

Preliminary report

Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice

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Abstract

Specific mixtures of prebiotic oligosaccharides showed immune modulatory effects in previous murine vaccination experiments, suggesting a shift towards T-helper 1 (Th1) immunity. These mixtures consisted of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) in a 9:1 ratio (Immunofortis), with or without pectin-derived acidic oligosaccharides (pAOS). To investigate whether these mixtures could suppress Th2-related responses, they were tested in an ovalbumin (OVA)-induced model for experimental allergic asthma in BALB/c mice. Supplementation with two mixtures of scGOS/lcFOS and scGOS/lcFOS/pAOS at approximately 1% (w/w% net oligosaccharides) in the diet, starting two weeks before OVA sensitization and lasting until the end of the experiment, decreased of several parameters of allergic asthma. The OVA-induced airway inflammation and hyperresponsiveness was significantly suppressed by both mixtures. Moreover, OVA-specific IgE titers were decreased by more than 25%, although this effect was not significant. The effects of the oligosaccharide mixture with pAOS appeared to be more pronounced than the effects of the scGOS/lcFOS mixture without pAOS, but a direct comparison between the mixtures was not made. Overall, the results further support the hypothesis that specific mixtures of oligosaccharides modulate the Th1/Th2 balance by enhancing Th1-related and suppressing Th2-related parameters.

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1. Introduction

Dietary oligosaccharides have been shown to modulate immune function in various recent reports. In the intestine, beneficial effects have been shown on parameters of inflammation [1–3] and modulatory effects have been shown on cell populations in Peyer's patches

(PP) [4–6]. A few reports on systemic immune modulatory effects have been published as well [7,8].

We have previously reported systemic immune modulatory effects after oral supplementation with specific mixtures of oligosaccharides, designed to mimic the prebiotic and immunological functions of breast milk oligosaccharides as closely as possible. These mixtures consisted of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) in a 9:1 ratio (Immunofortis), with or without pectin-derived acidic oligosaccharides (pAOS) that consist of partly unsaturated

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and partly methylated galacturonic acids [9,10]. Enhanced T-helper 1 (Th1) dependent delayed-type hypersensitivity responses after vaccination were shown repeatedly in an influenza vaccination model in C57BL/6 mice [11,12]. Additionally, inhibition of Th2 cytokine production was observed in pAOS supplemented animals [12], leading us to hypothesize that the dietary interventions induced a mild shift in the Th1/Th2 balance towards Th1 responses in healthy C57BL/6 mice.

To verify this hypothesis in a different animal model, the specific oligosaccharide mixtures were tested in a model for experimental allergic asthma in Th2-prone BALB/c mice. Since Th1 and Th2 responses are thought to be reciprocally regulated, it was hypothesized that dietary intervention with the specific oligosaccharide mixtures could decrease parameters of allergic asthma in this Th2-related model [13,14]. This hypothesis was tested in two separate experiments.

2. Materials and methods

2.1. Animals and diets

Male BALB/cByJlco SPF mice, 5–8 weeks of age with 20–25 g of weight, were obtained from Charles River (Somer, The Netherlands). The mice were accustomed to their new environment for at least 1 week before the start of the experiment. All experiments were approved by an independent animal experiments committee.

All animals received semi-purified AIN-93G-based diets (Research Diet Services, Wijk bij Duurstede, The Netherlands). All supplemented oligosaccharide products were exchanged for the same amount of total carbohydrates, mixed into the AIN-93G diet and pressed into pellets.

The active ingredients in the diets were a mixture of scGOS (Vivinal GOS, Borculo Domo, Zwolle, The Netherlands;) and lcFOS (Raftiline HP, Orafit, Wijchen, The Netherlands;) in a 9:1 ratio (Immunofortis). Additionally, pectin-derived pAOS were used (kindly provided by Südzucker AG, Mannheim, Germany). Two supplemented test diets were compared with AIN93G control diet in two separate experiments. The diets were described in detail and were shown to induce immune modulatory effects previously [12]. In short, one test diet contained a net amount of 1% (w/w) scGOS/lcFOS. The other test diet contained approximately 1% (w/w) net oligosaccharides, consisting of 83% scGOS/lcFOS + 17% pAOS. All mice were given control AIN-93G diets upon arrival at the animal facility for a minimum of 7 days. The dietary interventions in supplementation groups started 14 days before the primary sensitization and lasted during the entire experiment.

2.2. OVA sensitization and aerosol challenge

Mice were sensitized to ovalbumin by two intraperitoneal injections with 10 µg ovalbumin (OVA) grade V (Sigma Aldrich,

Zwijndrecht, The Netherlands) in 2.25 mg/0.1 ml AlumInject (Pierce, Perbio Science Nederland, Etten-Leur, The Netherlands) in PBS on day 0 and 7. Sham-sensitized animals received 0.1 ml PBS/AlumInject only. Each experiment consisted of four groups; the control and supplemented diets were tested both in sham and OVA-sensitized mice. All groups consisted of 9 animals.

OVA-sensitized and sham-sensitized mice were exposed to 10 mg/ml nebulized OVA aerosols in PBS using Pari LC Star (Pari IS-2, Pari-Werk GmbH, Starnberg, Germany). For each exposure, the animals were placed in an aerosol cabin for 20 min. Animals were aerosol challenged on day 35, 38 and 41 after the first sensitization.

2.3. Airway hyperresponsiveness

Airway responsiveness was measured *in vivo* 24 h after the last aerosol exposure in conscious unrestrained mice using a plethysmograph (Buxco, EMKA Technologies, France) as described by Hanemann et al. [15]. The mice were exposed for 3 min to doubling doses of aerosolized methacholine (Mch) ranging from 1.56 mg/ml to 50 mg/ml; after exposure to a methacholine dose lung function was measured for 3 min. From the lung function parameters peak expiratory flow (PEF), tidal volume (TV), expiratory time (Te) and frequency (f), the enhanced pause (PENH) parameter can be calculated with the formula $((Te/RT) - 1) \cdot (PEF/PIF)$. It has previously been demonstrated that PENH correlates strongly to airway resistance in this allergic asthma model in BALB/c mice [15–17].

2.4. Broncho-alveolar lavage

Broncho-alveolar lavage (BAL) was performed in the same animals that were used for *in vivo* airway hyperresponsiveness measurements. In pilot experiments it was found that combining these techniques had no effect on the total number of lavage cells. Mice received a lethal dose of pentobarbital sodium (300 mg/kg body weight intraperitoneally). The lungs were filled with 1 ml aliquots of pyrogen free saline (0.9% NaCl) supplemented with protease inhibitor cocktail tablet (1 tablet/100 ml) and bovine serum albumin (5 g/100 ml) of 37 °C *in situ*. Fluid was collected in a plastic tube on ice (4 °C) (totally 1 ml). This procedure was repeated 3 times with aliquots of pyrogen free saline (0.9% NaCl). The BAL cells were centrifuged (400 g, 4 °C, 5 min), and resuspended. All cells from one animal were pooled and counted by use of a Bürker-Türk chamber (magnification 100×).

2.5. Measurement of OVA-specific IgE in serum

Flat-bottom 96-well plates (Nunc Maxisorp, Life Technologies, Breda, The Netherlands) were coated overnight at 4 °C with capture antibody (1 µg/ml purified Rt×Ms IgE; Pharmingen, San Diego, CA, USA) in sodium carbonate buffer (pH 9.4). After coating and in between subsequent incubation steps, plates were washed with PBS containing 0.05% Tween-20. Plates were

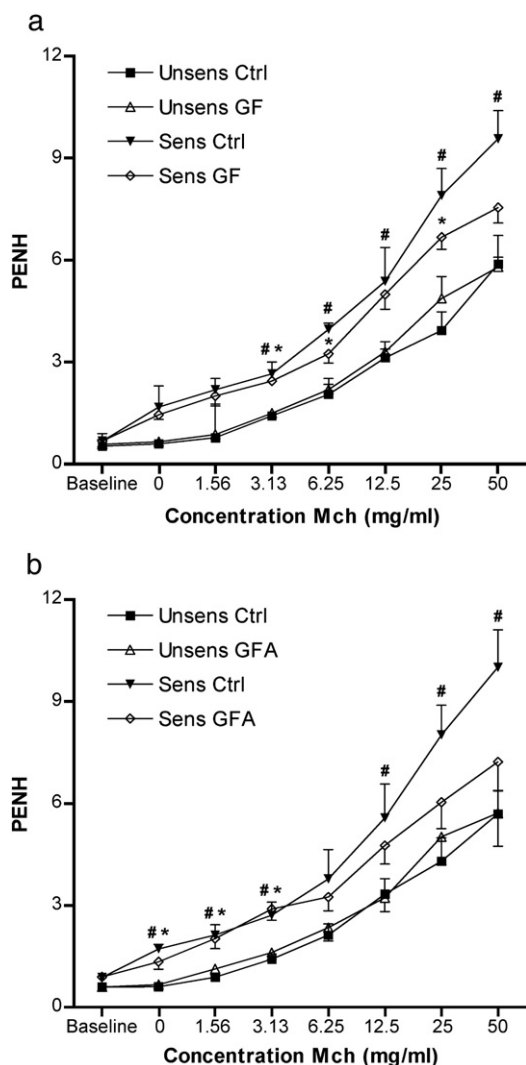


Fig. 1. Dietary intervention reduces airway hyperresponsiveness. The effects of dietary intervention with (a) scGOS/lcFOS in a 9:1 ratio (GF) and (b) scGOS/lcFOS/pAOS (GFA) were measured on the PENH parameter, measured in conscious unrestrained ovalbumin sensitized (Sens) and unsensitized mice (Unsens). After a baseline measurement, the mice were challenged with increasing doses of aerosolized Mch in PBS. Values are means $\pm$ SEM ($n=9$ per group); # indicates $p<0.05$ unsensitized control mice vs sensitized control mice; * indicates $p<0.05$ unsensitized control mice vs sensitized supplemented mice.

blocked (2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.5% BSA and 0.05% Tween-20, pH 7.2, 1 h, room temperature (RT)). Samples and standards (serum pool from OVA-immunized mice, CIP, Utrecht, NL) were applied (100 μ l/well, 2 h, RT), followed by OVA coupled to Digoxigenin (DIG; Roche Diagnostics, Mannheim, Germany; 100 μ l/well, 1 h, RT) and anti-DIG-peroxidase (Roche Diagnostics; 1 h, RT). After the final washing, 0.4 mg/ml *o*-phenylenediamine-dihydrochloride in PBS containing 0.04% hydrogen peroxide was added. The reaction was stopped after

approx. 5 min using 4 M H_2SO_4 . Optical density was measured at 492 nm.

2.6. Analysis and statistical calculations

Data were analyzed using Graphpad Prism 4.03 software and expressed as arithmetic average $\pm$ standard error of the mean (SEM). ANOVA and *post-hoc* Dunnet's test was performed to compare the airway hyperresponsiveness data from multiple groups with one control group. For direct comparisons between the sensitized control and sensitized supplemented groups, the one-sided Student's *T*-test (PENH and BAL inflammatory cells) or Mann–Whitney *U*-test (IgE titers) was used to evaluate whether supplementation reduced parameters of allergic asthma. A probability value of $p<0.05$ was considered significant.

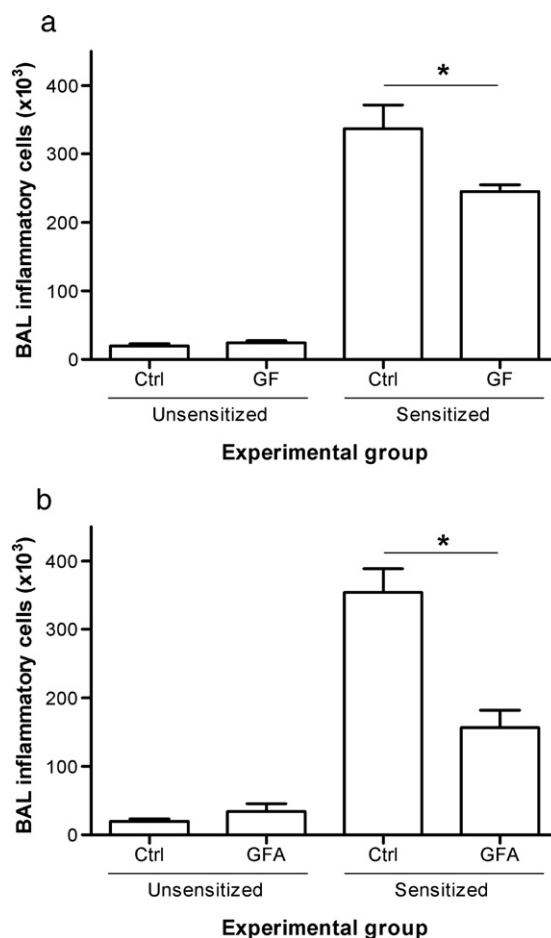


Fig. 2. Dietary intervention reduces the number of inflammatory cells in BAL fluid. The effects of dietary intervention with (a) scGOS/lcFOS in a 9:1 ratio (GF) and (b) scGOS/lcFOS/pAOS (GFA) are shown on the total number of inflammatory cells in BAL fluid. Values are means $\pm$ SEM ($n=9$ per group); * indicates $p<0.05$ sensitized control mice vs sensitized supplemented mice.

3. Results

3.1. Airway hyperresponsiveness

The airway responsiveness to increasing doses of Mch, as measured by PENH, was similar for unsensitized mice in both experiments, regardless of the diet. Airway hyperresponsiveness was observed in sensitized control animals in both experiments; the PENH was significantly higher compared to unsensitized control animals in five out of six concentrations of Mch and the largest effect occurred at the highest concentrations. Both supplemented diets suppressed the development of the OVA-induced airway hyperresponsiveness in sensitized mice (Fig. 1). In scGOS/lcFOS fed mice, the PENH was not significantly different from unsensitized control mice at 12.5 and 50 mg/ml Mch, in contrast to sensitized control mice. The same effect was observed in scGOS/lcFOS/pAOS supplemented mice at the three highest concentrations of Mch. Supplementation of sensitized mice did not affect the increased responses at lower Mch concentrations. However, the maximal responses were significantly decreased by 21% ($p=0.02$) and 27% ($p=0.03$) in the scGOS/lcFOS and scGOS/lcFOS/pAOS diet groups respectively, compared directly to the sensitized control group.

3.2. Pulmonary inflammatory cellular response

The effects of dietary supplementation with oligosaccharides on the number of inflammatory cells in BAL fluid are shown in Fig. 2. Low numbers of cells were observed in unsensitized animals irrespective of diet (range $19.2\text{--}33.9 \times 10^3$). OVA challenge dramatically increased BAL cellularity in the sensitized groups. Although airway inflammation was still present in both supplemented groups of sensitized mice, the number of cells in the BAL fluid was significantly decreased compared to the control diets (control vs scGOS/lcFOS: 336.8 ± 34.5 vs $244.9 \pm 9.8 \times 10^3$, $p=0.01$; control vs scGOS/lcFOS/pAOS: 353.9 ± 34.9 vs $156.6 \pm 25.1 \times 10^3$, $p<0.001$). The inflammatory cells were not differentiated in these experiments, but have been shown to consist of a large majority of eosinophils in previous comparable experiments [18].

3.3. IgE serum levels

OVA-specific IgE levels were expressed as arbitrary units (AU). Background levels were measured in unsensitized mice fed control and supplemented diets (range: 2.4–4.6 AU), but high titers were measured in all sensitized animals. The average IgE titers in mice that were fed scGOS/lcFOS and scGOS/lcFOS/pAOS containing diets were respectively 28% and 38% lower than in control diet fed animals, but the differences were not significant (control vs scGOS/lcFOS: 89.2 ± 17.7 vs 64.7 ± 4.4 AU, $p=0.13$; control vs scGOS/lcFOS/pAOS: 78.6 ± 17.8 vs 48.5 ± 6.7 AU, $p=0.17$) (Fig. 3).

4. Discussion

In accordance with the hypothesis, dietary intervention with scGOS/lcFOS in a 9:1 ratio (Immunofortis) and with

scGOS/lcFOS/pAOS decreased several parameters of allergic asthma in BALB/c mice. Significant effects were observed on airway hyperreactivity and the number of inflammatory cells in the BAL fluid. Additionally, OVA-specific IgE levels were decreased by more than 25% in both supplemented groups, although these differences were not significant. The basal airway reactivity in the OVA-sensitized and challenged groups was higher than in the unsensitized groups, suggesting that a methacholine-independent mechanism plays a role but was unaffected by the dietary intervention. It appears that the intervention only suppressed the Mch-dependent hyperresponsiveness, which is most evident at the highest concentrations. Overall, the results show that supplementation with the specific oligosaccharide mixtures decreases the manifestation of experimental allergic asthma in this model.

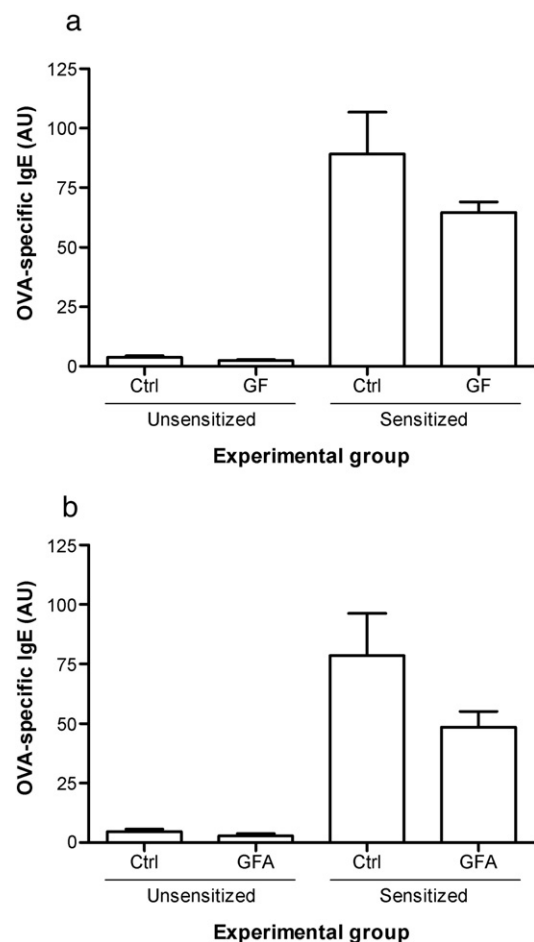


Fig. 3. The effect of dietary intervention on OVA-specific IgE titers in serum. The effects of dietary intervention with (a) scGOS/lcFOS in a 9:1 ratio (GF) and (b) scGOS/lcFOS/pAOS (GFA) on the OVA-specific IgE titers in serum, measured by ELISA. No significant differences were observed between sensitized control and sensitized supplemented groups.

These results fit well with data from previous murine vaccination experiments, in which enhanced Th-1 dependent DTH responses and reduced Th-2 cytokine production *ex vivo* were observed in healthy young adult C57BL/6 mice [11,12]. The combined data from all experiments are consistent with the notion that the specific mixtures of oligosaccharides induce a shift in the Th1/Th2 balance towards Th1 responses, in healthy or allergic mouse models using different strains. Although the effects shown in this paper were comparable for both test diets, the effects of the scGOS/lcFOS/pAOS containing diet appeared to be more pronounced than the effects of the scGOS/lcFOS diet. This difference corresponds to previous observations in the vaccination model, in which the combination of all three types of oligosaccharides was significantly more effective than scGOS/lcFOS or pAOS alone [12]. However, since both test diets were tested in two separate experiments in the allergic asthma model, no direct comparison could be made in the present study.

Anti-allergic effects of prebiotics have not been described widely in literature. However, Fujitani and coworkers have very recently demonstrated beneficial effects of 5% (w/w) short-chain FOS supplementation on parameters of OVA-induced food allergy in mice, leading to reduced numbers of mast cells and edema formation in the duodenum [19]. Additionally, Sonoyama et al. have shown that 5% (w/w) alpha-linked GOS and raffinose reduced airway eosinophilia and Th2 cytokine production in OVA sensitized rats [7].

In the current study, supplementation with the specific oligosaccharide mixtures in both test diets proved to be effective at a low dose that is relevant for various human applications, including infant formulas. It was previously shown that comparable doses of scGOS/lcFOS induced bifidogenic effects when applied in an infant formula, resulting in a composition of the intestinal microbiota that was closer to that of breast fed infants than of standard formula fed infants [20]. It has been described that the incidence of atopic dermatitis (AD) and early childhood wheezing is higher in standard non-hydrolyzed formula fed infants than in breast fed infants [21–24]. Similar to the effects on the microbiota, the current results suggest that addition of the specific mixtures of oligosaccharides to infant formula may help to make it closer to breast milk with regard to the development of atopy and wheezing. This notion is supported by the first clinical results that are available: in children that received scGOS/lcFOS-containing formula during the first 6 months of life, several allergic symptoms were reduced significantly, even until these children were two years of age [25,26]. Additional clinical studies are currently ongoing.

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