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Effect of Dietary Lactosucrose (4^G-β-D-Galactosylsucrose) on the Intestinal Immune Functions in Mice

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Keiko Hino,[1] Mayumi Kurose,[1] Takeo Sakurai,[1] Shin-ichiro Inoue,[1] Kazuyuki Oku,[1,*] Hiroto Chaen[1] and Shigeharu Fukuda[1]

[1]*Glycoscience Instituite, Research Center, Hayashibara Biochemical Laboratories, Inc. (675–1, Fujisaki, Okayama 702–8006, Japan)*

Abstract: Lactosucrose (4^G-β-D-galactosylsucrose, LS) is suggested to be an oligosaccharide required for the proliferation of Bifidobacteria in the intestine. We have examined the dietary effects of LS on the intestinal immune function of mice. BALB/c mice were fed with 2 and 5% LS for 4 weeks, and the intestinal mucosal immune responses were determined. In the 2 and 5% LS fed groups, the amounts of IgA in feces and in cecum contents were significantly increased. In addition, IgA, transforming growth factor-β1 (TGF-β1) and interleukin-6 (IL-6) secretion by Peyer's patch (PP) cells were enhanced in LS fed mice. In LS fed mice, pH in the cecum was decreased. LS, in addition, suppressed serum IgG1. These results suggest that LS supplementation changes the intestinal environment of microflora, and indirectly enhances the immune function in the gut, and suppresses the systemic immune response to the dominant type 2 helper T (Th2).

Key words: lactosucrose, IgA, TGF-β1, IL-6, IgG1

Recently, much attention has been focused on the mucosal immune system in the gut, and the elucidation of the system is underway. The intestinal tract is one of the internal organs, which are always exposed to various kinds of pathogens and antigens including pathogenic bacteria, viruses and allergens. For this reason, the mucosal membrane in the intestinal tract act, as an important frontline defense barrier, plays important roles. Probiotics[1] is defined as microbial food supplements that beneficially affect the host by improving its intestinal microbial balance. Prebiotics,[2] such as dietary indigestible oligosaccharides, also beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of health-promoting indigenous flora. Both prebiotics and probiotics have been shown to reduce the incidence and severity of gastrointestinal disorders and prevent the development of allergic diseases.[3–5]

Lactosucrose (4^G-β-D-galactosylsucrose, LS), which is composed of both lactose and sucrose moieties in a molecular structure, has been synthesized using a transfructosylation catalyzed by β-fructofuranosidase.[6–8] Because hydrolysis of LS is not undertaken by the digestive enzymes in the small intestine, most of the LS taken with meals reaches the cecum, where fermentation will take place.[9] It has been reported that the proportion of Bifidobacteria in fecal microflora is remarkably enhanced during the period of LS intake in human subjects.[10,11]

On the other hand, Park *et al*. have reported that *Bifidobacterium bifidum* significantly induced total IgA and IgM synthesis by both mesenteric lymph nodes (MLN) and Peyer's patch (PP) cells.[12] It has been reported that oral probiotic bacterial administration (*Bifidobacterium bifidum* and *Lactobacillus casei*) suppressed allergic responses in an ovalbumin-induced allergy mouse model (C3H/HeJmice).[13] However, it remains to be clarified whether intake of LS potentiates the mucosal immune function. In this study, we have investigated the effect of oral LS administration on the intestinal mucosal immunity in mice.

MATERIALS AND METHODS

Animals and diets. Female 6-week-old BALB/c mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan), and were housed in a room at 23–25 °C and 50–60% relative humidity with a 12 h light-dark cycle. After acclimatizing for 7 days, mice were divided into 5 groups (5 animals per group), and were provided with the experimental diets and water *ad libitum*. The diets were prepared according to the recommendation of the American Institute of Nutrition AIN-93G. Table 1 shows the composition of the experimental diets. LS (containing lactosucrose 91.8%, Lactose 2.9%, sucrose 4.1% and other oligosaccharides 1.2 %) was prepared in our laboratory. The mice consumed the diets for 4 weeks. We collected serum from tails under non-fasting conditions every 2 weeks. After 4 weeks of feeding on experimental diets, the mice were euthanized.

The experimental procedures used in this study met the guidelines of the Animal Usage Committee of Hayashibara Biochemical Laboratories, Inc.

Material. Concanavalin A (Con A) was purchased from Sigma (St. Louis, MO) and dissolved in phosphate buff-

*Corresponding author (Tel. +86–276–3142, Fax. +86–276–8670, E-mail: okukaz@hayashibara.co.jp).

Abbreviations: LS, lactosucrose; IgA, immunoglobulin A; TGF-β1, transforming growth factor-β1; IL-6, interleukin-6; PP, Peyer's patch; NK, natural killer; Con A, Concanavalin A; PBS, phosphate buffered saline; ELISA, enzyme-linked immunosorbent assay.

Table 1. Composition of experimental diets.

(g/kg diet)

Ingredient	Cont.	LS2*	LS5*
Corn starch	397	375	342
Casein	200	200	200
α-Con starch	132	132	132
Sucrose	100	100	100
Soybean oil	70	70	70
Mineral mixture	35	35	35
Vitamin mixture	10	10	10
L-Cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
tert-Butylhydroquinone	0.014	0.014	0.014
Cellulose	50	50	50
Lactosucrose	0	22	55

*LS2 and 5, experimental diet groups containing 2 and 5% of lactosucrose (>91.8% purity).

ered saline (PBS). Enzyme-linked immunosorbent assay (ELISA) kits for quantitative analysis of immunoglobulin A (IgA) and immunoglobulin G1 (IgG1) were purchased from BETHYL (Montgomery, TX). ELISA kits for quantitative analysis of Interleukin-6 (IL-6) and Transforming Growth Factor-β1 (TGF-β1) were purchased from R & D System (Minneapolis, MN), and from Promega (Madison, WI), respectively.

Analysis of feces. Fecal samples were collected every 4 weeks and diluted 5-fold with PBS containing 50 mM EDTA and 0.1 mg/mL trypsin inhibitor, and were then centrifuged at 9000×*g* for 10 min at 4°C. The supernatants were subjected to the IgA ELISA.

Analysis of cecum contents. The weight of the cecum content was obtained by subtracting the weight of the cecum wall from the total weight of the cecum with wet contents. To measure the pH of the cecum, the contents were carefully taken out and then added into a 5-fold volume of water. The pH values were measured using a pH meter (TOA Electronics Ltd., HM-11P). The suspended solution of cecum contents was centrifuged at 10,000×*g* for 10 min at 4°C. The supernatants were subjected to the IgA ELISA.

Cell preparation. Peyer's patch (PP) cells were collected by density gradient centrifugation with Percoll™/Redi Grad™ (Amarsham Biosciences, Sweden), and washed three times with RPMI 1640 medium (Sigma Chemicals Co., St. Louis, MO) supplemented with 10% FCS (Thermo Trace Ltd., Melbourne, Australia), 60 μg/mL of Penicillin, 50 μg/mL of streptomycin and 10 mM HEPES. PP cells were then resuspended in the above-mentioned medium supplemented with 5×10^{-5} M 2-mercaptoethanol (complete medium) at a cell density of 1 $\times10^{6}$ cells/mL. Cells were stimulated with 2 μg/mL Con A for 48 h at 37°C. After the incubation period, culture supernatants were collected and stored at −70°C until cytokine and IgA assays.

Serum analysis. We measured serum IgG1 and IgG2a levels using by ELISA kits as follows.

Enzyme-linked immunosorbent assay (ELISA). The amounts of IgA, TGF-β, IL-6, IgG1 and IgG2a in these preparation samples were measured with the above-mentioned ELISA kits.[1] The detection limits for IgA, TGF-β, IL-6, IgG1 and IgG2a were 15.6 ng/mL, 31.2 pg/mL, 15.6 pg/mL, 15.6 ng/mL and 3.9 ng/mL, respectively.

Statistical analysis. Results were expressed as the mean ±SD. An analysis of variance (ANOVA) was used to determine differences between the control mice and the treated mice. When statistically significant differences ($p<0.05$ or $p<0.01$) were found between the groups, the *t*-test was used to determine the level of significant differences between the control group and the treated group.

RESULTS

Increases in intestinal IgA secretion by LS intake.

To examine the effect of LS feeding on the intestinal mucosal immunity, diets containing 2 and 5% of LS were given to BALB/c mice. There was no significant increase in food intake or body weight gain among the 5 groups (Table 2). First, we determined the IgA secretion from the intestinal mucosa. Total IgA contents in the feces were significantly higher in the group given 2 and 5% LS than those of the control group in the 1st to 4th week (Fig. 1).

IgA and cytokine production by PP cells.

We then examined the IgA production by PP cells,

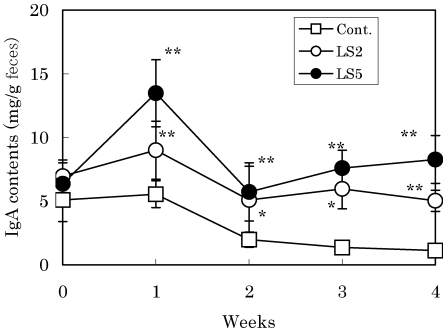

Fig. 1. The amounts of IgA in the fecal samples collected from mice that were fed with varying doses of oligosaccharides.

The amounts of IgA in the fecal samples were determined every week by ELISA. Values represent the means±SD of 5 mice for each group. *$p<0.05$, **$p<0.01$, significantly different from the mean value of the control group.

Table 2. Influence of experimental diet intake on the body weight gain of mice.

Dietary group	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Food intake (g/day)	Food efficiency (g gain/g intake)
Cont.	20.36±1.21	22.78±0.99	2.42±0.50	3.06±0.48	0.028
LS2	20.42±1.04	23.04±1.54	2.62±0.78	3.09±0.23	0.030
LS5	20.20±0.73	22.90±1.11	2.70±0.62	3.08±0.37	0.031

Experimental diets were prepared as shown in Table 1 and given to BALB/c mice. After 4 weeks, food intake and body weight gain were determined for each group. Values represented the mean±SD of 5 mice for each group.

which were collected from mice fed the experimental diet for 4 weeks. The PP cells were stimulated with Con A *in vitro*, and the amounts of IgA in the culture supernatants were measured. Increased secretion of IgA by PPcells was observed in the LS-administered group in a dose-dependent manner (Table 3). Since both TGF-β and IL-6 play essential roles in IgA production,[16] we determined the levels of these cytokines in the culture supernatants. The amounts of TGF-β and IL-6 significantly increased in the 5% of LS group, compared with those of the control group (Table 3).

Weight and pH in the cecum content.

The weight of the cecum content increased in the LS-administered group in a dose-dependent manner (Table 4), suggesting that most of LS is not digested and passes into the large intestine. The pH of the cecum content in the 2 and 5% LS groups was significantly lower than that of the control group (Table 4). These results suggest that indigestible LS was fermented to cause a change in the composition of intestinal microflora.

Table 3. Effects of orally administered LS on the IgA secretion and the cytokine production by PP cells.

Dietary group	IgA (mg/mL)	IL-6 (pg/mL)	TGF-β (pg/mL)
Cont.	1.46±0.45	32.7±8.9	296.3±234.2
LS2	1.91±0.50	35.1±13.6	399.0±240.2
LS5	4.50±2.60*	84.1±38.5*	657.0±127.7*

PP cells were derived from mice that were fed with the experimental diet for 4 weeks, and were stimulated with Con A for 48 h at 37°C. The amounts of IgA and cytokines in the culture supernatants were determined every week by ELISA. Values represent the means±SD of 5 mice for each group. *$p<0.05$, significantly different from the mean value of the control group.

Table 4. pH and weight of cecum content.

	pH	Weight (g)
Cont.	7.80±0.23	0.127±0.032
LS2	7.41±0.24*	0.148±0.017
LS5	6.63±0.27*	0.224±0.051*

BALB/c mice were fed with the experimental diets for 4 weeks. pH and the weight of cecum content were determined as described in the "MATERIALS AND METHODS" section. Values represent the means±SD of 5 mice for each group. *$p<0.05$, significantly different from the mean value of the control group.

Table 5. Effects of orally administered LS on serum IgG1 and IgG2a levels.

Dietary group	Experimental days (week)		
	0	2	4
	Serum IgG1 levels (μg/mL)		
Cont.	115.7±23.7	132.9±42.4	89.1±25.2
LS2	98.2±16.6	111.2±58.3	64.5±8.20*
LS5	89.1±12.6	85.0±21.0	62.4±15.1*
	Serum IgG2a levels (μg/mL)		
Cont.	44.1±15.1	35.9±10.5	62.9±25.2
LS2	43.8±18.9	36.9±28.7	69.3±16.7
LS5	52.3±14.7	34.2±18.4	63.6±18.3

BALB/c mice were fed with the experimental diets for 4 weeks. Values represent the means±SD of 5 mice for each group. *$p<0.05$, significantly different from the mean value of the control group.

Suppression of Th2 type antibody in the sera due to LS administration.

To examine the immunoregulatory effects of LS administration in the systemic immune system, we measured the levels of IgG1 and IgG2a in sera. IgG2a levels did not differ significantly between these groups (Table 5). IgG1 level was significantly lower in the groups given LS than in the control group at the 4th week (Table 5). The IgG1 subclass reflected a Th2 type immune response predominantly in the serum and the Th2 type antibody response was suppressed by LS administration *in vivo.*

DISCUSSION

The present study was undertaken to explore the dietary effects of Lactosucrose (4^G-β-D-galactosylsucrose, LS) on the intestinal immune function of mice.

It has been reported that the digestive enzymes in the small intestine do not hydrolyze LS, and most of the LS taken with meals reaches the cecum where fermentation will take place.[14] In our results, a fall in the pH of the cecum contents in the LS group was observed after 4 weeks' administration (Table 4). This result suggests that LS supplementation changes the intestinal environment of microflora.

Actually, Hara *et al.* reported that LS was fermented by all Bifidobacteria species including *B. bifidum* and Lactobacilli species including *L. casei*.[11] Additionally, Kim *et al.* reported that administration of *Escherichia coli, B. bifidum* BGN4, or *L. casei* decreased the OVA-induced allergy response in C3H/HeJ mice.[13] The administration of *B. bifidum* alone significantly induced total IgA and IgM synthesis by both mesenteric lymph nodes (MLN) and PP cells.[12]

PP are major IgA inductive sites in mammals. The regulation of IgA production *in vitro* by T cells in PP has already been reported.[15] In our experiment, the IgA, TGF-β and IL-6 production by PP cells from LS-fed groups of mice was up-regulated *in vitro* (in unpublished data). It is well known that TGF-β induces B cell activation and Ig isotype switching to IgA.[16,17] IL-6 also plays an important role in the terminal stage of mucosal immune responses.[18] IgA secretion from B cells in PP is promoted by human recombinant IL-6, and IL-6 antibodies abrogate it. Thus, it is reasonable to speculate that up-regulation of IgA production by PP cells from LS-fed mice is due to the increased production of TGF-β and IL-6.

Jutel *et al.* reported that TGF-β and IL-10 cooperate in suppression of the immune response to aeroallergens and control allergic inflammation due to mucosal allergen exposure.[19] TGF-β is also an effecter molecule of the Th3-type cells that plays important roles in oral immune tolerance.[20] These results suggest that intake of LS may be effective for the induction of oral immune tolerance by Th3 cells, resulting in the prevention of food allergy.

Sudo *et al.* reported that adequate probiotic (lactic acid-producing bacteria) intervention after antibiotic treatment may improve the intestinal ecosystem, and thereby pre-

vent the Th2-shifted immune responses induced by neonatal antibiotic use.[1] As for the systemic immune response, total IgG1 and IgG2a concentrations in the serum were measured for comparison with the mucosal immune response. We have shown that LS supplementation can suppress serum IgG1 levels, as a Th2-type reaction (Table 4). Although mucosal IgA production was affected in the 1st week, systemic immune responses were remarkable in the 4th week of LS administration. This indicates that LS changes the microbial environment in the gut and upregulates mucosal immune response for protective immunity; then it affects systemic immune responses.

The balance of intestinal flora is known to play an important role in the health and biological defense of its host.[4] Water-soluble dietary fiber such as pectin and glucomannan, and indigestible carbohydrates such as fructooligosaccharides have been reported to exert biological effects as the prebiotics.[21−23] These prebiotics are expected to increase IgA secretion and prevent allergic responses in the intestinal tract. These results together with our findings presented in this study suggest that LS as a prebiotic exerts immunopotentiating effects in the intestinal mucosal membranes through interacting with intestinal microflora. Our results further imply that LS creates better intestinal environments and activates the mucosal immune system, resulting in the efficient prevention of pathogenic bacterial invasion and allergic responses.

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マウスにおける腸管免疫反応に及ぼすラクトスクロース (4^G-β-D-ガラクトシルスクロース) 連続摂取の影響

日野恵子[1]，黒瀬真弓[1]，桜井岳夫[1]，井上紳一郎[1]
奥　和之[1]，茶圓博人[1]，福田恵温[1]
[1] 株式会社林原生物化学研究所研究センター糖質研究部門
(702−8006 岡山市藤崎 675−1)

ラクトスクロース (4^G-β-D-ガラクトシルスクロース，以下 LS) は，腸管内において *Bifidobacterium* に選択的に資化されるオリゴ糖である．本研究では，マウスにおける腸管免疫反応に及ぼす LS 連続摂取の影響を検討した．BALB/c マウスに LS2% または 5% 添加飼料を 4 週間摂取させ，小腸粘膜の免疫応答性を調べた．LS2% または 5% 摂取群で，糞および盲腸内容物中の IgA 量が有意に増加した．パイエル板細胞からの IgA，TGF-β，IL-6 分泌も増加した．また，LS 摂取マウスの盲腸内容物中の pH が低下した．さらに血清中の IgG1 が LS 摂取群で有意に低下した．以上の結果から，LS は腸内細菌叢の変化を促すことにより，間接的に消化管内の免疫反応に影響を及ぼすことが示唆された．さらに，LS 摂取による全身免疫系の Th2 応答抑制作用が期待された．