

Further Evidence Regarding the Effect of Dietary Protein on Oral Tolerance against Beta-Lactoglobulin through Th1-Mediated Immune Response in Mice

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Summary Oral tolerance is a potential strategy for preventing or minimizing aberrant immune responses. Although, oral tolerance has been extensively studied, to date the effects of dietary protein on the induction of oral tolerance are poorly understood. We have previously shown that restricted dietary protein induces oral tolerance to ovalbumin. This study was designed to investigate whether or not such tolerance occurs with beta-lactoglobulin (BLG) instead of ovalbumin (OVA) and if the tolerance resulting from this feeding regimen involves Th1-mediated immune response. Female BALB/c mice fed either 20% or 5% dietary protein were given 5 mg BLG or water orally for four consecutive days and then immunized intraperitoneally (ip) twice with BLG at 3-wk intervals. Oral tolerance induction was compared in BLG-fed and water-fed mice by measuring total IgE, BLG-specific antibodies, footpad reactions, splenocyte proliferation, and cytokine production. When mice were given BLG orally before ip immunization, the Th1-mediated immune responses (production of IL-2, IFN- γ , and IgG2a) were significantly reduced, whereas the Th2-mediated immune responses (production of IL-4 and IgG1) were unchanged. The Th1-mediated immune responses were markedly down-regulated in mice fed 5% protein as compared to those in mice fed 20% protein. Moreover, the production of total IgE, BLG-specific IgE, splenocyte proliferation, and footpad reactions were more reduced in mice fed 5% protein than those in mice fed 20% protein. The present study provides evidence that dietary protein plays an important role in the induction of oral tolerance against BLG as the result of, clear down-regulation of Th1 helper activity accompanied by a reduction in IgE.

Key Words oral tolerance, dietary protein, BLG, Th1

Oral tolerance is a biological pathway for the induction of systemic immunological hyporesponsiveness against a specific food antigen to prevent or minimize aberrant immune responses (1–3). Ingested food proteins are normally degraded to their smaller components with digestive enzymes in the gastrointestinal tract and are absorbed into the intestinal epithelium. But some dietary foods escape from the degradation process and are absorbed considerably as allergenic epitopes. Thus, a failure in or defectiveness of oral tolerance may lead to an adverse reaction(s) to dietary food antigens (i.e., food allergy), resulting in the production of specific antibodies including IgE, a key molecule of anaphylaxis (4).

Oral tolerance is characterized by a systemic hyporesponsiveness to ingested food antigens and can be explained by at least two possible mechanisms depending upon the antigen dose and frequency of administration (5–7). A high dose of antigen has been demonstrated to lead to T helper 1 (Th1) anergy, whereas multiple low

doses of antigen supported active suppression by down-regulating the regulatory T-cells secreting cytokines. Other factors such as aging (3), genetic background (8), and environment are also critical for the induction or maintenance of oral tolerance. Although, it has been reported that nutritional status and some nutrients affect cellular and humoral immune responses (9, 10), to date the effects of nutritional status on oral tolerance to dietary antigens are poorly understood. 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 over the past few decades allergies have been frequently considered to be a social problem caused by recent changes in lifestyle and dietary habits (11–13). Although Asian countries used to have a low rate of allergic diseases as compared to Western countries, increasing prevalence has been reported in Japan, Hong Kong, and Taiwan, with wide variations within the continent. Next to egg allergy, cow's milk allergy is a common issue in view of its high incidence of 2–10% among children (14, 15) and 1% among the adult population (16). Therefore, we tried to investigate whether the manipulation of nutri-

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tional status affects the induction of oral tolerance to dietary food antigens. We have previously shown that, when mice were maintained on restricted dietary protein, a marked oral tolerance was induced to ovalbumin, a major allergen that causes egg allergy. The observations in this study, however, may look unique to the experimental system examined *in vitro* and *in vivo* and therefore could not be indicative of other antigens to induce such tolerance. BLG is a major allergen in cow's milk, and there are few reports describing the effects of dietary protein on the induction of oral tolerance to it. Hence, the objective of this study is to investigate whether or not restricted dietary protein induces oral tolerance to BLG and if the resulting tolerance is selectively limited to Th1-mediated immune responses. Our data indicate that oral tolerance was established more markedly in mice fed restricted dietary protein, which helps us understand nutritional conditions to reduce adverse food reactions.

MATERIALS AND METHODS

Animals, diets, and husbandry. Specific pathogen-free female BALB/c mice 6 wk of age and weighing 15–20 g were purchased from Charles River Japan (Tokyo, Japan). They were maintained for a week on a cow's milk free standard laboratory diet (Oriental Yeast Company Ltd., Tokyo, Japan.). Mice were then randomly divided into two groups: one was given a 20% protein diet and the other a 5% protein diet. The 20% and 5% protein diets were iso-caloric (Table 1). The diets, containing 20% or 5% egg white (% of w/w) as the sole source of protein, were prepared by a previously published method (17). Each diet was made into dough with one third of its weight in water. All experimental mice were kept under standard animal housing conditions (18) with free access to the food and water. Every day in the morning, food and water were renewed, and food intake and body weight of the mice were measured. All experiments were approved by the Animal Research Ethics Committee at The University of Tokushima, Japan.

Induction of oral tolerance and immunization. The experimental design for the induction of oral tolerance and immunization is shown in Fig. 1. To induce oral tolerance, mice were given 5 mg of BLG (Sigma Chemical Co., St. Louis, MO, USA) in 0.5 mL of distilled water for four consecutive days by gastric intubation using an 18-gauge plastic feeding tube with a round tip. Control mice received distilled water alone. Four days after the last oral administration of BLG, all mice were immunized twice *ip* at 3-wk intervals with 100 μ g (0.5 mL) of alum-precipitated BLG dissolved in phosphate-buffered saline (PBS). Mice were given BLG or distilled water orally 3 wk after they started to receive the experimental diets and maintained on the same diet until they were sacrificed.

Collection of serum. One week after the last immunization, blood and spleens were collected from different groups of mice under anesthesia. Sera were separated by centrifugation and kept at -70°C until use for the

Table 1. Composition of experimental diets.

Constituent (g/kg)	Dietary egg white (%)	
	20	5
Egg white	200	50
Carbohydrate ¹	670	820
Cellulose	20	20
Mineral mixture ²	50	50
Vitamin mixture ³	10	10
Corn oil	50	50
Total energy (kcal/kg)	3,930	3,930

¹ Starch : sucrose, 2 : 1 ratio.

² Obtained from Oriental Yeast Co. Ltd., Tokyo, Japan. The mixture consists of (mg/kg diet): $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, 7,280; KH_2PO_4 , 12,860; NaH_2PO_4 , 4,680; NaCl , 2,330; Ca-lactate , 17,550; Fe-citrate , 1,590; MgSO_4 , 3,590; ZnCO_3 , 55; $\text{MnSO}_4 \cdot 4-6\text{H}_2\text{O}$, 60; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 15; KI , 5.

³ Obtained from Oriental Yeast Co. Ltd. The composition is expressed in units or milligrams of vitamins per kg of diet: thiamin-HCl, 12; riboflavin, 40; pyridoxine-HCl, 8; vitamin B_{12} , 0.005; ascorbic acid, 300; D-biotin, 0.2; 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.

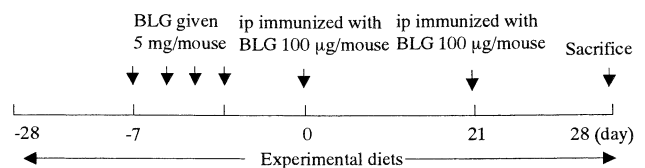


Fig. 1. Experimental design for the induction of oral tolerance and/or immunization of mice. Groups of mice were given 5 mg/d of BLG or distilled water orally for four consecutive days and then immunized *ip* twice at 3-wk intervals with 100 μ g/mouse BLG. Experimental diets were given to the mice 3 wk before the oral administration of BLG started and continued until they were sacrificed on day 28 to collect blood and spleens for analysis.

analysis of total and specific antibodies against BLG.

Determination of Total IgE in serum. The amount of total IgE in the serum was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (18). Briefly, 96-well microtiter plates (Greiner Labortechnik, GmbH) were coated with 100 μ L/well of rat anti-mouse IgE monoclonal antibody (Yamasa Corporation, Chiba, Japan) and incubated overnight at 4°C . The non-specific protein binding sites were blocked by incubating for 90 min at room temperature with 200 μ L/well of 3% BSA (Sigma) dissolved in Tris-HCl buffer (25 mM, pH 7.4) containing 0.04 M NaCl, 5 mM KCl (TBS), and 0.1% (w/v) NaN_3 . Microtiter plates were washed three times with TBS containing 0.5% Tween-20 (TBS-T). They were dispensed in duplicate with serum samples (10-fold dilutions), and serially diluted standard IgE in Tris-HCl buffer (20 mM, pH 7.2) containing 0.01 mM PMSE, 0.01 mM leupeptin, and 2 mM

EDTA, and incubated for 90 min at room temperature. For a calibration curve, an antidinitrophenyl monoclonal mouse IgE (Yamasa Corporation) was used as the standard IgE. After being washed three times with TBS-T, the plates were reacted for 90 min at room temperature with 100 μ L/well of biotinylated anti-mouse IgE rat monoclonal antibody (1 μ g/mL) in PBS. They were washed and then incubated for 90 min at room temperature with an avidin-biotin-alkaline phosphatase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). After four washings, the plates were incubated for 20 min at room temperature with 100 μ L/well of a substrate mixture, *p*-nitrophenyl phosphate (PNPP, Kirkegaard & Perry Laboratory Inc., Gaithersburg, MD, USA). A solution of 150 mM EDTA was used to stop the enzymatic reaction and the resulting color was measured at 415 nm in a microplate reader (Hitachi F-3010, Japan). The total IgE concentrations were determined by referring to a standard curve constructed using serial dilutions of standard monoclonal IgE.

Measurement of specific antibodies against BLG in serum. Serum samples were collected from different groups of mice and assayed for specific antibodies by indirect ELISA (18). Briefly, microtiter plates (Greiner) were coated with 100 μ L/well of BLG (100 μ g/mL) in carbonate buffer (50 mM, pH 9.6) and left overnight at 4°C. The non-specific protein binding sites were blocked by incubating with 200 μ L/well of 3% BSA (Sigma) in TBS for 90 min at room temperature. Four washings with TBS-T were performed before each reagent was successively reacted. For the determination of BLG-specific IgE, IgG1, and IgG2a, the plates were dispensed in duplicate with 100 μ L/well (10-, 300- or 20-fold dilutions) serum samples and incubated for 90 min at room temperature. Then the microtiter plates were incubated for 90 min at room temperature with 100 μ L/well biotinylated anti-mouse IgE rat monoclonal antibody (Yamasa Corporation), anti-mouse IgG1 rat monoclonal antibody (BD PharMingen, San Diego, CA, USA), or anti-mouse IgG2a rat monoclonal antibody (BD PharMingen). They were finally reacted with an avidin-biotin-alkaline phosphatase complex and a substrate mixture (PNPP) and treated further in the same way for the determination of total IgE.

Preparation of splenocytes. Spleens from different experimental groups of mice were removed 1 wk after the last immunization with BLG and placed in RPMI-1640 (Sigma) containing Antibiotic-Antimycotic (GIBCO BRL Life Technologies, Inc., Grand Island, NY, USA) and 25 mM HEPES. Single-cell suspensions were prepared as described previously (10) with some modifications. In brief, spleens were cut into pieces and cell suspensions were prepared by rubbing them through a stainless steel mesh and passing the resulting suspension through two layers of sterile cloth. Red blood cells were depleted by treatment with a Tris-ammonium chloride lysing buffer in an ice bath. After three washes with medium, cells were resuspended in RPMI-1640 containing Antibiotic-Antimycotic (GIBCO BRL), 5×10^{-5}

M 2-mercaptoethanol, and 10% heat-inactivated FBS (Sigma Chemical Co., St. Louis, MO, USA). The viability of cells was determined by the trypan blue exclusion method and kept on ice until use on the same day.

Specific proliferation against BLG. The single-cell suspensions described above were resuspended at a final concentration of 2.5×10^6 cells/mL in a medium containing Antibiotic-Antimycotic (GIBCO BRL), 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated FBS (Sigma). Then the cells (5×10^5 cells/well) were cultured in triplicate wells for 72 h in either medium alone or in medium with 100 μ g/mL BLG, 5 μ g/mL ConA, or 10 μ g/mL LPS at 37°C under a humidified atmosphere of 5% CO₂. They were then pulsed with 1 μ Ci/well tritium thymidine (specific activity: 74 GBq/mM or 2 Ci/mM, Amersham Pharmacia Biotech., England) for the last 17 h. After harvesting the cells onto fiberglass filters, radioactivity was counted for the incorporation of ³H-thymidine using a direct beta counter (MATRIX™ 9600, PACKARD, A Canberra Company, USA).

Cytokine production to BLG. For the cytokine assay, spleen cells were prepared as described above. They were resuspended at a final concentration of 1×10^7 cells/mL in RPMI-1640 medium containing Antibiotic-Antimycotic (GIBCO BRL), 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, and 10% FBS. Cells (5×10^6 cells/well) were then cultured in 24-well culture plates with or without BLG at 37°C under a humidified atmosphere of 5% CO₂. Culture supernatants were collected after 72 or 96 h of incubation by centrifugation at $400 \times g$ for 5 min to determine IFN- γ and IL-2 or IL-4, respectively. They were then aliquotted and stored at -70°C until assayed.

The cytokine level in the culture supernatants was quantified using an ELISA technique described previously (18). Briefly, 96-well microtiter plates (Greiner) were incubated overnight at 4°C with 100 μ L/well anti-mouse IL-4 rat monoclonal antibody (2 μ g/mL, PharMingen) or anti-mouse IFN- γ rat monoclonal antibody (2 μ g/mL, PharMingen). After washing, the immunoplates were treated with 100 μ L/well 3% BSA for 90 min at room temperature to block nonspecific binding sites. They were then dispensed in duplicate with 100 μ L/well culture supernatant and serial dilutions of recombinant standard IL-4 or IFN- γ (PharMingen) and incubated overnight at 4°C. The plates were washed four times with buffer and then reacted for 90 min at room temperature with 100 μ L/well biotinylated anti-mouse IL-4 or IFN- γ rat monoclonal antibody (1 μ g/mL, PharMingen). They were washed again and reacted for 90 min at room temperature with an avidin-biotin-alkaline phosphatase complex (Vectastain). After five washings, the plates were reacted a final time for 90 min at room temperature with 100 μ L/well substrate mixture (PNPP). A solution of 150 mM EDTA was used to stop the enzymatic reaction and the resulting color was measured at 415 nm in a microplate reader (Hitachi F-3010, Japan). The concentration of IL-4 or IFN- γ in the culture supernatants was calculated by

plotting the readings of immunoplate wells on the calibration curve prepared above.

The IL-2 level in the culture supernatants was measured by cytokine ELISA kits (PharMingen) following the manufacturer's instructions.

Footpad reactions. Spleens were collected from the mice given BLG orally or control mice, which received water instead of BLG. Single-cell suspensions were prepared and pooled according to groups of treatment. Splenocytes (1×10^7 cells/mouse) in 100 μ L of saline were transferred into BALB/c mice via the tail vein. After transfer, 100 μ g/foot BLG was injected into the left hind footpad to elicit a DTH reaction. Footpad swelling was measured with a dial caliper (Ozaki, Co. Ltd., Tokyo, Japan) 48 h after the injection. The right hind footpad was injected with saline and served as the control. Results were calculated by subtracting the measurements of control mice injected with saline from those of the BLG-injected mice.

Statistical analysis. The results were presented as mean $\pm$ SD. Statistical significance of difference between the values was analyzed by Student's *t*-test followed by Duncan's multiple range test. Results were considered significant at $p < 0.01$ or $p < 0.05$.

RESULTS

Food consumption and body weight

Mice were given free access to a 5% or 20% protein diet for the whole experimental period. Consumption of food and gain of body weight of mice were significantly affected by the protein content in the diets. During the experimental period, the average food intake was 2.86 ± 0.21 and 2.55 ± 0.25 g/d for mice receiving the 5% and 20% protein diets, respectively. After 7 wk on the experimental diets, food intake per day was significantly higher for mice receiving the 5% protein diet as compared to those mice receiving the 20% protein diet. The average initial body weight of mice receiving the 5% and 20% protein diets was 18.4 ± 0.1 g and 18.0 ± 0.1 , respectively. However, on the day of sacrifice, the average body weight of the mice receiving the 20% and 5% protein diets was 24.1 ± 0.3 and 21.7 ± 0.3 g, respectively. The difference between the two groups was statistically significant, with a $p < 0.05$.

Total IgE in serum

As shown in Fig. 2, the level of total IgE in the sera of water-fed mice immunized ip with BLG was elevated in both the 20% and 5% protein groups. There was no statistical difference in the concentration of total IgE between the animal groups fed the 20% and 5% protein diets. When mice were orally given soluble BLG before ip immunization, both the 20% and 5% protein groups showed a significantly lower concentration of total IgE than those of mice given water orally instead of BLG. Moreover, in the BLG-fed groups the 5% protein group also showed a significantly lower concentration of total IgE as compared to the 20% protein group.

Specific antibodies against BLG in serum

BLG-specific serum antibodies IgE, IgG1, and IgG2a in mice fed the 20% and 5% protein diets are shown in

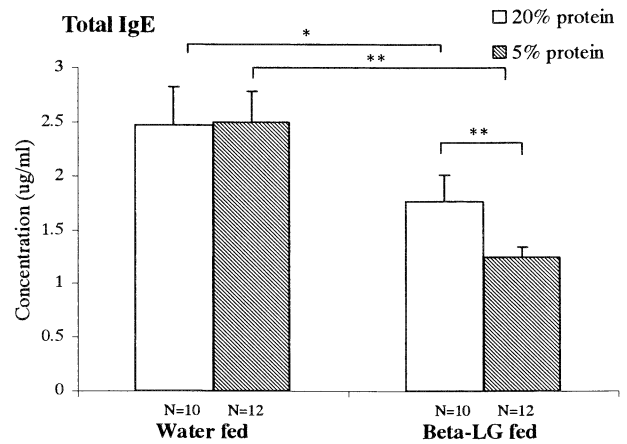


Fig. 2. Total IgE in the sera of mice fed 20% and 5% protein diets. The mice were given 5 mg/d of BLG or water orally for four consecutive days and then immunized twice with alum-precipitated BLG (100 μ g/mouse) at 3-wk intervals. Serum samples were collected 1 wk after the last immunization and their total IgEs were measured by ELISA as described in Materials and Methods. The results are expressed as mean $\pm$ SD from 10 or 12 mice per group. Open and crossed columns represent the mice fed 20% and 5% dietary protein, respectively. Single and double asterisks indicate the statistical significance of difference with p values of < 0.05 and 0.01 , respectively.

Fig. 3. Remarkable levels of IgE, IgG1, and IgG2a were produced in both 20% and 5% protein groups when the mice received water plus ip immunization of 100 μ g BLG (water-fed). The oral administration of soluble BLG for four consecutive days before ip immunization with the same antigen showed a different effect on antibody production. In the BLG-fed group, both mice in the 20% and 5% protein groups showed significantly lower levels of IgE antibody against BLG as compared to the water-fed control groups. In the BLG-fed groups, the IgE level was moderately lower in mice fed the 5% protein diet as compared to those fed the 20% protein diet when they were given soluble BLG orally.

BLG-specific IgG1 and IgG2a isotypes in the sera were also determined. Both 20% and 5% protein groups showed significantly lower titers of IgG2a in the BLG-fed groups than those in the water-fed control groups. Moreover, in the BLG-fed groups, the 5% protein group also showed a significantly lower titer of IgG2a as compared to the 20% protein group. In contrast, both the 20% and 5% protein groups did not show any statistical difference in BLG-specific IgG1 between the water and BLG-fed groups.

Proliferation of splenocytes

The proliferation of lymphocytes from different groups of experimental mice was examined by the incorporation of ^{3} H-thymidine into splenocytes upon in vitro stimulation with BLG, ConA, or LPS (Fig 4). In the 5% protein groups, mice fed BLG before ip immunization showed significantly lower responses to BLG, ConA, and LPS than those of mice given water orally instead of BLG. In contrast, for the mice fed 20% protein, there

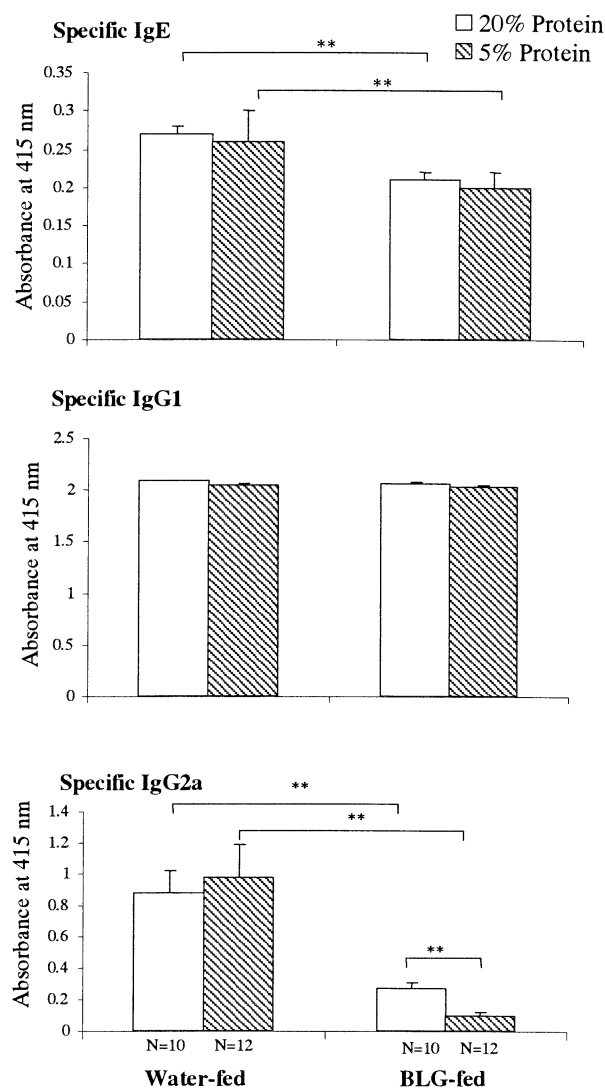


Fig. 3. Specific antibody titers against BLG in the sera of mice fed a 20% or 5% protein diet. The mice were treated as described in Fig. 1. Serum samples were collected 1 wk after the last immunization and assayed by ELISA as described in Materials and Methods. Specific antibody titers are expressed as mean $\pm$ SD taken from 10 or 12 mice per group. Open and crossed columns represent the mice fed 20% and 5% dietary protein, respectively. Double asterisks indicate the statistical significance of difference with a p value of <0.01 .

was no statistical difference between the water-fed and BLG-fed groups in the levels of proliferation in terms of specific and non-specific stimulation.

Cytokines responses to BLG

The effects of dietary protein on the production of some regulatory cytokines from spleen cells were examined following *in vitro* stimulation with BLG (Fig. 5). Without the oral administration of BLG (water-fed), production of IL-2 and IFN- γ was moderately higher in the 5% protein group as compared to the 20% protein group. It was significantly reduced in the 5% protein group, whereas it remained unchanged in the 20% protein group when the animals were given BLG orally. On the other hand, a substantial amount of IL-4 was detectable even in the 5% protein group as well as

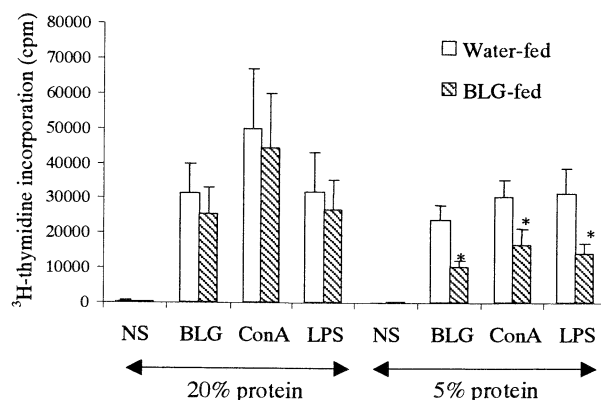


Fig. 4. BLG-specific and non-specific (ConA and LPS) proliferative responses of mice fed 20% or 5% dietary protein. Spleen cell suspensions were prepared as described in Materials and Methods. In the presence of BLG (100 μ g/mL), ConA, LPS, or medium only, cells (5×10^5 cells/well) were cultured in triplicate wells for 55 h and a further 17 h with ^{3} H-thymidine. The proliferative responses were measured by counting the radioactivity of ^{3} H-thymidine incorporated in cells from each animal group. The results are expressed as mean (cpm) $\pm$ SD taken from 10 or 12 mice per group. The single asterisk indicates the statistical significance as compared to the water-fed control group with a p value of <0.01 .

the 20% protein group upon BLG administration. Moreover, in both the BLG-fed and water-fed groups, the production of IL-4 was significantly lower in the 5% protein group than that in the 20% protein group.

Footpad reactions to BLG

To test whether or not the effect of dietary protein on spleen cell proliferation *in vitro* is reflected in immunological reaction *in vivo*, we examined footpad reactions in the mice. As shown in Fig. 6, the 5% protein group showed significantly lower footpad swelling than that of water-fed control mice. In contrast, in the 20% protein group, footpad swelling was not significantly different between the BLG-fed and water-fed control groups.

DISCUSSION

Oral tolerance is a state of immunological unresponsiveness induced by prior feeding of specific food antigens. When food antigens are presented, antigen-specific cells develop in different ways depending on the dose, frequency, and nature of the antigen, resulting in different states of oral tolerance. Nutritional status may also be a major factor to ensure the induction of oral tolerance (19). Although dietary protein was shown to affect the induction of oral tolerance against ovalbumin, a major allergen that causes egg allergy (18), in our previous study, it is not clear yet whether this tolerance occurs with other antigens through Th1 and/or Th2-mediated immune responses. To address the effects of dietary protein on oral tolerance, groups of mice were maintained on two different diets prepared in such a way that they provided the same amount of calories with different amounts of protein. In this study, the mice that were maintained on a low dietary protein ate

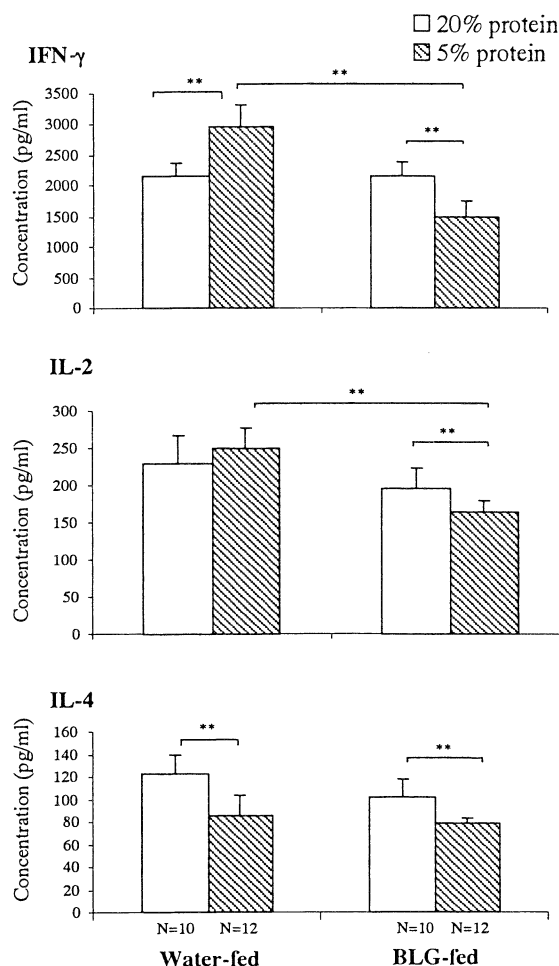


Fig. 5. Cytokine production against BLG by spleen cells of mice fed 20% or 5% dietary protein. Cell suspensions were prepared as described in Materials and Methods. Cells (5×10^6 cell/well) were cultured in triplicate wells with or without BLG ($500 \mu\text{g/mL}$). After 72 or 96 h of incubation, the concentration of IL-4, IFN- γ , and IL-2 in the culture supernatants was determined by ELISA. The levels of cytokines against BLG were calculated by subtracting the readings of the control cultures from those of the BLG-stimulated cultures. The results are expressed as mean $\pm$ SD taken from 10 or 12 mice per group. Open and crossed columns represent the mice fed 20% and 5% dietary protein, respectively. Double asterisks indicate a statistical significance of difference with a p value of <0.01 .

more food than the mice on the protein-sufficient diet in order to meet their physiological needs. Moreover, mice fed the 5% protein diet had a lower gain in body weight and fewer lymphocytes in their spleens despite the adequate consumption of food. These results were consistent with a previous study from our laboratory (18) and the results of others (10, 17).

Oral tolerance was induced by exposing mice to BLG followed by ip immunization with the same antigen and then evaluated. To investigate the *in vivo* level of tolerance generated by multiple feedings of BLG, the production of total IgE and specific IgE, IgG2a, and IgG1 antibodies against BLG were measured. First, the marked effects shown in mice fed the 5% dietary protein were characterized by down-regulated total IgE when com-

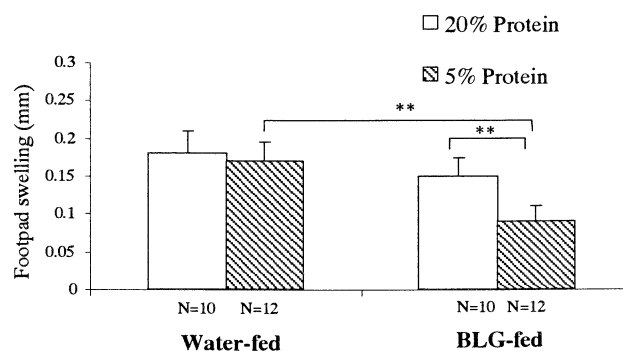


Fig. 6. Footpad reactions in mice after the transfer of spleen cells from tolerant mice fed 20% or 5% dietary protein. Spleens were collected from the mice given BLG or water orally and subsequent ip immunization as shown in Fig. 1. Single-cell suspensions were prepared and pooled according to group of treatment. Naïve mice received spleen cells intravenously (1×10^7 cells/mouse) from each group of mice. The mice were then tested for footpad reaction to BLG as described in Materials and Methods. Footpad swelling was measured 48 h after the injection of BLG. The results are expressed as mean $\pm$ SD taken from 10 or 12 mice per group. Open and crossed columns represent the mice fed 20% and 5% dietary protein, respectively. Double asterisks indicate a statistical significance of difference with a p value of <0.01 .

pared to those in mice fed the 20% protein diet. The oral administration of antigen before ip immunization showed different effects on the production of specific antibody titers against BLG. The different antibody levels seen in this study are attributed to the regulatory cytokines secreted from different T-cells; IgE and IgG1 are antibodies dependent on Th2 cytokine IL-4, while the production of IgG2a requires the participation of Th1 cytokine IFN- γ (20). In fact it was reported that IFN- γ knockout mice (lacking Th1 cells) orally immunized with a high dose of OVA did not produce any IgG2a antibody (21). IgG2a thus represents Th1-mediated response, which might also be demonstrable by using IL-2 knockout mice. In our study, specific IgG2a antibody against BLG was markedly down-regulated in the sera of tolerant mice fed the 5% dietary protein, whereas no effect was observed in specific IgG1 antibody. It is interesting to note that, in contrast to specific IgG1, the IgE titer was significantly lower in the sera of tolerant mice fed the 5% dietary protein. Although both IgE and IgG1 antibodies are regulated by the same Th2 cells cytokines, their production in the sera of tolerant mice fed 5% protein suggests the involvement of a distinct regulatory mechanism. This result is consistent with a previous study showing a decrease in IgE with no effect on IgG1 in systematically challenged mice (22).

IL-4 is a necessary molecule for the induction of both IgE and IgG1 isotypes, as demonstrated in IL-4 deficient mice (23). It has also been reported that less IL-4 is required for the production of a peak IgG1 compared with that for the peak IgE response (24). Mice on a low dietary protein without any presensitization and/or immunization showed suppressed production of IL-4 from

their spleen cells when compared to the mice fed normal dietary protein (10). The low level of IL-4 in tolerant mice fed 5% dietary protein was sufficient for down-regulating specific IgE, but did not affect specific IgG1.

To demonstrate the effects of dietary protein on oral tolerance at the cellular level by a more direct means, we examined some cytokines produced from spleen cell cultures and the proliferation of splenocytes from each group of mice after restimulation with BLG. While IL-4, IL-2, and IFN- γ were secreted specifically in response to BLG in the spleen cells of control mice, the secretion of IL-2 and IFN- γ , but not IL-4, was significantly reduced in the supernatants from the spleen cells of tolerant mice. In addition, reduction of IL-2 and IFN- γ was more effective in the mice fed the 5% protein than that in the mice fed the 20% protein. It is well documented that CD4⁺ Th1 cells secrete IFN- γ and IL-2 for cell-mediated immunity and delayed type hypersensitivity, whereas CD4⁺ Th2 cells preferentially secrete IL-4, IL-5, and IL-10, which provide help for antibody response (25). Thus, in this study, it was shown that mice fed the 5% protein diet developed a selective tolerance of Th1-mediated immune response with lower production of IgG2a, IL-2, and IFN- γ , whereas the Th2-mediated response remained intact with no effect on IL-4 and IgG1 antibody production in vivo.

The oral tolerance established in the present study was also examined for specific and non-specific proliferation of spleen cells upon stimulation in vitro with BLG and ConA/LPS, respectively. Both specific and non-specific proliferation to BLG, and, ConA and LPS, respectively, were significantly reduced in tolerant mice fed the 5% dietary protein, whereas no statistically significant differences were observed between the tolerant and control groups of mice fed the 20% dietary protein. These findings indicate that both specific and bystander suppression to antigen might be induced by multiple feedings of BLG to mice as described by Pecquet S et al. (26).

The mechanism(s) and a factor(s) involved in the induction of oral tolerance still remain unclear. There is considerable evidence that cytokines play an important role in inducing systemic oral tolerance. The interaction of the CD28 molecule with the B7 family on antigen-presenting cells is essential for T-cell activation (1). Both B7-1 and B7-2 molecules are known to costimulate the production of IL-2 and IFN- γ , whereas B7-2 costimulates the production of IL-4 more effectively (27). Thus, in our system, the restricted dietary protein might have some suppressive effects on the B7-1 molecule, which reduced the production of IL-2 and IFN- γ , but not that of IL-4, and led to the anergy of Th1 cells.

The present study has provided evidence that restricted dietary protein plays an important role in the induction of oral tolerance through the hyporesponsiveness of Th1 helper functions. BLG is a major allergen in milk protein and can cause type I allergy with a possibility of type IV allergic reactions as manifested by footpad reactions. Considering the increasing prevalence of allergic conditions, several approaches are currently

under evaluation to overcome the immunological disorders (7, 26). The results also indicate that multiple exposure of BLG to mice successfully suppresses the Th1-mediated immune responses accompanied with the down-regulation of specific IgE by restricted dietary protein, which might be of great benefit for improving immune deviation.

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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, Aiba 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 H, 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) Zhang S, Petro TM. 1997. The effect of moderate protein malnutrition on murine T cell cytokine production. *Nutr Res* **17**: 51–64.
- 11) 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.
- 12) Lee SH, Shin MH, Lee HB, Lee JS, Son BK, Koh YY, Kim KE, Ahn YO. 2001. Prevalence of symptoms of asthma and other allergic diseases in Korean children: a nationwide questionnaire survey. *J Korean Med Sci* **16**: 155–164.
- 13) Davies RJ, Rusznak C, Devalia JL. 1998. Why is allergy increasing?—Environmental factors. *Clin Exp Allergy*

- 28** (Suppl 6): 8–14.
- 14) Kleinman RE, Bahna S, Powell GF, Sampson HA. 1991. Use of infant formula in infants with cow's milk allergy. *Pediatr Allergy Immunol* **4**: 146–155.
 - 15) Host A, Halken S. 1990. A prospective study of cow's milk allergy in Danish infants during the first 3 years of life. Clinical course in relation to clinical and immunological type of hypersensitivity reaction. *Allergy* **45**: 587–596.
 - 16) Bjornsson E, Janson C, Plaschke P, Norrman E, Sjoberg O. 1996. Prevalence of sensitization to food allergens in adult Swedes. *Ann Allergy Asthma Immunol* **77**: 327–332.
 - 17) Ota F, Yamamoto S, Takeuchi S, Akiyama M, Kido Y, Kishi K, Hirota K, Fukui K. 1992. Effects of protein intake on immune parameters in mice surviving streptococcal infection. *FEMS Microbiol Immunol* **89**: 111–122.
 - 18) Satter MA, Sakai K, Ahmed S, Yoshino K, Yamamoto S, Shimizu Y, Ota F. 2002. Low-protein diet induces oral tolerance to ovalbumin in mice. *J Nutr Sci Vitaminol* **48**: 51–58.
 - 19) 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.
 - 20) Snapper CM, Mond JJ. 1993. Towards a comprehensive view of immunoglobulin class switching. *Immunol Today* **14**: 15–17.
 - 21) Kweon MN, Fujihashi K, VanCott JL, Higuchi K, Yamamoto M, McGhee JR, Kiyono H. 1998. Lack of orally induced systemic unresponsiveness in IFN- γ knockout mice. *J Immunol* **160**: 1687–1693.
 - 22) Holt PG. 1994. Immunoprophylaxis of atopy: Light at the end of the tunnel? *Immunol Today* **15**: 484–489.
 - 23) Kuhn R, Rajewsky K, Muller W. 1991. Generation and analysis of interleukin-4 deficient mice. *Science* **254**: 707–710.
 - 24) Snapper CM, Finkelman FD, Stefany D, Conred DH, Paul WE. 1988. IL-4 induces co-expression of intrinsic membrane IgG1 and IgE by murine B-cells stimulated with lipopolysaccharide. *J Immunol* **141**: 489–498.
 - 25) Mosmann TR, Coffman RL. 1989. Th1 and Th2 cells: Different Patterns of lymphokine secretion lead to different functional properties. *Ann Rev Immunol* **7**: 145–173.
 - 26) Pecquet S, Pfeifer A, Gauldie S, Fritsche R. 1999. Immunoglobulin E suppression and cytokine modulation in mice orally tolerized to β -lactoglobulin. *Immunology* **96**: 278–285.
 - 27) Freeman GJ, Boussiotis VA, Anumanthan A, Bernstein GM, Ke XY, Rennet PD, Gray GS, Gribben JG, Nadler LM. 1994. B7-1 and B7-2 do not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity* **2**: 523–532.