Low-Protein Diet Induces Oral Tolerance to Ovalbumin in Mice

Mohammed A. SATTER1, Kentaro SAKAI2, Sherin AHMED1, Kenji YOSHINO2, Shigeru YAMAMOTO2, Yuji SHIMIZU3 and Fusuo OTA1,*

1Department of Food Microbiology and 2Department of Applied Nutrition, School of Nutrition, Faculty of Medicine, The University of Tokushima, Tokushima 770–8503, Japan
3First Department of Internal Medicine, School of Medicine, Gunma University, Gunma 371–8511, Japan

(Received August 30, 2001)

Summary The suitable development of oral tolerance against ingested dietary foods is of critical importance to escaping food allergy. Using mice as an animal model for oral tolerance against ovalbumin (OVA) as a dietary antigen, we investigated the effects of dietary protein on their immunological tolerance. Female BALB/c mice fed either a 20% or 5% protein diet were orally administered 5 mg of OVA for four consecutive days, then immunized intraperitoneally with 100 µg of OVA. The immunized group of mice were fed and treated in the same manner, except that they received orally distilled water for four consecutive days before receiving intraperitoneal immunization with the antigen. Immunization alone with OVA elevated the total IgE and induced the production of OVA-specific antibodies IgE, IgG, IgG1, and IgG2a in the sera of both the 20% and 5% protein diet groups. The oral administration of OVA to mice before intraperitoneal immunization significantly reduced the total IgE and OVA-specific antibodies in mice fed 5% protein diet, but it had hardly any effect on those in mice fed a 20% protein diet. When spleen cells from these groups of mice were cultured with OVA as a mitogen, they responded substantially to OVA in the immunized groups fed 20% and 5% protein diets and in the presensitized group fed 20% protein, but those from the presensitized group fed a 5% protein diet did not respond. Furthermore, when IL-4 was assayed in the spleen cell cultures of the 20% and 5% groups, mice in the presensitized group fed a 5% protein diet produced a significantly less amount of IL-4 than those fed a 20% protein diet. Moreover, irrelevant to the protein amount in the diet, the production of IFN-γ from spleen cell cultures dramatically decreased in the group without presensitization and profoundly increased in the presensitized group of mice fed a 5% protein diet. These findings suggest that a low-protein diet leads to an induction of oral tolerance against dietary antigens; this appears to involve a clear down-regulation of Th2 cytokine, IL-4.

Key Words oral tolerance, low protein diet, OVA, IgE, IL-4

Oral tolerance is equipped in the gastrointestinal mucosa for the physiological pathway of systemic immune hyporesponsiveness against dietary food antigens to prevent undesirable immune responses (1–3). Ingested food proteins are usually degraded to their minimal components, such as amino acids and monosaccharides, with digestive enzymes in the gastrointestinal tract. But some dietary foods escaping from the degradation process may remain as considerably allergenic epitopes and are absorbed into the intestinal epithelium. Therefore a defectiveness of oral tolerance in the gastrointestinal immune system may lead to hyperresponsiveness to dietary food antigens, called food allergy, resulting in the production of specific antibodies that include IgE, a key molecule of anaphylaxis (4).

Oral tolerance has been understood as unresponsive-ness of the immune response to dietary food antigens with at least two possible mechanisms (5–7); one is a clonal deletion and/or anergy of T-cells, and the other is an activation of regulatory T-cells producing antigen nonspecific cytokines. Some other factors affecting oral tolerance are known to be aging (3) and genetic background (8). Although there has been accumulated evidence that nutritional status and some nutrients affect cellular and humoral immune responsiveness in humans (9, 10) and rodents (11–13), few or perhaps no reports are present regarding the effects of nutritional status on oral tolerance to dietary antigens. In particular, nutritional status appears to be an influential factor for increased allergic reactions, judging from the rarity of allergic diseases until World War II and that they frequently have been considered a social problem caused by recent changes of life and dietary habits in the past three decades (14–16). In Japan and other developed countries, egg albumin has long been considered the major antigen for food allergy in infants (17,18). Therefore we were to investigate whether the manipulation of nutritional status affects the induction of oral tolerance to dietary food antigens. We report here that...
Fig. 1. Experimental design for the induction of oral tolerance in mice. Groups of mice (N, I, O, and T) were treated as indicated above. The mice were orally given 5 mg of OVA/water for four consecutive days and/or immunized twice at 3-wk intervals with 100 μg of OVA in alum. Experimental diets were given to the mice at 3 wk before the oral administration of OVA started and continued until they were sacrificed on day 28 to get the spleens and blood for analysis. The number in the parenthesis represents the number of mice in each group.

A low-protein diet induces oral tolerance to OVA in mice, which helps us to appropriately understand nutritional conditions to reduce food allergy.

MATERIALS AND METHODS

Animals, diets, and husbandry. Specific pathogen-free female BALB/c mice at 6 wk of age, weighing 15–20 g were purchased from Japan SLC, Inc., Shizuoka, Japan, and fed on a commercial standard diet (Oriental Yeast Co. Ltd., Tokyo, Japan) for 1 wk. They were then randomly divided into two groups; one was given a 20% protein diet and the other received a 5% protein diet. The experimental mice fed 20% and 5% protein diets were further subdivided into four groups, N, I, O, and T, as shown in Fig. 1. The 20% and 5% protein diets were isocaloric (Table 1). The diets, containing 20% or 5% casein (% of w/w) as a sole source of protein, were prepared by a previously published method (19). Each diet was made into a dough with half its weight in water. The casein used in the present study consisted of 84.3% protein. All mice were kept in an air-conditioned room at 22 ± 2 °C with 12 h light period from 8 a.m. to 8 p.m. Every day at 9 a.m., food intake and body weight of the mice were measured, and food and water were renewed. Unless otherwise stated, the mice were anesthetized with diethyl ether in a jar before any surgical procedure. This study was approved by the Animal Research Ethics Committee at The University of Tokushima, Japan.

Induction of oral tolerance. The mice were orally given 5 mg of OVA (5 × crystallized, Seikagaku Corp., Tokyo, Japan) in 0.5 mL of water for four consecutive days with a plastic gavage needle 3 wk after they started to receive the experimental diets. Four days after the last oral administration of OVA, the mice were immunized twice intraperitoneally at 3-wk intervals with 100 μg (0.5 mL) of alum-precipitated OVA dissolved in phosphate-buffered saline. The control mice (group I) were immunized with diethyl ether in a jar before any surgical procedure. This study was approved by the Animal Research Ethics Committee at The University of Tokushima, Japan.

Table 1. Composition of experimental diets.

| Constituent (g kg⁻¹) | Dietary casein 5% | 20% |
|----------------------|------------------|-----|
| Casein¹              | 50               | 200 |
| Carbohydrate²        | 820              | 670 |
| Cellulose            | 20               | 20  |
| Mineral mixture³     | 50               | 50  |
| Vitamin mixture⁴     | 10               | 10  |
| Soya bean oil        | 50               | 50  |
| Total energy (kcal kg⁻¹) | 3,930          | 3,930 |

¹ Crude protein was 84.3%.
² Starch : sucrose, 2 : 1 ratio.
³ Obtained from Oriental Yeast Co. Ltd., Tokyo, Japan. The mixture consists of (mg/kg of diet): CaHPO₄ · 2H₂O, 7,280; KH₂PO₄, 12,860; NaH₂PO₄, 4,680; NaCl, 2,330; Ca-lactate, 17,550; Fe-citrate, 1,590; MgSO₄, 3,590; ZnCO₃, 55; MnSO₄ · 4-H₂O, 60; CuSO₄ · 5H₂O, 15; KI, 5.
⁴ Obtained from Oriental Yeast Co., Ltd., Japan. The composition is expressed in units or milligrams of vitamins per kilogram of diet: thiamine-HCl, 12; riboflavin, 40; pyridoxine-HCl, 8; vitamin B₁₂, 0.005; ascorbic acid, 300; b-biotin, 0.02; folic acid, 2; calcium pantothenate, 50; p-aminobenzoic acid, 50; niacin, 60; inositol, 60; choline chloride, 2,000; retinol acetate, 5,000; ergocalciferol, 1,000 IU; tocopherol acetate, 50; menadione, 52.

Collection of blood. One week after the last immunization, blood was collected from the postcaval vein of mice under anesthesia. Serum was separated by centrifugation and kept at −40°C until analysis.

Measurement of total IgE in serum. The amount of total IgE in serum was determined by direct enzyme-linked immunosorbent assay (ELISA). Microtiter plate wells (Greiner Labortechnik, GmbH) were coated overnight at 4°C with rat antimouse IgE monoclonal antibody (Yamasa Corporation, Chiba, Japan) at a final concentration of 2 μg/mL in carbonate buffer (50 mM, pH 9.6). The plate wells were then incubated for 90 min at room temperature with 3% (w/v) BSA dissolved in Tris-HCl buffer (25 mM, pH 7.4) containing 0.14 mM NaCl, 5 mM KCl (TBS), and 0.1% (w/v) NaN₃. The wells were washed three times with TBS containing 0.5% Tween 20 (TBS-T). They were incubated for 90 min at room temperature with serial dilutions of serum sam-
samples and a standard IgE in Tris-HCl buffer (20 mM, pH 7.2) containing 0.01 mM PMSE, 0.01 mM leupeptin, and 2 mM EDTA. For a calibration curve, an antidiintrophenyl monoclonal mouse IgE (Yamasa Corporation) was used as the standard IgE. The wells were washed three times and reacted for 90 min at room temperature with 100 µL of biotinylated antioimmunoglobulin IgE rat monoclonal antibody (1 µg/mL) in PBS containing 0.1% Tween 20 (PBS-T). They were washed and then incubated for 90 min at room temperature with avidin-biotin-alkaline phosphatase complex (Vecostain ABC Kit, Vector Laboratories, Burlingame, CA). After four washings, the plate wells were incubated for 20 min at room temperature with 100 µL of a substrate mixture, p-nitrophophosphate (PNPP, Kirkegaard & Perry Laboratory Inc., Gaithersburg, MD). The reaction was terminated by adding 50 µL of 150 mM EDTA. Absorption was measured at 415 nm in a microplate reader (Hitachi F-3010, Japan).

**OVA-specific antibodies in serum.** The levels of OVA-specific antibodies in serum from experimental mice were also determined by indirect ELISA. Microwell plate wells (Greiner) were coated by overnight incubation at 4°C with 100 µL of OVA (100 µg/mL) in carbonate buffer (50 mM, pH 9.6). The wells were treated with 3% BSA to block the residual nonspecific protein binding sites. For a determination of OVA-specific IgE, IgG1, IgG1, and IgG2a, the wells were incubated for 90 min at room temperature with 100 µL of various dilutions (10, 300, 300, or 20) of serum samples. After four washings with PBS-T, the wells were incubated for 90 min at room temperature with 100 µL of biotinylated antioimmunoglobulin IgE rat monoclonal antibody (Yamasa Corporation), anti-mouse IgG horse serum (Vecostain), antioimmunoglobulin IgG1 rat monoclonal antibody (BD PharMingen, San Diego, CA) or antimouse IgG2a rat monoclonal antibody (BD PharMingen). They were then reacted with avidin-biotin-alkaline phosphatase complex and a substrate mixture (PNPP) and treated further in the same way for total IgE determination.

**Lymphocyte proliferation assay.** Spleens were removed 1 wk after the last immunization with OVA, and cell Suspensions were prepared as described by Zhang and Petro (12) with some modifications. Cells were suspended to give 2.5 × 10⁶ cells/mL in RPMI-1640 medium containing Antibiotic-Antimycotic (GIBCO BRL Life Technologies, Inc., Grand Island, NY), 25 mM HEPES, 5 × 10⁻⁵ M 2-mercaptoethanol, and 10% CPSR-3 (Sigma Chemical Co., St. Louis, MO). Antimouse CD3 hamster monoclonal antibody (3 µg/mL, R&D System Inc., USA) was used for the pretreatment of some tissue culture plate wells. Cells (5 × 10³ cells/well) were incubated into triplicate wells and cultured for 72 h with or without 100 µg/mL of OVA at 37°C in a humidified atmosphere of 5% CO₂. They were incubated further with 1 µCi of thymidine tritium (specific activity: 74 GBq/mmol or 2 Ci/mmol, Amersham Pharmacia Biotech, England) for 24 h under the same condition. The cultures were harvested and counted for radioactive thymidine incorporation with a direct beta counter (MATRIX™ 9600, PACKARD, A Canberra Company, USA).

**Cytokine production from splenocytes.** Spleen cells were prepared as described above. They were suspended to give 5 × 10⁶ cells/well in RPMI-1640 medium containing Antibiotic-Antimycotic (GIBCO BRL), 25 mM HEPES, 5 × 10⁻⁵ M 2-mercaptoethanol, and 10% CPSR-3. They were cultured with or without OVA at 37°C in a CO₂ incubator under an atmosphere of 5% CO₂ and 95% air. Culture supernatants were collected by centrifugation at 400 × g for 5 min at 72 and 96 h to determine IFN-γ and IL-4, respectively. After collection, they were aliquotted and stored at -40°C until assayed.

IL-4 and IFN-γ were assayed by ELISA following the conventional method. Briefly, microwell plate wells (Greiner) were incubated overnight at 4°C with 100 µL of antimouse IL-4 rat monoclonal antibody (2 µg/mL, PharMingen) or antimouse IFN-γ rat monoclonal antibody (2 µg/mL, PharMingen). The immunoplate wells were washed four times with PBS-T and treated with 3% BSA for 90 min at room temperature to block nonspecific binding sites. They were then incubated overnight at 4°C with samples of culture supernatants stored above. For a calibration curve, the immunoplate wells were incubated in the same way with serial dilutions (5 to 1,000 pg/mL) of recombinant standard IL-4 or IFN-γ (PharMingen). The wells were washed four times with buffer, then reacted for 90 min at room temperature with biotinylated antioimmunoglobulin antibody, IL-4 or IFN-γ (PharMingen). The wells were washed again and reacted for 90 min at room temperature with avidin-biotin-alkaline phosphatase complex (Vestastein). They were finally washed five times with buffer and reacted for 20 min at room temperature with a substrate mixture (PNPP). The reaction was terminated by the addition of 150 mM EDTA and subjected to a measurement of absorbance at 415 nm in the microplate reader. The amounts of IL-4 and IFN-γ in the samples were calculated by plotting the readings of immunoplate wells on the calibration curve prepared above.

**Statistical analysis.** The results were expressed as means ± SD taken from at least two experiments, and the statistical differences were determined by Student’s t-test.

**RESULTS**

**Food consumption and body weight of mice**

Food intake and body weight gain in mice were significantly affected by dietary protein content. During the experimental period, mice receiving a 20% or 5% protein diet ate a total of 113.0 ± 5.4 g and 142.2 ± 12.7 g, respectively. The average initial body weight was 17.4 ± 0.1 g in both dietary groups, which increased until immunization and reduced in all mice after immunization. At the end of the experiment, the average body weight of the mice was 22.6 ± 0.1 g for those fed on a 20% protein diet and 20.7 ± 0.4 g for those on a 5% protein diet. The difference between the two groups was statistically significant, with a p value of <0.05.
Fig. 2. Total IgE levels in the sera of mice fed 20% and 5% protein diets. For four consecutive days, the mice received oral administration of OVA (5 mg/mouse/d) and/or intraperitoneal challenge twice of alum-precipitated OVA (100 µg/mouse) at 3-wk intervals. Serum samples were collected 7 d after the last immunization, and their total IgEs were measured by ELISA as described in Materials and Methods. The results are expressed as means ± SD taken from four or six mice per group. Data were taken from at least two experiments. Crossed and dotted columns represent the mice fed with 20% and 5% protein diets, respectively. Single and double asterisks indicate the statistical significance of difference with p values of 0.05 and 0.01, respectively. N, I, O, and T represent the groups of mice as described in the legend for Fig. 1. Bars on the columns represent SD.

Total IgE in serum

The total IgE in groups N and O ranged from 0.40 to 0.97 µg/mL, as shown in Fig. 2. Intraperitoneal immunization of mice with OVA raised the serum total IgE to nearly 2.5 µg/mL in both mice groups fed 20% and 5% protein diets (group I). No statistical differences were found between the 20% and 5% protein diet groups, except for the mice receiving oral administration and subsequent intraperitoneal immunization with OVA (group T). Also, no significant difference in total IgE was observed between groups I and T fed a 20% protein diet. In contrast, when mice were fed a 5% protein diet and orally given OVA before intraperitoneal immunization with the antigen, they showed a statistically significant suppression to 1.3 ± 0.2 µg/mL. This suppression was also statistically significant when compared to that in the mice fed a 20% protein diet in group T.

OVA-specific antibody in serum

OVA-specific antibody titers of IgE, IgG, IgG1, and IgG2a in serum are shown in Fig. 3. An oral administration of OVA before immunization with this antigen showed different effects on the antibody titers. Almost no detectable titers of all classes and subclasses of antibody examined were observed in the sera of mice fed both 20% and 5% protein diet (groups N and O in Fig. 3, A, B, C, and D), but considerable levels of these antibodies were found in the sera of immunized mice fed 20% and 5% protein diet (groups I and T in Fig. 3, A, B, C, and D). The titers of OVA-specific IgG, IgG1, and IgG2a were similar in group I when the mice were fed with 20% and 5% protein diet. The mice were fed, pretreated orally with OVA, and immunized as described in Fig. 1. Serum samples were collected 7 d after the last immunization and assayed by ELISA for the titers of OVA-specific IgE (A), IgG (B), IgG1 (C), and IgG2a (D) as described in Materials and Methods. Specific antibody titers are expressed as means ± SD taken from four or six mice per group. Data were from at least two experiments. Crossed and dotted columns represent the mice fed with 20% and 5% protein diets, respectively. Single and double asterisks indicate the statistical significance of difference with p values of 0.05 and 0.01, respectively. N, I, O, and T represent the groups of mice as described in the legend for Fig. 1. Bars on the columns represent SD.
20% and 5% protein diets, whereas OVA-specific IgE was moderately lower in the mice fed a 5% protein diet than that in the mice fed a 20% protein diet. Although OVA-specific IgE was similar in the mice of groups I and T fed a 20% protein diet, the mice fed 5% protein and receiving oral OVA in group T showed a significantly lower titer than those in group I (Fig. 3A). Moreover, in group T the mice fed a 5% protein diet also showed a significant difference when compared to those mice fed a 20% protein diet. Similar differences in OVA-specific IgG and IgG1 titers were observed between group I and T, though the titers were slightly different among the antibody classes (Fig. 3, B and C). On the other hand, specific IgG2a was generally lower in titer than the other antibodies IgG and IgG1. It was significantly higher in group T, compared to that in group I, when mice were fed a 20% protein diet (Fig. 3D). Moreover, as in other antibodies, OVA-specific IgG2a was also suppressed significantly in the mice fed 5% protein in group T when compared with the mice fed 5% protein in group I.

**Proliferation of splenocytes**

To know if the suppression of IgE and other classes or subclasses of antibodies is a reflection of specific immune response to OVA, the proliferation of lymphocytes was examined for the incorporation of \(^{3}H\)-thymidine into splenocytes upon in vitro stimulation with OVA. As shown in Fig. 4, when splenocytes were stimulated with nonspecific anti-CD3 antibody, all mice fed a 20% protein diet responded normally, whereas those fed a 5% protein diet responded poorly, except the mice in group I. They also responded with some additional effects in the same manner when stimulated with anti-CD3 plus OVA.

**Cytokine production from splenocytes**

Among many cytokines, IL-4 and IFN-γ are known to control antibody switching, in particular specific for IgE. Their production was examined in the supernatant of spleen cell cultures taken from each group of mice after stimulation with OVA as a mitogen. As shown in Fig. 5A, IL-4 was not detectable in the mice fed 20% and 5% protein diets and not receiving intraperitoneal immunization with OVA (groups N and O). In contrast, groups I and T produced sizable amounts of IL-4, but with significantly lower titers in group T when compared with those in mice of group I fed a 5% protein diet. On the other hand, in mice fed a 20% protein diet there was no statistical difference in IL-4 production between groups I and T. Furthermore, the mice fed a 5% protein diet and receiving an oral administration of OVA before intraperitoneal immunization showed significantly lower levels of IL-4 than those fed a 20% protein diet and pretreated orally with OVA.

In a manner similar to IL-4, the production of IFN-γ was generally higher in the mice of groups N and O than those in groups I and T (Fig. 5B). In comparison with group I, IFN-γ production was significantly higher in the mice receiving an oral administration of OVA before intraperitoneal immunization (group T). Moreover, the mice fed a 5% protein diet also showed a significantly higher titer than those of the mice fed a 20% protein diet.

**DISCUSSION**

Nutritional status may be a critical factor in the induction of oral tolerance (20). To address the effects of nutritional manipulation on oral tolerance by the administration of OVA via an oral route, two diets were prepared for mice in such a way that they provided the same amount of calories, but different amounts of protein. Normally, young animals procure energy from protein and are able to match their protein intake closely to their requirements (21). Thus in the present study, it is reasonable that mice fed 5% protein diet ate more food than the mice fed a 20% protein diet to meet their physiological need of protein. Moreover, mice fed a 5% protein diet resulted in reduced body weight gain and number of lymphocytes in their spleens. These results were consistent with the previous findings (19).

In this study, immunological tolerance or hyporesponsiveness was judged by three parameters: immunoglobulin production, lymphocytes proliferation, and cytokine production from splenocytes. First, oral tolerance was shown in mice fed a 5% protein diet by down-regulated total IgE when compared with those in mice fed a 20% protein diet. A virtually identical down-regulated production of OVA-specific IgE as well as IgG, IgG1, and IgG2a were also observed in mice fed a 5%
Fig. 5. Effects of OVA stimulation on the cytokine production by splenocytes. Spleen cell suspensions were prepared as described in Materials and Methods. Cells giving $5 \times 10^6$ cells/well were cultured in triplicate with or without OVA (400 µg/mL). Supernatants were collected after 96 and 72 h of incubation for measurement by ELISA of IL-4 (A) and IFN-γ (B), respectively. The levels of OVA-specific cytokine production were calculated by subtracting the readings of control cultures from those of OVA-stimulated cultures. The results shown are means±SD taken from four or six mice per group. The data were taken from at least two experiments. Crossed and dotted columns represent the mice fed with 20% and 5% protein diets, respectively. Single and double asterisks indicate a statistical significance of difference with $p$ values of 0.05 and 0.01, respectively. N, I, O, and T represent the groups of mice as described in the legend for Fig. 1. Bars on the columns represent SD.

It is known that the synthesis of IgE is critically dependent on the regulatory cytokines secreted from CD4+ T helper cells (22). A CD4+ T-cell mediated response to specific antigens results in the dominance of Th2 cells that produce IL-4, a necessary molecule to facilitate the isotype switching of IgE and IgG1 antibodies (23). It has been reported that mice on a low-protein diet but without presensitization and/or immunization show a suppression of production of IL-4 from their spleen cells when compared with mice fed a normal protein diet (12), supporting its role for reduced antibody production in mice fed a 5% protein diet. On the other hand, IgG2a in the sera of mice fed 20% protein was moderately up-regulated after an oral administration of antigen. The regulatory role of Th1 and Th2 cytokines for antibody production can explain different antibody levels seen in this study; IgE and IgG1 are antibodies regulated by Th2 cytokines, and IgG2a is regulated by Th1 cytokines (24). It has also been reported that a high dose of OVA (orally 250 mg/mouse) in BALB/c mice has more profound and persistent tolerance on Th2 mediated immune response than Th1 response (25). This is why the production of OVA-specific-IgG2a was increased in tolerant (group T) mice fed a 20% protein diet. Thus the results indicated that a low-protein diet renders oral tolerance at the humoral level.

The induction of oral tolerance at the humoral level shown in the present study can not exclude a possibility that it is a result of T-cell tolerance, since the tolerance is regulated by a T-cell mediated mechanism (26). This hypothesis was confirmed in this study by a marked reduction of OVA-specific proliferative responses of splenic cells from tolerant mice (group T) fed 5% protein but not from those fed 20% protein when compared with that in an immunized group (group I). The absence of proliferation is only a partial indication of tolerance in which nondividing activated cells continue to produce cytokines (27, 28). Thus to further confirm a state of tolerance in T lymphocytes, the production of cytokines was determined after in vitro OVA stimulation. Early studies have demonstrated that enhanced IL-4 and reduced IFN-γ production are associated with the elevated serum IgE level (29, 30). It has also been reported that the capacity of mouse T-cell clones to induce IgE and IgG1 synthesis is directly correlated with the ratio of IL-4 to IFN-γ (31). It is argued that a higher secretion of IFN-γ with reduced or no IL-4 in the naive mice (group N) lead to reduced IgE and IgG1, but a reduced IFN-γ and greatly elevated level of IL-4 resulted in a higher production of IgE and IgG1 in immunized groups (group I). This variation of cytokine production was probably due both to a selective amplification of Th2 cells and a regulatory activation of Th1 and Th2 cells. In this study, it was also shown that the tolerant mice (group T) fed a 5% protein diet significantly suppressed IL-4 and enhanced IFN-γ production in vitro with a concomitantly reduced production of antibodies.
in vivo when compared with the immunized groups, but these effects were less in the mice fed a 20% protein diet. The results presented here directly indicated a Th2-selective oral tolerance in the mice fed a 5% protein diet after the feeding of OVA for four consecutive days. Thus we speculate that restricted dietary protein may balance the production of IL-4 and IFN-γ in such a ratio that tolerance may be brought about, helping the host to escape from allergic reactions to the antigen.

A possible mechanism contributing to the observed decrease in IL-4 with a concomitant low production of antibodies in tolerant mice (group T) fed a 5% protein diet might be an impaired expression of costimulatory molecules on antigen-presenting cells (APC). An APC costimulatory signal through the interaction of the CD28 molecule with the B7 family is essential for T-cell activation (1). Even with an optimal TCR stimulation, unless the costimulatory signal is provided, T-cells are rendered tolerant because of their partial activation (32). It is shown that APC costimulatory molecules B7-1 and B7-2 effectively induce the production of IFN-γ and IL-4 after a differential activation of Th1 and Th2 cells, respectively (33, 34). Elevated B7-1 and reduced B7-2 on APC were reported in mice fed a low-protein diet (12), which might be responsible for decreased IL-4 and increased IFN-γ in tolerant mice fed a 5% protein diet. Judging from these observations along with the present results, we speculate that the most effective costimulatory molecule, B7-2, might have some obligatory role for the hyporesponsiveness of Th2 cells in the tolerant mice fed 5% protein with a marked reduction of proliferation and IL-4 production as well as antibody production.

The present study has provided evidence that a low-protein diet plays an important role in the induction of oral tolerance by Th2 cells against dietary antigen. The Th2 subset has a direct relationship to allergic responses, including the activation of inflammatory cells and the production of antigen-specific IgE antibody (35, 36), which is successfully tolerated in mice fed a 5% protein diet. Some attempts to regulate Th2 cytokines and to enhance Th1 cytokines have been made as a fundamental therapeutic approach to cure the IgE-mediated allergies and asthma and have been achieving promising results (37). Thus as a guide to control the balance of Th1/Th2 as a therapy for different types of autoimmune diseases and allergies by restricted dietary protein, the findings from this study should provide encouraging information.

Taken altogether, the present results warrant further study to investigate how and where OVA is absorbed, leading finally to oral tolerance, how long it continues, and whether it is observed with other food antigens.

Acknowledgments
This work was supported in part by Grants-in-Aid (Nos. 11691211 and 13670066) from the Ministry of Education, Science, Sports and Culture of Japan, and also partly supported by a grant from Yakult Ltd., Japan.

REFERENCES
1) Strobel S. 1997. Oral tolerance: immune responses to food antigens. In: Food Allergy Adverse Reactions to Foods and Food Additives (Metcalf DD, Sampson HA, Simon RA, eds), p 107–135. Blackwell Science, Boston.
2) Weiner HL. 1997. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. Immunol Today 18: 335–343.
3) Faria AMC, Weiner HL. 1999. Oral tolerance: Mechanisms and therapeutic applications. Adv Immunol 73: 153–264.
4) Sudo N, Sawamura S, Tanaka K, Alba Y, Kubo C, Koga Y. 1997. The requirement of intestinal flora for the development of an IgE production system fully susceptible to oral tolerance induction. J Immunol 159: 1739–1745.
5) Chen Y, Inobe JI, Marks R, Gonnella P, Kuchroo VK, Weiner HL. 1995. Peripheral deletion of antigen-reactive T cells in oral tolerance. Nature 376: 177–180.
6) Melamed D, Friedman A. 1994. In vivo tolerization of Th1 lymphocytes following a single feeding with ovalbumin: anergy in the absence of suppression. Eur J Immunol 24: 1974–1981.
7) Chen Y, Kuchroo VK, Inobe JI, Hafler DA, Weiner HL. 1994. Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis. Science 265: 1237–1240.
8) Yoshida O, Yoshida I, Iwamoto H, Nishino K, Fukushima A, Ueno H. 1999. Genetic background determines the nature of immune responses and experimental immune-mediated blepharoconjunctivitis (EC). Current Eye Res 18: 117–124.
9) Chandra, RK. 1988. Nutrition, immunity and outcome: past, present, and future. Nutr Res 8: 225–237.
10) Edelman R, Suskind R, Sirishinha S, Olson R. 1973. Mechanisms of defective delayed cutaneous hypersensitivity in children with protein-calorie malnutrition. Lancet 1: 506–508.
11) Rose AH, Turner KJ. 1980. Effect of a low protein diet on IgE antibody responses in Balb/c mice. Int Arch Allergy Appl Immunol 61: 271–277.
12) Zhang S, Petro TM. 1997. The effect of moderate protein malnutrition on murine T cell cytokine production. Nutr Res 17: 51–64.
13) Mengheri E, Bises G, Gaetani S. 1988. Differentiated cell-mediated immune response in zinc deficiency and in protein malnutrition. Nutr Res 8: 801–812.
14) Hijazi N, Abalkhail B, Seaton A. 2000. Diet and childhood asthma in a society in transition: a study in urban and rural Saudi Arabia. Thorax 55: 775–779.
15) Lee SII, Shin MH, Lee HB, Lee JS, Son BK, Koh YY, Kim KE. Abh YN. 2001. Prevalence of symptoms of asthma and other allergic diseases in Korean children: a nationwide questionnaire survey. J Korean Med Sci 16: 155–164.
16) Davies RJ, Rusznak C, Devallia JL. 1998. Why is allergy increasing?—Environmental factors. Clin Exp Allergy 28 (Suppl 6): 8–14.
17) Pascual CY, Crespo JF, Perez PG, Esteban MM. 2000. Food allergy and intolerance in children and adolescents, an update. Eur J Clin Nutr 54 (Suppl 1): S75–S78.
18) Ahmed T, Kamota T, Sumazaki R, Shibasaki M, Hirano...
T, Takita H. 1997. Circulating antibodies to common food antigens in Japanese children with IDDM. *Diabetes Care* 20: 74–76.

19) Yamamoto S, Ota F, Akiyama M, Takeuchi S, Ikemoto S, Shizuka F, Kishi K, Fukui K, Inoue G. 1988. Optimal protein intake estimated by the resistance to streptococcal infection and the nutritional indices in mice. *J Nutr Sci Vitaminol* 34: 423–432.

20) Lamont AG, Gordon M, Ferguson A. 1987. Oral tolerance in protein-deprived mice. Evidence of normal 'gut processing' of ovalbumin, but suppressor cell deficiency, in deprived mice. *Immunology* 61: 339–343.

21) Leathwood PD, Ashley DVM. 1983. Strategies of protein selection by weaning and adult rats. *Appetite: J Intake Res* 4: 97–112.

22) Finkelman FD, Holmes J, Katona IM, Urban JE, Beckmann MP, Park LS, Schooley KA, Coffman RL, Mosmann TR, Paul WE. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu Rev Immunol* 8: 303–333.

23) Siebenkotten G, Esser C, Wabl M, Radbruch A. 1992. The murine IgG1/IgE class switch program. *Eur J Immunol* 22: 1827–1834.

24) Stevens TL, Bossie A, Sanders VM, Botran RF, Coffman RL, Mosmann TR, Vitetta ES. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T-cells. *Nature* 334: 255–258.

25) Kang B, Kim KM, Kang CY. 1999. Oral tolerance by a high dose OVA in BALB/c mice is more pronounced and persistent in Th2-mediated immune responses than in Th1 responses. *Immunology* 200: 264–276.

26) Melamed D, Friedman A. 1993. Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin. *Eur J Immunol* 23: 935–942.

27) Sloan-Lancaster J, Evavold BD, Allen PM. 1993. Induction of T-cell anergy by altered T-cell receptor ligand on live antigen presenting cells. *Nature* 363: 156–159.

28) Jenkins MK, Johnson LG. 1993. Molecules involved in T-cell costimulation. *Curr Opin Immunol* 5: 361–367.

29) Jujo K, Renz H, Abe J, Gelfand EW, Leung DYM. 1992. Decreased interferon gamma and increased interleukin-4 production in atopic dermatitis promotes IgE synthesis. *J Allergy Clin Immunol* 90: 323–331.

30) Rouxset F, Robert J, Andary M, Bonnin JP, Souillet G, Chretien I, Briere F, Pene J, de Vries JE. 1991. Shifts in interleukin-4 and interferon-γ production by T cells of patients with elevated serum IgE levels and the modulatory effects of these lymphokines on spontaneous IgE synthesis. *J Allergy Clin Immunol* 87: 58–69.

31) Coffman RL, Carter J. 1986. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. *J Immunol* 136: 949–954.

32) Hackett CJ, Dickler HB. 1999. Immunologic tolerance for immune system-mediated disease. *J Allergy Clin Immunol* 103: 362–370.

33) Kuchroo VK, Das MP, Brown JA, Ranger AM, Zamvil SS, Sobel RA, Weiner HL, Nabavi N, Glimcher LH. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: Application to autoimmune disease therapy. *Cell* 80: 707–718.

34) Freeman GJ, Boussiotis VA, Anumanthan A, Bernstein GM, Ke XY, Rennert PD, Gray GS, Gribben JC, Nadler LM. 1995. B7-1 and B7-2 do not deliver identical co-stimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity* 2: 523–532.

35) Van Halteren AGS, Van der Cammen MJF, Biewenga J, Bleumink HJ, Kraal G. 1997. IgE and mast cell responses on intestinal allergen exposure: A murine model to study the onset of food allergy. *J Allergy Clin Immunol* 99: 94–99.

36) Scott DE, Agranovich I, Inman J, Gober M, Golding B. 1997. Inhibition of primary and recall allergen-specific T helper cell type 2-mediated responses by a T helper cell type 1 stimulator. *J Immunol* 159: 107–116.

37) Vogel G. 1997. New clues to asthma therapies. *Science* 276: 1643–1646.