We examined how dietary melibiose affected the T-helper (Th) cell responses induced by an orally fed antigen in ovalbumin (OVA)-specific T cell receptor transgenic mice (OVA 23-3). Dietary melibiose markedly decreased the Th2 type responses as shown by a significant decrease in the interleukin (IL)-4 production and T cell proliferative response induced by sensitization from the 7-d oral administration of OVA. It was additionally observed that the Th1 type responses tended to decrease. We therefore examined the effect of melibiose feeding on the induction of immunological tolerance induced by the oral administration of an antigen (oral tolerance). The Th cell responses induced in BALB/c mice by subcutaneous immunization with OVA were suppressed by the prior oral administration of OVA. Such responses in the OVA-fed and immunized mice were further diminished by dietary melibiose. These results suggest that dietary melibiose strongly affected the Th cell responses to an ingested antigen, and further demonstrate the potential of melibiose to enhance the induction of oral tolerance.

Key words: melibiose; oral tolerance; T cell response; allergic disease

It has been revealed that there are two different types of T helper (Th) cell subsets, Th1 and Th2, and that the cytokines derived from each Th cell regulate each other. Th1-type cells produce interleukin (IL)-2 and interferon (IFN)-γ which activate macrophages and induce delayed-type hypersensitivity reactions. In contrast, Th2-type cells produce IL-4, IL-5 and IL-10 which induce IgE production. Thus, allergic disorders occur when the balance of Th1 and Th2 shifts toward Th2.

Lactic bacteria such as lactobacilli are well known to enhance the Th1-type immune system by IL-12 induction and suppress the Th2-type response. Kalliomaki M et al. have suggested that oral administration of lactobacilli was effective for preventing early atopic diseases in children. Bjorksten B. et al. have reported that infants who developed allergy were less often colonized with bifidobacteria during the first year of life than healthy infants and that indigenous intestinal flora might play important roles in the development of and protection from allergy. On another front, prebiotics such as indigestible oligosaccharides are known to increase indigenous lactic bacteria, especially bifidobacteria in humans. We have previously reported that dietary raffinose, an indigestible oligosaccharide, suppressed the Th2-type immune response against an oral antigen. This finding implied that the various physiological functions of raffinose might make their contribution in the form of melibiose.

Melibiose (6-o-D-galactopyranosyl-D-glucose) exists in natural plants such as cacao beans, and has also been found in processed soybeans. We have reported that the functions of melibiose included increasing lactic bacteria, especially bifidobacteria, and improving the stool condition in humans, as has been recognized with the other oligosaccharides. We also performed a clinical test to investigate whether melibiose was useful for atopic dermatitis. These results indicated that melibiose had an effect on the immune system and could
be useful for improving the symptoms of allergic diseases.

In the present study, we investigated the effects of dietary melibiose on immune responses, especially focusing on the Th cell response to an orally administered protein antigen.

Materials and Methods

Animals. Female BALB/c mice were purchased from Clea Japan (Tokyo, Japan). OVA23-3 transgenic mice with a BALB/c genetic background expressing an OVA-specific I-A<sup>d</sup> restricted αβ-T cell receptor were originally established by Sato et al.¹⁵ Female transgenic mice were used for all the experiments. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Tokyo.

Culture medium. Lymphocytes were cultured in an RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with heat-inactivated 10% fetal calf serum (Sigma, St. Louis, MO, USA), 100 U/ml of penicillin, 100 µg/ml of streptomycin, 5 × 10⁻⁵ M 2-mercaptoethanol and 2 mM L-glutamine.

Antigen. OVA (5 × crystallized OVA; Seikagaku-kogyo, Tokyo, Japan) was used for adding to the culture medium and for needle feeding and immunization of BALB/c mice. As a food supplement, OVA (albumin from eggs, containing 500 g of OVA/kg) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Diets. The composition of the purified basal diet is shown in Table 1. The OVA diet was prepared by partly substituting OVA for casein in the basal diet. The melibiose diet and the melibiose-OVA diet were prepared by adding melibiose to the basal diets instead of cornstarch. The diets were prepared in pellets and vacuum-sealed in plastic bags by Funabashi Farm (Chiba, Japan). Melibiose was used as melibiose monohydrate (Nippon Beet Sugar, Tokyo, Japan).

### Table 1. Composition of the Diets (g/kg)

<table>
<thead>
<tr>
<th></th>
<th>basal diet</th>
<th>OVA diet</th>
<th>melibiose diet</th>
<th>melibiose-OVA diet</th>
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<td>120</td>
</tr>
<tr>
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<td>80</td>
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<tr>
<td>melibiose</td>
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<td>—</td>
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</table>

Effects of melibiose on cytokine production from transgenic mice fed with OVA. Female OVA23-3 mice at 4–5 weeks of age were maintained with the basal diet or the melibiose diet for 14 d. The basal diet was changed to the OVA diet, and the melibiose diet was changed to the melibiose-OVA diet. The mice were maintained for 0, 4, 7 and 10 d with each diet. Subsequently, the mice were sacrificed and their spleen and MLN cells were prepared from individual mice as a single cell suspension, and CD4<sup>+</sup> T cells were purified from MLN cells by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-CD4 beads.

Effects of the oral administration of melibiose on the T cell hyporesponsiveness (oral tolerance) induced in OVA-fed BALB/c mice. BALB/c mice (4 weeks of age) were fed with the basal diet or melibiose-containing diet for 14 d and assigned to three groups, the basal diet group, the basal diet plus OVA-fed group and the melibiose diet plus OVA-fed group. The mice in the basal diet plus OVA-fed and the melibiose diet plus OVA-fed groups were orally given 1 mg of OVA in saline by needle feeding, while the animals in the basal diet group were only given saline. Seven days after the oral administration of OVA/saline, all the BALB/c mice were subcutaneously (sc) immunized into the footpads and base of the tail with 50 µg of OVA in complete Freund’s adjuvant (CFA, DIFCO, Detroit, MI, USA). Mice were maintained for another 7 d and finally sacrificed. Their inguinal lymph nodes were dissected and separate cell suspensions were prepared.

Cell culture for cytokine production. Spleen cells and MLN cells (2.5 × 10<sup>6</sup>/ml) from OVA 23-3 mice were cultured with OVA in 48-well plates (Costar, Cambridge, MA, USA) for the detection of IL-2 (for 36 h), and IL-4 and IFN-γ (for 60 h). In addition, MLN-CD4<sup>+</sup> T cells (5.0 × 10<sup>5</sup>/ml) were cultured for cytokine production with OVA and with antigen-presenting cells (APC; 2.0 × 10<sup>6</sup>/ml) over a suitable incubation time for each cytokine as already described. APC were prepared from the splenocytes of BALB/c mice maintained on a
commercial diet, and the splenocyte cell suspensions were incubated with mitomycin C (50 μg/ml, 45 min at 37°C; Sigma, St. Louis, MO, USA). Inguinal lymph node cells (2.5 × 10^6/ml) from BALB/c mice were cultured with OVA for 24 h to detect the levels of IL-2. The cells were also incubated for 65 h under the same conditions for IFN-γ detection.

**Determination of the lymphocyte proliferative response.** The lymphocyte proliferative response was determined by using a commercial 5-bromo-2′-deoxyuridine (BrdU) labeling and detection kit β (Roche Diagnostics, Mannheim, Germany). CD4^+^ T cells of MLN (5.0 × 10^5/ml) were plated with APC (2 × 10^6/ml) into 96-well plates, and inguinal lymph node cells (2.5 × 10^6/ml) were also plated for cell culture. Each cell was then cultured with OVA for 38 h, and BrdU was added on hour 24. The detection of BrdU-incorporated cellular DNA was carried out according to the manufacturers’ protocol. Briefly, the cultured cells were fixed with 0.5 M ethanol/HCl on to the plates, and a peroxidase-labeled anti-BrdU antibody was added. After adding the substrate, the absorbance at 405 nm was measured and therefore the amount of BrdU incorporated into the intracellular DNA could be evaluated.

**ELISA for cytokines.** The IL-2, IL-4, and IFN-γ levels were detected by sandwich ELISA. Rat anti-mouse IL-2, IL-4 and IFN-γ monoclonal antibodies were used as capture antibodies, with biotinylated rat anti-mouse IL-

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**Fig. 1.** Effect of Dietary Melibiose on the Cytokine Production and Proliferation Response of MLN CD4^+^ T Cells during Oral Sensitization with the OVA Antigen for Various Periods in TCR Transgenic Mice.

OVA23-3 mice (4 weeks old) were fed on the basal diet or the melibiose-containing diet for 14 d. Subsequently, each diet was changed to the respective OVA-containing diet, and the mice were maintained for another 0, 4, 7, or 10 d. CD4^+^ T cells of MLN were prepared and cultured with OVA (1.0 mg/ml) to detect the proliferative response and cytokine production. Columns indicate the mean values and circles represent the values for individual mice. ○ and unfilled column, basal diet group; ● and hatched column, melibiose diet group. The level of significance was set at *P < 0.05.
2, IL-4 and IFN-γ respectively as the detection antibodies. All monoclonal antibodies were purchased from Pharmingen, San Diego, CA, USA.

Statistical analysis. Each result is presented as the mean value with SD. The differences in cytokine levels and cell proliferative activity between the experimental groups were analyzed with Student’s t-test. Significant differences between values were measured by using Stat View (Ver 5.0). The level of significance was set at \( P < 0.05 \).

Results and Discussion

The aim of this study was to examine the effects of dietary melibiose on the immune response induced by an orally fed antigen. We demonstrate that dietary melibiose strongly suppressed the Th2 response and properly enhanced the oral tolerance induced by oral administration of the antigen OVA.

We evaluated the effects of melibiose on the T cell differentiation induced by the gradual oral administration of OVA to OVA 23-3 mice. OVA 23-3 mice express T-cell receptor αβ-chain genes derived from a clone of the OVA-specific I-A\(^d\) restricted CD4\(^+\) T cell and are therefore useful for observing the T cell responses induced by the oral administration of antigen.\(^{15}\) The mice have already been evaluated for the changes in cytokine production from their splenic T-cells after a long-term administration of OVA.\(^{10}\) In that study, the IL-4 production increased and conversely IL-2 and IFN-γ production decreased after one week of ingesting OVA, showing the typical cytokine production patterns of Th2-type response.\(^{16}\) However, it was confirmed that the splenic T cell cytokine production diminished after the OVA diet had been fed for more than 2 weeks.

We first observed the changes of cytokine production and T cell proliferative response at several stages of OVA sensitization. The mice in both the basal diet and melibiose diet group were further assigned to 4 groups (finaly 8 groups, \( n = 3 \)), and OVA was orally administered for 0, 4, 7, or 10 d, respectively. Spleen cells and MLN cells from the mice were incubated with OVA, and each cytokine level was measured. CD4\(^+\) T cells were purified from MLN and incubated with APC for cytokine detection, because the IL-4 production level from whole MLN cells was negligible even in the presence of OVA in vitro. The cytokine production and proliferative response of the CD4\(^+\) T cells from MLN are shown in Fig. 1. The cytokine production and proliferative response of CD4\(^+\) T cells increased from the start of administration of OVA (day 0) to around day 7 and then started to decrease. These overall changes observed during the Th cell sensitization caused by OVA intake in the mice are consistent with the previous findings.\(^{16}\) It was then noted that T cell proliferation in the melibiose diet group was significantly lower \( (P < 0.05) \) than that in the basal diet group and that the IL-4 production level tended to decrease \( (P = 0.08) \) on day 7. The IFN-γ production level also slightly decreased on day 7 compared with the basal diet group. Similar results were observed with spleen cells (data not shown).

We next performed an additional experiment in the same manner to observe the difference in cytokine production between the basal diet group \( (n = 10) \) and the melibiose diet group \( (n = 10) \) with the focus on day 7 (Fig. 2). As the representative results of two experiments, CD4\(^+\) T cells of MLN from the melibiose diet group produced a significantly lower level of IL-4 than from the basal diet group on day 7 \( (P < 0.05) \). Spleen cells also showed a much more decreased IL-4 production level \( (P < 0.01) \) (data not shown). These results indicate that dietary melibiose strongly suppressed the Th2-type response induced by the oral administration of OVA. Furthermore, in both cells, IFN-γ production derived from the Th1 cell type tended to be lower in the melibiose diet group than in basal diet group. There was no significant difference in IL-2 production between the two groups.

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**Fig. 2.** Effect of Dietary Melibiose on Cytokine Production by MLN CD4\(^+\) T Cells of TCR Transgenic Mice Fed with OVA for 7d. 
- (unfilled column), basal diet group;  (hatched column), melibiose diet group. Four-week old OVA23-3 mice were fed on the basal diet or melibiose-containing diet for 14 d. Subsequently, each diet was changed to the OVA-containing basal diet or OVA-containing melibiose diet, respectively, and the mice were maintained for another 7 days. CD4\(^+\) T cells from MLN were prepared and cultured with the antigen (OVA) for a cytokine assay. Each result is presented as the mean value plus or minus SD. The level of significance was set at \( ^*P < 0.05 \).
Several researchers have reported on food components which had been expected to improve allergic disorders, and most of their effects depended on regulation of the Th1/Th2 balance. Our present results show that dietary melibiose inhibited the Th2 response, but in addition to this, that the T cell proliferation was diminished and the Th1 response tended to decrease. Although there appeared to be a shift in the Th1/Th2 balance, the effects of melibiose on T-cell responses could not be explained solely by the regulation of the Th1/Th2 balance. It is also well known that the ingestion of a food antigen can cause systemic hyporesponsiveness to the antigen, this being called oral tolerance. Oral tolerance is one of the most important inherent immune-regulation systems and helps prevent a strong immune response to a dietary antigen that would be potentially harmful to the individual. It has been demonstrated in animals that such responses as specific antibody production, delayed-type hypersensitivity and the cytokine secretion of antigen-specific Th cells of the spleen or MLN were decreased by oral tolerance. We therefore explored the possibility that dietary melibiose also had some effects on the induction of oral tolerance through the administration of an antigen by using another evaluation model with BALB/c mice.

![Fig. 3. Effect of Dietary Melibiose on the T Cell Hyporesponsiveness (Oral Tolerance) Induced by Oral Administration of OVA in BALB/c Mice.](image)

Three-four-week-old mice were fed on the basal diet or the melibiose-containing diet for 14 d and then 1 mg of OVA in saline or saline only was orally administrated by needle feeding. The mice were assigned to three groups: the basal diet group (unfilled column), the basal diet plus OVA-fed group (hatched column) and the melibiose diet plus OVA-fed group (filled column). Seven days after the oral administration of OVA/saline, all the mice were immunized subcutaneously (sc) with 50 μg of OVA in complete Freund’s adjuvant and maintained for another 7 d. Their inguinal lymph nodes were dissected, and each cell suspension was prepared and cultured with antigen OVA for detecting the cell proliferative response and IL-2 and IFN-γ production. Each result is presented as the mean value plus or minus SD. The level of significance was set at *P < 0.05.
We performed a preliminary experiment to elucidate the dose and times of oral administration of OVA which were able to induce T cell hyporeactivity before examining the effects of melibiose on the T cell response with this experimental system. We were able to find the appropriate conditions for intragastric administration with 1 mg of OVA and then set up the additional experimental groups. The BALB/c mice were assigned to three groups, the basal diet group (n = 6), basal diet plus OVA-fed group (n = 8) and melibiose diet plus OVA-fed group (n = 8). After the administration schedule, the lymph node cell-proliferative response and cytokine production in each group were measured and compared (Fig. 3). The cell-proliferative response of the basal diet plus OVA-fed group tended to be lower than that of the basal diet group, the IL-2 response being significantly lower in the former than in the latter group. This shows that the Th cell responses normally induced when the mice are subcutaneously immunized with OVA were suppressed by a prior oral administration of OVA. Additionally, the response in the melibiose diet plus OVA-fed group was significantly lower than in the basal diet or basal diet plus OVA-fed group. Concerning IL-2 production, this showed almost the same pattern of that of the cell-proliferative activity, the differences observed between the groups actually being much clearer. As for IFN-γ production, no difference was apparent between the basal diet and basal diet plus OVA-fed group. However, the melibiose diet plus OVA-fed group demonstrated a lower IFN-γ level than that in the other groups. These results suggest that the phenomenon considered as the induction of oral tolerance was enhanced by dietary melibiose. Although not shown by the data, we also performed a supplemental experiment to examine whether melibiose decreased the Th response without oral administration of OVA. The cell proliferative response and cytokine production induced by subcutaneous immunization of OVA were no different between the basal diet group and the melibiose diet group without prior OVA feeding. This indicates that dietary melibiose only suppressed the Th response when the antigen was administrated orally.

At present, the mechanism by which dietary melibiose affects the Th cell response and oral tolerance remains unclear. However, many reports have stated that intestinal bacterial flora may affect immune responses; for instance, in germ-free mice which lack intestinal bacteria, IgA secretion is less than that of normal mice and oral tolerance is not induced.25–27 Furthermore, it has also been reported that the kinds of T cell induced by invading bacteria or an antigen are different from those in normal mice. The fact that melibiose improved the intestinal flora as a prebiotic might lead to the hypothesis that dietary melibiose affects the intestinal immune system by modulating the intestinal flora and thereby regulating the systemic immune responses. There have also been some recent reports that oligosaccharides, which possess α-galactosyl binding activity, suppressed allergic airway eosinophilia and that the effect did not appear to be mediated by the intestinal microflora.28-29 Although we have also examined in vitro whether melibiose had a direct influence on immune cells, no clear results have been obtained. In either case, further research to elucidate the mechanism of melibiose action is necessary.

In conclusion, we have shown that dietary melibiose significantly suppressed the Th2 response and enhanced oral tolerance induced by an orally fed antigen. These findings indicate the possibility that melibiose would be useful for preventing or improving the symptoms of allergic disease by regulating the Th cell response and that melibiose could well be a noteworthy food ingredient which could reinforce oral tolerance.

Acknowledgment

We thank Drs. S. Habu and T. Sato at Tokai University for originally providing the OVA 23-3 mice.

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