



## Effects of Dietary Lactosucrose (4<sup>G</sup>-β-D-galactosylsucrose) on the IgE Response in Mice

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In this study, we examined the effects of dietary lactosucrose (LS, a non-digestible oligosaccharide) on the IgE response in mice immunized with ovalbumin (OVA)/alum. In addition to IgG1 and IgG2a responses, the anti-OVA IgE response in mice fed LS diets was dose-dependently suppressed, as compared with the control mice, while the serum total IgG levels were comparable. Moreover, dietary LS feeding inhibited antigen-specific IgE and IgG1 productions even after a second immunization. Regarding with cytokine production, when stimulated *in vitro* with OVA, splenocytes obtained from LS-fed mice produced a similar level of IFN-γ, and lower levels of IL-4 and IL-5, as compared with the control mice. But IL-10 production by OVA-stimulated splenocytes was augmented in LS-fed mice, suggesting that IL-10 producing cells are responsible for the immunoregulatory effect of LS. Our findings indicate the further possibility that dietary LS supplementation can be used to prevent IgE-mediated allergic diseases.

**Key words:** lactosucrose; oligosaccharides; immunoglobulin E (IgE); Cry j 1; IL-10

Allergic diseases such as atopic dermatitis, allergic rhinitis, and asthma are associated with immunoglobulin (Ig) E production specific to the antigens contained in food and environmental substances. A therapeutic strategy of down-regulating IgE production using anti-IgE humanized antibody has, therefore, proven to be useful against allergic diseases.<sup>1)</sup> Recent epidemiological findings have demonstrated the important role of intestinal microflora in the prevention of allergic diseases.<sup>2)</sup> For instance, an epidemiological study comparing intestinal microflora, as between allergic and non-allergic children showed characteristic micro-

flora in the allergic children, with a lower frequency of *lactobacilli* and *bifidobacteria* and higher counts of aerobes.<sup>3-5)</sup> It has been reported that antibiotic use in infancy might be associated with an increased risk of developing atopic disorders.<sup>6)</sup> In fact, the Th2 immune response was predominant in germ-free mice, but serum IgE was suppressed by oral administration of *Bifidobacterium* in infants.<sup>7)</sup> Furthermore, elimination of indigenous microflora by kanamycin treatment during infancy increased the serum levels of IgE in mice.<sup>8)</sup>

Lactosucrose (4<sup>G</sup>-β-D-galactosylsucrose, LS), which is composed of both lactose and sucrose moieties in a molecular structure, has been synthesized using transfructosylation catalyzed by β-fructofuranosidase.<sup>9-11)</sup> Because hydrolysis of LS is not undertaken by digestive enzymes in the small intestine, most of the LS taken with meals reaches the cecum, where fermentation takes place.<sup>12)</sup> It has been reported that the proportion of *Bifidobacteria* in fecal microflora is remarkably enhanced during the period of LS intake in human subjects.<sup>13,14)</sup> These results indicate that LS has functional characteristics of prebiotics similar to other non-digestible oligosaccharides.<sup>15,16)</sup> Recently it was shown that LS supplementation caused a change in the intestinal environment of microflora and indirectly enhanced IgA in the gut,<sup>17)</sup> but little is known about the effects of LS supplementation on IgE production. In the present study, we found that LS intake suppressed IgE response induced by intraperitoneal immunization with OVA/Alum in a mice model.

## Materials and Methods

**Animals and diets.** BALB/c female mice, 6 weeks of age, were purchased from Charles River Japan (Kagawa, Japan), and were housed in a room at 23–25 °C at

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Abbreviations: LS, lactosucrose; OVA, ovalbumin; IgE, immunoglobulin E; ELISA, enzyme-linked immunosorbent assay; ip, intraperitoneally

**Table 1.** Composition of Experimental Diets

|                       | Control | LS 5%   |
|-----------------------|---------|---------|
| Casein                | 20 g    | 20 g    |
| Soybean oil           | 7       | 7       |
| Mineral mixture*      | 3.5     | 3.5     |
| Vitamin mixture*      | 1       | 1       |
| Cellulose powder      | 5       | 5       |
| L-cystin              | 0.3     | 0.3     |
| t-Bt hydroquinone     | 0.0014  | 0.0014  |
| Corn starch           | 40      | 33      |
| $\alpha$ -Corn starch | 13.2    | 13.2    |
| Sucrose               | 10      | 10      |
| Lactosucrose**        | 0       | 9.083   |
| Total                 | 100.0 g | 102.0 g |

The LS 2% diet was prepared by mixing the control diet with the LS 5% diet.

\*Mineral mixture and vitamin mixture were obtained from Oriental Yeast according to the AIN-93G formula.

\*\*Lactosucrose syrup (Nyuka Oligo 700, 77% w/w), which contains 71.4% LS, 7.1% lactose, and 12.3% sucrose, was obtained from Hayashibara Shoji (Okayama, Japan).

50–60% relative humidity on a 12 h light-dark cycle. The mice were provided with a control diet and water *ad libitum*. After acclimatization for 7 d, the mice were used. The experimental procedures used in this study met the guidelines of the Animal Usage Committee of Hayashibara Biochemical Laboratories, Inc.

The diets were prepared according to the recommendations of the American Institute of Nutrition, AIN-93G. Table 1 shows the composition of the control and experimental 2% and 5% LS containing diets. Dietary components were obtained from Oriental Yeast (Tokyo). Lactosucrose syrup (Nyuka Oligo 700, 77% w/w), which contains 71.4% LS, 7.1% lactose, and 12.3% sucrose, was obtained from Hayashibara Shoji (Okayama, Japan).

**Antigens.** Ovalbumin (OVA, gradeV) was purchased from Sigma Chemicals (St. Louis, MO). Cry j 1, purified Japanese cedar allergen, and biotin-conjugated Cry j 1 for ELISA were purchased from Seikagaku Corporation (Tokyo). Alum (inject alum) was purchased from Pierce Biotechnology (Rockford, IL).

**Experimental schedule 1.** The mice were divided into three groups (5–7 animals per group). They were intraperitoneally (ip) injected with 20  $\mu$ g of OVA and 4.5 mg of alum in a total volume of 0.2 ml on days 7 and 21. In another experiment, Cry j 1 was injected into the mice instead of OVA. LS diets or the control diet were fed for 35 d starting at day 1. On day 35, whole blood was drawn from the abdominal aorta under anesthesia with diethyl ether, after which the spleens were quickly excised for cytokine production assay. Sera were stored at  $-80^{\circ}\text{C}$  until antibody titers were measured.

**Experimental schedule 2.** In exp. 2, we examined the regulatory effect of dietary LS on further production in

IgE-producing mice. BALB/c female mice, 7 weeks of age, were immunized ip with OVA/Alum 2 times at a 1-week interval. Two weeks after the second immunization, blood was sampled from the tail vein. Based on the results of IgE titers in sera, the mice were divided into three groups (5 animals per group). LS diets or the control diet were fed to the OVA-immunized mice for 10 weeks starting on the day of grouping. During the experimental period, serum IgE anti-OVA titers were monitored at 2, 4, and 6 weeks. After the measurement of IgE titers at 6 weeks, the mice were given a booster injection with OVA/Alum. Serum antibody titers were determined at 2 and 4 weeks after the booster injection.

**Measurement of serum Igs.** Antigen-specific IgE and IgG2a titers in serum were measured by captured ELISA and antigen-specific IgG1 titers were measured by indirect ELISA, as described previously.<sup>18,19)</sup> In the captured ELISA, 96-well plates were coated with rat anti-mouse IgE mAb (R35-72, BD PharMingen, San Diego, CA) or with rat anti-mouse IgG2a mAb (R11-89, BD PharMingen) for 3 h at room temperature. IgE captured by anti-IgE mAb was detected with biotin-conjugated antigen (5  $\mu$ g/ml) and HRPO-conjugated Streptavidin (Zymed, San Francisco, CA). In the indirect ELISA, 96-well plates were coated with OVA or Cry j 1 for 3 h at room temperature. Antigen-specific IgG1 bound to 96-well plates was detected with HRPO-conjugated anti-mouse IgG1 (Zymed).

Titers of the standards for OVA-specific IgE, IgG1, and IgG2a ELISA, which were prepared from the sera of mice immunized with OVA, were tentatively assigned as 1,760 U/ml, 128,000 U/ml, and 7,040 U/ml respectively. Titers of the standards for Cry j 1-specific IgE and IgG1 ELISA, prepared from the sera of mice immunized with Cry j 1, were tentatively assigned as 1,600 U/ml and 500,000 U/ml respectively. The amounts of total IgG in the serum were determined by sandwich ELISA. Goat anti-mouse IgG (Fc) and HRPO-conjugated anti-mouse IgG (Fc) were purchased from MP Biomedicals (Irvine, CA). Standard mouse IgG was purchased from Sigma Chemicals.

**Cytokine measurement.** For cytokine production assay, single-cell suspensions of spleen were prepared in RPMI 1640 medium containing 10% FCS and  $5 \times 10^{-5}$  M 2-ME. Spleen cells ( $1 \times 10^6$  cells/ml) were incubated for 2 or 4 d at  $37^{\circ}\text{C}$  in 1-ml aliquots in 24-well plates in the presence or absence of OVA (10  $\mu$ g/ml). Supernatants were harvested for the determination of cytokine levels by sandwich ELISA. Cytokine concentrations in the culture supernatants were measured by sandwich ELISA, as described previously.<sup>18,19)</sup> Cytokine standards and mAbs used for the cytokine (IL-2, IL-4, IL-5, IL-10) ELISAs were obtained from BD PharMingen. Mouse IFN- $\gamma$  standard and rabbit anti-mouse IFN- $\gamma$  pAb were prepared in our laboratories.<sup>20)</sup> HRPO-conjugated Streptavidin was purchased from Zymed.

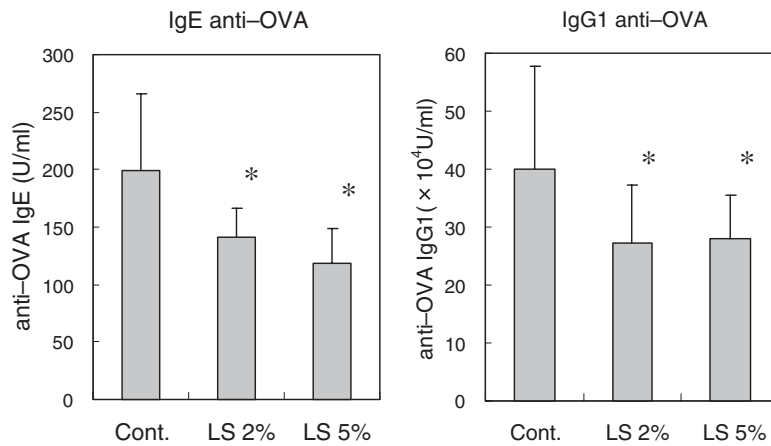


**Fig. 1.** Effects of LS Feeding on Serum Anti-OVA IgE and IgG1 Antibody Responses.

BALB/c mice were fed 2% and 5% LS-containing experimental diets for 5 weeks. On days 7 and 21, the mice were intraperitoneally immunized with OVA/Alum. Sera were collected on day 35, and titers of anti-OVA IgE and IgG1 antibodies were determined by ELISA. Data represent the mean  $\pm$  SD for 11 mice per group. An asterisk indicates a significant difference from the control group ( $p < 0.05$ ).

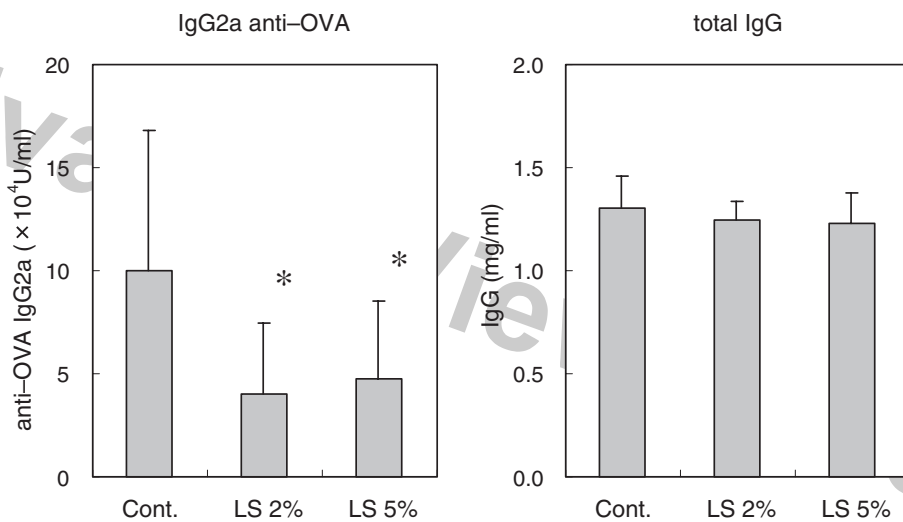


**Fig. 2.** Effects of LS Feeding on Serum Anti-OVA IgG2a and IgG Antibody Responses.

Mice were treated as described in Fig. 1. Titers of serum anti-OVA IgG2a antibody and total IgG concentrations on day 35 were determined by ELISA. Data represent the mean  $\pm$  SD for 11 mice per group. An asterisk indicates a significant difference from the control group ( $p < 0.05$ ).

The lower limits of detection, as determined using standard titration curves, were as follows: for IL-2, 25 pg/ml; IL-4, 12.5 pg/ml; IL-5, 25 pg/ml; IL-10, 31.3 pg/ml; and IFN- $\gamma$ , 0.2 IU/ml.

**Statistical analysis.** Results were expressed as means  $\pm$  SD. Statistical differences in antibody levels in the sera were analyzed by Dunnett's test. For evaluation of cytokine data, either Student's or Welch's T test was employed after examination by the F test. A value of  $p < 0.05$  was considered to be statistically significant.

## Results

### *Dietary LS induced down-regulation of OVA-specific immune response in normal mice*

To examine the effect of dietary LS supplementation

on antibody responses, mice were fed a 2–5% LS-containing diet and immunized with OVA/Alum on days 7 and 21. Then OVA-specific antibody responses were determined on day 35. The experiments were performed twice independently, and the combined results are shown in Fig. 1. In mice fed LS diets, the anti-OVA IgE response was significantly and dose-dependently suppressed, by 30–40%, as compared with the control group mice. The anti-OVA IgG1 response was also significantly suppressed in the LS-fed mice. Since both IgE and IgG1 antibody production is mediated through Th2 cells in mice, these data suggest that LS feeding suppressed Th2-type immune responses.

We further measured OVA-specific IgG2a levels in the sera of the LS-fed mice. The anti-OVA IgG2a response was also significantly suppressed in the LS-fed mice (Fig. 2). Since IgG2a production is considered to be a Th1-type immune response, these results suggest

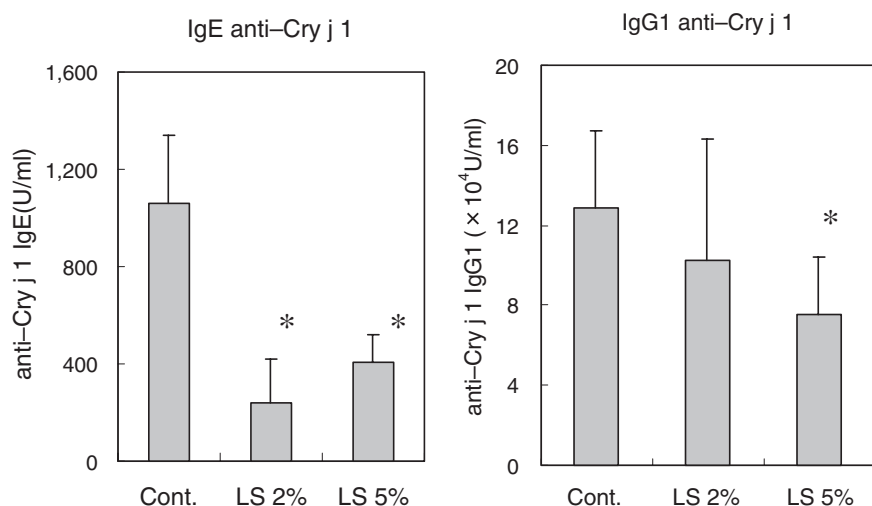


**Fig. 3.** Effects of LS Feeding on Serum Anti-Cry j 1 IgE and IgG1 Antibody Responses.

BALB/c mice were intraperitoneally immunized with Cry j 1/Alum instead of OVA/Alum as in Fig. 1. Sera were collected on day 35, and titers of anti-Cry j 1-IgE and IgG1 antibodies were determined by ELISA. Data represent the mean  $\pm$  SD for 5 mice per group. An asterisk indicates a significant difference from the control group ( $p < 0.05$ ).

that LS feeding down-regulated both Th1 and Th2 immune responses in the immunized mice. Moreover, total IgG concentrations in the sera of the LS-fed mice were comparable with those of the control group (Fig. 2), suggesting that dietary LS supplementation down-regulates antibody responses in an antigen-specific manner.

#### *Dietary LS induces Cry j 1-specific down-regulation of immune response in normal mice*

In the next experiments, we examined whether LS feeding would suppress IgE responses to Cry j 1, a representative of Japanese cedar allergens. As shown in Fig. 3, in mice fed a 5% LS containing diet, the anti-Cry j 1 IgE and IgG1 responses were significantly suppressed, by 30–40%, as compared with the control group. Since the primary structure of Cry j 1 shows no homology with that of OVA, the effects of LS feeding were not restricted to a specific antigen. LS feeding might down-modulate any immune response elicited by antigen injections.

#### *Dietary LS feeding after the second immunization effectively inhibited the serum anti-OVA IgE and IgG1 antibody responses in mice*

Next, we examined whether dietary LS supplementation would exert any down-modulatory effects on further IgE production in IgE-producing mice. For this purpose, OVA-immunized mice were divided into three groups based on the IgE titers in the sera, and fed LS-containing diets for 10 weeks. During the experimental period, anti-OVA IgE titers in serum were monitored at 2, 4, and 6 weeks. As shown in Fig. 4, no significant change in the serum on-going IgE titers was observed in any of the three groups during the 6-week period. These results suggest that IgE-suppressive effects due to LS feeding might not be a direct effect on B cells.

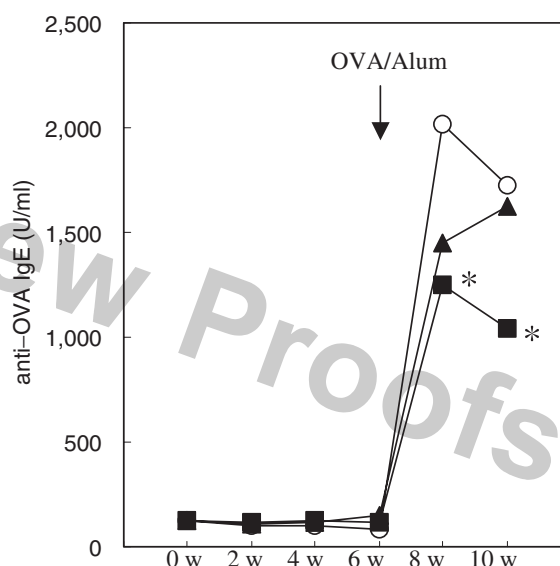


**Fig. 4.** Effects of LS Feeding on Serum Anti-OVA IgE Responses in IgE-Producing Mice.

IgE anti-OVA antibody producing mice were divided into three groups, which were provided with a control diet (○), or 2% (▲) or 5% (■) LS-containing diets for 10 weeks. At 6 weeks, the mice were given a booster injection of OVA/Alum. Sera were collected on the indicated weeks post-feeding, and the titer of anti-OVA IgE was determined by ELISA. Data represent the mean for 5 mice per group. An asterisk indicates a significant difference from the control group ( $p < 0.05$ ).

After 6 weeks, the mice were given a booster injection with OVA/Alum. Serum antibody titers were determined at another 2 and 4 weeks after the booster injection. As shown in Fig. 4, the anti-OVA IgE response was significantly suppressed, by 30–40%, as compared with the control mice. Anti-OVA IgG1 and IgG2a responses were also significantly suppressed in 5% LS-fed mice (Fig. 5). These results suggest that the

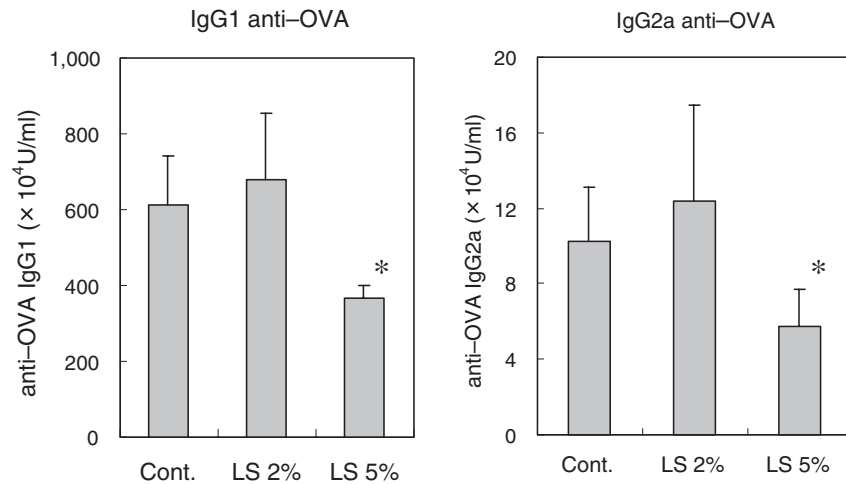


**Fig. 5.** Dietary LS Feeding after the Second Immunization Effectively Inhibited Serum Anti-OVA IgG1 and IgG2a Antibody Responses in Mice. The mice were treated as described in Fig. 4. Sera were collected 2 weeks after the booster injection, and titers of anti-OVA IgG1 and IgG2a antibodies were determined by ELISA. Data represent the mean  $\pm$  SD for 5 mice per group. An asterisk indicates a significant difference from the control group ( $p < 0.05$ ).

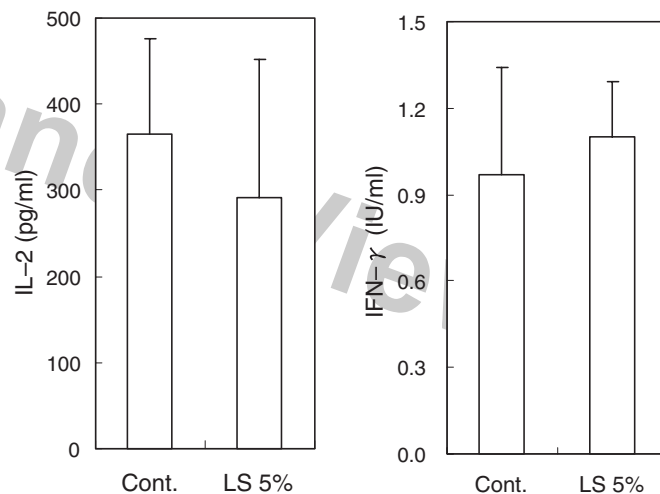


**Fig. 6.** *In Vitro* IL-2 and IFN- $\gamma$  Production by Splenocytes in LS-Fed Mice.

The mice were treated as described in Fig. 1. Splenocytes were collected on day 35 and were stimulated with OVA (final 10  $\mu$ g/ml) for 2 d. The amounts of IL-2 and IFN- $\gamma$  in the culture supernatants were measured by ELISA. Data represent the mean  $\pm$  SD for 7 mice per group.

regulatory effects of dietary LS feeding were also effective as to the IgE, IgG1, and IgG2a responses even after the second immunization.

#### *Effects of dietary LS on cytokine production by splenocytes*

To investigate the mechanisms involved in LS-induced inhibition of antibody production in mice, we examined cytokine production by spleen cells stimulated with OVA *in vitro*. As shown in Fig. 6, there was no apparent difference in the production of Th1 cytokines, such as IFN- $\gamma$  and IL-2, between the LS-fed and the control mice. In contrast, the Th2 cytokines, IL-4 and IL-5, produced by spleen cells after a 4-d incubation period, were significantly inhibited by 5% LS feeding, while the levels of IL-4 produced after a 2-d incubation period were comparable, as between 5% LS-fed mice

and the control mice (Fig. 7). Interestingly, the production of IL-10 by spleen cells in response to OVA was augmented after a 4-d incubation period in the LS-fed mice (Fig. 8).

## Discussion

In the present study, we found a suppressive effect of dietary LS on antigen-specific IgE production in mice, which is responsible for allergic diseases. In addition to the suppression of IgE production, antigen-specific IgG1 and IgG2a production was also decreased in LS-fed mice. Production of IgE and IgG1 has been found to be dependent on Th2 cells, whereas production of IgG2a is required for the participation of Th1 cells.<sup>21)</sup> Since these three immunoglobulin isotypes were down-regulated in the LS-fed mice, it is unlikely that the mechanism

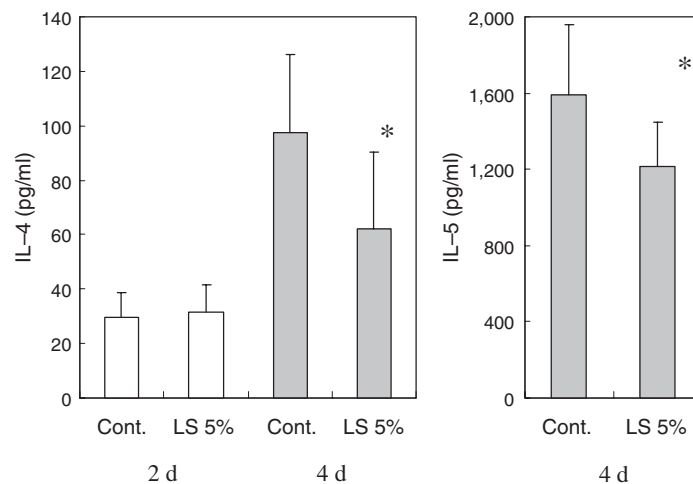


**Fig. 7.** Effects of LS Feeding on *in Vitro* IL-4 and IL-5 Production by Splenocytes in Mice.

The mice were treated as described in Fig. 1. Spleen cells were collected on day 35, and were stimulated with OVA (final 10 µg/ml) for 2 and 4 d. The amounts of IL-4 and IL-5 in the culture supernatants were measured by ELISA. Data represent the mean ± SD for 7 mice per group. An asterisk indicates a significant difference from the control group ( $p < 0.05$ ).

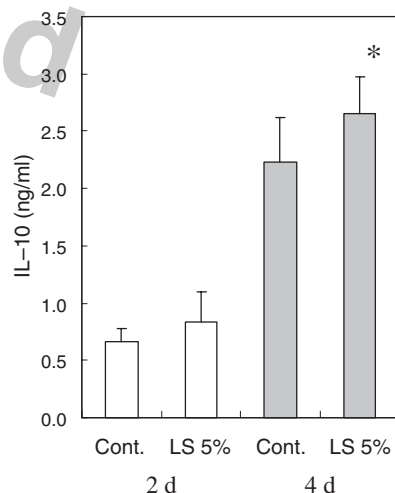


**Fig. 8.** Effects of LS Feeding on *in Vitro* IL-10 Production by Splenocytes in Mice.

The mice were treated as described in Fig. 1. Spleen cells were collected on day 35, and were stimulated with OVA (10 µg/ml) for 2 and 4 d. The amounts of IL-10 in the culture supernatants were measured by ELISA. Data represent the mean ± SD for 7 mice per group. An asterisk indicates a significant difference from the control group ( $p < 0.05$ ).

involved in the down-regulation of antibody responses by LS feeding is due to regulation of the Th1/Th2 balance. Furthermore, there was no difference in the serum levels of total IgG and on-going IgE production as between LS-fed mice and control mice. These results suggest that dietary LS supplementation induces antigen specific but not systemic immunosuppression.

To determine the mechanisms involved in the inhibition of antibody production, we examined cytokine production by spleen cells stimulated with OVA *in vitro*. There were no apparent differences in the production of Th1 cytokines. The production of Th2 cyto-

kines was lower in LS-fed groups than in the control group. But the production of IL-10, which plays a pivotal role in the regulation of immune responses, was augmented in 5% LS-fed mice. It is well recognized that IL-10 is produced by Tr1 cells as well as by Th2 cells.<sup>22,23</sup> Tr1 cells suppress both Th1 and Th2 types of effector T cell responses through secretion of IL-10 and TGF- $\beta$ .<sup>22</sup> In this regard, Hino *et al.*<sup>17</sup> reported that Peyer's patch (PP) cells derived from LS-fed mice produced significantly higher amounts of TGF- $\beta$  in response to Con A as compared with control diet-fed mice. Furthermore, Nomura *et al.* reported that *Bifidobacterium* proliferated in LS-administered mice.<sup>24</sup> Recently, Giacinto *et al.* reported that oral administration of a probiotic mixture including *Bifidobacterium* ameliorated the severity of recurrent colitis by inducing IL-10 and IL-10-dependent TGF- $\beta$ -bearing regulatory T cells.<sup>25</sup> These reports prompt us to speculate that Tr1-like cells were induced by dietary LS intake in the antigen/Alum-immunized mice, resulting in suppression of IgE and IgG1 production as well as IgG2a production. Further studies are needed to confirm this hypothesis.

In studies concerning the effects of non-digestible oligosaccharides on the immune system, Nagura *et al.* found an immunosuppressive effect of dietary raffinose.<sup>15</sup> Dietary raffinose suppressed an increase in serum IgE levels induced by oral feeding of OVA in OVA-specific T-cell receptor transgenic (Tg) mice. Furthermore, they found that dietary raffinose decreased IL-4, but increased IL-2 production by CD4<sup>+</sup> T cells in mesenteric lymph nodes prepared from OVA-specific Tg mice orally fed OVA. In their study, higher levels of IL-12 were observed in cultures with PP cells from raffinose-fed mice than with control mice. From these results, they concluded that dietary raffinose suppresses the serum IgE response against oral antigens by inhibiting the Th2-type immune response. Their results

are inconsistent with ours as to Th1 cytokine (IL-2 and IFN- $\gamma$ ) production by OVA-stimulated splenocytes, which was not augmented in mice fed LS. This might attributed be to the difference in types of mice (Tg vs. SPF mice), and/or to the administration route for the antigen (oral vs. ip injection with alum).

There are a number of reports showing that intestinal bacteria (e.g., *Lactobacillus*) suppress the development of Th2-type immune responses.<sup>26,27</sup> It was also reported that serum IgE levels in germ-free mice, which show the Th2-dominant immune response, the Th2 response was reduced by oral administration of *Bifidobacterium* in infants.<sup>7</sup> Furthermore, the elimination of indigenous microflora by kanamycin treatment during the infancy of the mice increased the serum levels of IgE.<sup>8</sup> In our previous study of the biological effects of dietary LS, we observed that anaerobic bacteria, including *Bifidobacterium* in the cecum, proliferated in LS-fed mice.<sup>24</sup> Furthermore, in LS-fed mice, the amounts of short-chain fatty acids in the cecum increased, and pH in the cecum decreased.<sup>17</sup> These results indicate that indigestible LS was fermented, leading to a change in the composition of the intestinal microflora. Since there was no effect of LS on OVA-specific IgE secretion in OVA stimulated splenocyte cultures *in vitro* (data not shown), we suppose that the immunoregulatory effect of dietary LS is not a direct action on immune cells, but rather an indirect one that acts by improving intestinal microflora.

It should be noted that both antigen-specific IgE and IgG1 production was inhibited when dietary LS feeding was initiated after the second ip immunization with antigen/Alum. These results, together with the finding that Cry j 1-specific IgE production was suppressed by dietary LS feeding, might provide a rationale for clinical evaluation of LS in the treatment of IgE-mediated allergic diseases, such as Japanese cedar pollinosis.

In conclusion, we found a novel function of the non-digestible oligosaccharide lactosucrose, which suppressed the specific IgE response against OVA and Cry j 1 that was elicited by immunization with alum adjuvant.

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Advance View Proofs