G., Sorghi, M., and Testoni, P. A., *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. *Dig. Dis. Sci.*, **45**, 1462–1464 (2000).
- 12) Yamashita, A., Hashimoto, H., Kitahata, S., Kikuchi, E., Fujita, K., Okada, M., Mizutani, A., Mori, S., Amano, Y., and Kanda, T., Production of α -linked galactooligosaccharide (α -GOS) by α -galactosidase from *Aspergillus niger* APC-9319 and its physical and physiological properties. *J. Appl. Glycosci.*, **51**, 115–122 (2004).
- 13) Sonoyama, K., Watanabe, H., Watanabe, J., Yamaguchi, N., Yamashita, A., Hashimoto, H., Kishino, E., Fujita, K., Okada, M., Mori, S., Kitahata, S., and Kawabata, J., Allergic airway eosinophilia is suppressed in ovalbumin-sensitized Brown Norway rats fed raffinose and α -linked galactooligosaccharide. *J. Nutr.*, **135**, 538–543 (2005).
- 14) Watanabe, H., Sonoyama, K., Watanabe, J., Yamaguchi, N., Kikuchi, H., Nagura, T., Aritsuka, T., Fukumoto, K., and Kasai, T., Reduction of allergic airway eosinophilia by dietary raffinose in Brown Norway rats. *Br. J. Nutr.*, **92**, 247–255 (2004).
- 15) Fujitani, S., Ueno, K., Kamiya, T., Tsukahara, T., Ishihara, K., Kitabayashi, T., and Itabashi, K., Increased number of CCR4-positive cells in the duodenum of ovalbumin-induced food allergy model Nc/jic mice and antiallergic activity of fructooligosaccharides. *Allergol. Int.*, **56**, 131–138 (2007).
- 16) Vos, A. P., van Esch, B. C., Stahl, B., M'Rabet, L., Folkerts, G., Nijkamp, F. P., and Garssen, J., Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice. *Int. Immunopharmacol.*, **7**, 1582–1587 (2007).
- 17) Watanabe, J., Sasajima, N., Aramaki, A., and Sonoyama, K., Consumption of fructo-oligosaccharide reduces 2,4-dinitrofluorobenzene-induced contact hypersensitivity in mice. *Br. J. Nutr.*, in press.
- 18) Venge, P., Monitoring the allergic inflammation. *Allergy*, **25**, 26–32 (2004).
- 19) Zuany-Amorim, C., Leduc, D., Vargaftig, B. B., and Pretolani, M., Characterization and pharmacological modulation of antigen-induced peritonitis in actively sensitized mice. *Br. J. Pharmacol.*, **110**, 917–924 (1993).
- 20) Das, A. M., Flower, R. J., and Perretti, M., Eotaxin-induced eosinophil migration in the peritoneal cavity of ovalbumin-sensitized mice: mechanism of action. *J. Immunol.*, **159**, 1466–1473 (1997).
- 21) Johnston, B., and Butcher, E. C., Chemokines in rapid leukocyte adhesion triggering and migration. *Semin. Immunol.*, **14**, 83–92 (2002).
- 22) Reeves, P. G., Nielsen, F. H., and Fahey, G. C., Jr., AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *ad hoc* writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.*, **123**, 1939–1951 (1993).
- 23) Yamashita, A., Hashimoto, H., Fujita, K., Okada, M., Mori, S., and Kitahata, S., Reverse reaction of *Aspergillus niger* APC-9319 α -galactosidase in a super-saturated substrate solution: production of α -linked galactooligosaccharide (α -GOS). *Biosci. Biotechnol. Biochem.*, **69**, 1381–1388 (2005).
- 24) Renzi, P., and Ginns, L. C., Analysis of T cell subset in normal adults: comparison of whole blood lysis technique to Ficoll-Hypaque separation by flow cytometry. *J. Immunol. Methods*, **98**, 53–56 (1987).
- 25) Ashmore, L. M., Shopp, G. M., and Edwards, B. S., Lymphocyte subset analysis by flow cytometry: comparison of three different staining techniques and effects of blood storage. *J. Immunol. Methods*, **118**, 209–215 (1989).
- 26) Ajuebor, M. N., Das, A. M., Virág, L., Flower, R. J., Szabó, C., and Perretti, M., Role of resident macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10. *J. Immunol.*, **162**, 1685–1691 (1999).
- 27) Mackay, C. R., Chemokines: immunology's high impact factors. *Nat. Immunol.*, **2**, 95–101 (2001).
- 28) Moro, G., Arslanoglu, S., Stahl, B., Jelinek, J., Wahn, U., and Boehm, G., A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch. Dis. Child.*, **91**, 814–819 (2006).
- 29) Osborn, D. A., and Sinn, J. K., Probiotics in infants for prevention of allergic disease and food hypersensitivity. *Cochrane Database Syst. Rev.*, **4**, CD006474 (2007).