

Oral Administration of IL-12 Abrogates the Induction but Not the Maintenance of Oral Tolerance

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Key Words

Oral tolerance · Interleukin-12 · Antibody production ·
Antiallergic food

Abstract

Background: IL-12 is a Th1-inducing cytokine and known to be induced by some food factors. This function of such foods is expected to be applied for antiallergic materials. However, the influence of IL-12 on the immune responses has not been fully investigated. Oral tolerance is an immunologically unresponsive state induced by orally administered food antigens. Although a failure in the induction of oral tolerance would result in food allergy, the mechanisms for the induction and abrogation of oral tolerance have not been clarified. IL-12 induced by food factors may also affect the induction of oral tolerance. In this study we examined the effect of the oral administration of IL-12 on the induction and maintenance of oral tolerance. **Methods:** BALB/c mice were fed β -lactoglobulin with or without IL-12. After immunizing them with the antigen, the serum antibody titer of these mice was measured to evaluate the induction of oral tolerance. **Results:** The induction of oral tolerance was prevented in the mice that had been simultaneously fed the antigen and IL-12. On the other hand IL-12 did not abrogate the already established oral tolerance when it was administered after feeding the antigen. We also found that oral tolerance could be induced normally in mice that had failed to induce such toler-

ance by simultaneous feeding of IL-12 and the antigen. **Conclusions:** The results indicate that IL-12 induced in the intestine by some food factors is involved in the regulation of oral tolerance.

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Introduction

IL-12 is a cytokine which induces a Th1 response and contributes to enhanced cellular immunity. Modifications of some immune responses by IL-12 would be especially useful for many people troubled with allergic, infectious and autoimmune diseases or tumors. Recent studies have revealed that not only pathogens but also some food factors induced IL-12 production [1–4]. There have been several reports on the effect of antiallergic, antiviral and antitumor foods [1–4]. We have also shown that alginic acid oligosaccharide (ALGO), a lyase-lysate obtained from natural edible polysaccharide, inhibited antigen-induced IgE production, and that this function was mediated by the enhancement of IL-12 production [5]. We consider that a daily intake of ALGO would help prevent allergic disorders in humans. However, the influence of IL-12 on other immune responses such as oral tolerance has not been investigated. IL-12 might be able to efficiently enhance oral tolerance or to prevent it.

Oral tolerance is a state of immunological unresponsiveness to orally administered antigens. Although oral tolerance has been reported since the 1960s [6, 7], the mechanism for its induction, maintenance and abrogation has not been clarified. It has been reported that T cells were important to maintain the state of oral tolerance [8, 9]. Furthermore, the anergy of CD4⁺ T cells specific to oral antigens has been reported as a critical mechanism [8, 10], while active suppression mediated by regulatory T cells has also been shown to be involved [11, 12]. However, the mechanism involved in the induction of T cell anergy and regulatory T cells has not been demonstrated *in vivo*. An investigation of the actions involved in oral tolerance would contribute to the prevention and treatment of food allergy because oral tolerance against a food antigen is considered to play a critical role in inhibiting the hyperimmune responses leading to food allergy [13]. Furthermore the application of oral tolerance can be expected for treating autoimmune diseases [14]. If the mechanisms for the induction and abrogation of oral tolerance can be clarified, oral tolerance could be applied more efficiently and safely to such treatment.

Previous studies have shown that some factors regulated the induction of oral tolerance, although the mechanisms for such responses have not been clarified. Cholera toxin is well known as a mucosal adjuvant to induce a strong immune response against a simultaneously administered oral antigen [15]. On the other hand, a conjugate of the B subunit of cholera toxin and the protein antigen has been reported to induce oral tolerance more efficiently than native protein [16]. Tsuji et al. [17] have reported that the oral administration of lipopolysaccharide inhibited the induction of oral tolerance to a simultaneously ingested oral antigen, while λ -carrageenan did not, even if both of them had the same function for modulating the immune response toward dominant Th1. Furthermore, several reports have indicated that the administration of IL-12 negatively modulated the induction of oral [18–20] and other tolerances [21]. However, the effect of orally administered IL-12 on oral tolerance has not been clarified. The effect might change depending on the method of administration because the transference of IL-12 to the site involved in the induction of oral tolerance, which has not been revealed, might be influenced by the administration method. It would be necessary to investigate the effect of orally administered IL-12 on oral tolerance to understand the influence of such an antiallergic food as ALGO. In addition, the effect of IL-12 would also change depending on the administration conditions, such as the timing. We therefore need to examine in de-

tail the effect of IL-12 under various conditions in order to understand the actual influence of antiallergic foods.

In the present study we examine the uptake effect of orally administered IL-12 on the induction and maintenance of oral tolerance. IL-12 mediates the IgE-inhibiting function of ALGO [5] and may also be involved in the antiallergic, anti-infectious and antitumor activity of many other food factors [22–25]. We therefore examined the effect of IL-12 on the intestine by orally administering IL-12. Our results show that the oral administration of IL-12 abrogated the induction of oral tolerance, but not its maintenance. Oral tolerance was established in mice that had had its induction blocked by IL-12. Our results clearly demonstrate both the risk and safety of ALGO and some other food factors and will contribute to their application to humans.

Materials and Methods

Mice

Female BALB/c mice (6 weeks old) were purchased from Clea Japan (Tokyo, Japan). All the mice were maintained and used in accordance with the guidelines for the care and use of experimental animals of the Tokyo University of Agriculture and Technology.

Antigen

Crude bovine β -lactoglobulin (β -LG; genotype AA) was prepared from fresh milk of a Holstein cow according to the method of Armstrong et al. [26]. The crude β -LG was purified in a DEAE-Sephacrose fast-flow column (2.5 ID $\times$ 50 cm; Amersham Pharmacia Biotech, Buckinghamshire, UK) by the method previously described [27]. The purity of β -LG was confirmed by polyacrylamide gel electrophoresis (PAGE) performed by the method of Davis [28].

Oral Administration of the Antigen and IL-12

BALB/c mice were orally administered with β -LG (200 μ g) or IL-12 (100 ng; Genzyme/Techne, Minneapolis, Minn., USA) dissolved in 200 μ l of PBS by a feeding needle, β -LG and IL-12 being simultaneously administered in some experiments. One cycle of feeding involved administering it 4 times, and each administration was performed at 2-day intervals.

Immunization

The mice were intraperitoneally immunized with 100 μ g of β -LG emulsified in Freund's complete adjuvant (CFA; Difco Laboratories, Detroit, Mich., USA) 2–3 days after the last feed and boosted with 100 μ g of β -LG in Freund's incomplete adjuvant (IFA; Difco Laboratories) 2 weeks after the first immunization. These mice were bled 10 days after the second immunization. The blood samples were centrifuged, and the sera were collected and stored at -30°C until needed. In the experiment to confirm active suppression, the mice were subcutaneously immunized with 100 μ g of β -LG emulsified in CFA H37Ra (Difco Laboratories).

Ten days after the immunization the inguinal and popliteal lymph nodes were obtained from the sensitized mice, and single-cell suspensions were prepared.

Cell Culture

The erythrocyte-depleted splenocytes of mice fed 4 times 200 µg of β-LG were cocultured with the lymph node cells obtained from β-LG-sensitized mice in the presence of the antigen at 5 µM. The lymph node cells were plated at a concentration of 2×10^6 cells/ml, and the splenocytes were cocultured at a concentration of 1, 2 or 4×10^6 cells/ml. Three days after stimulating the antigen the proliferative response was measured by a bromodeoxyuridine (BrdU) cell proliferation ELISA kit (Roche Diagnostics, Basel, Switzerland). The proliferative response measured by this method mainly represents the proliferation of the lymph node cells, because the splenocytes of unimmunized mice hardly proliferated in response to antigen stimulation. The splenocytes just modulated the proliferation of the lymph node cells through cytokines or any other mechanism if suppression was induced [11, 29]. All cells were cultured in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal calf serum (JRH Biosciences, Lenexa, Kans., USA).

Serum Antibody Detection

The β-LG-specific antibody titer in the serum was measured by ELISA. Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with a 0.01% β-LG solution. After washing and blocking the serum sample with standards was added to a plate. The bound antibody was detected with the alkaline phosphatase-labeled anti-mouse immunoglobulin antibody (total Ig; Dako, Glostrup, Denmark), the biotin-labeled anti-mouse IgG1 antibody (Zymed, San Francisco, Calif., USA) or the biotin-labeled anti-mouse IgG2a antibody (Zymed). Alkaline phosphatase-streptavidine (Zymed) was subsequently incubated when the biotin-labeled antibodies were used for detection. A substrate (*p*-nitrophenyl-phosphate) was then added, and the absorbance was determined at 405 nm. The antibody concentration was calculated relative to that in the standard serum. The standard serum for total Ig, IgG1 and IgG2a was prepared from mice that had been immunized with β-LG in CFA and boosted with the antigen in IFA. The standard serum for IgE was prepared from mice that had been immunized with β-LG in an alum adjuvant.

Epitope Mapping

A series of overlapping 15-mer peptides moving 1 amino acid residue at a time in accordance with the amino acid sequence of β-LG were synthesized on activated polyethylene gears in each pin, using a five-in-one B and T cell epitope scanning kit (Chiron Mimotopes, Clayton Australia) as described previously [30]. The B cell epitopes were scanned by ELISA, using the overlapping synthetic peptides on each pin as the antigen on a solid phase as described previously [30]. The pins were reused after the bound antibodies had been stripped off according to the procedure described in the manual for the epitope scanning kit.

To determine the epitope profiles, several criteria were used to evaluate whether or not the observed response was significant. The peptides adopted as positive were (1) those which showed reactivity greater than the mean value plus 3 standard deviations below the median absorbance in the case of peptides binding to the antibodies in anti-β-LG antisera [31], (2) those which showed

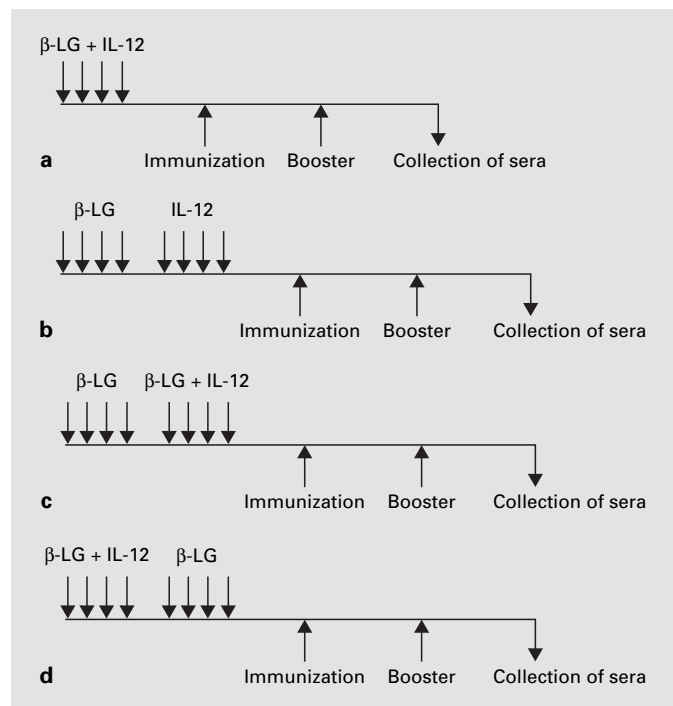


Fig. 1. Experimental scheme for this study. BALB/c mice were orally administered with β-LG and IL-12, individually or simultaneously (a). The mice that had received β-LG were further fed IL-12 (b) or β-LG and IL-12 (c). Similarly the mice that had received β-LG and IL-12 were further fed β-LG (d). One cycle of feeding involved administering it 4 times. These mice were immunized with β-LG 2–3 days after the last feed with the antigen or IL-12 and boosted 2 weeks after the first immunization. The sera of these mice 10 days after the second immunization were collected and used to determine the antibody titer.

positive reactivity with at least 2 consecutive overlapping peptides [32] and (3) those which showed positive reactivity in 2 of 3 individual mice [33].

Statistical Analysis

The difference among each group was analyzed by the Student *t* test, *p* < 0.05 being considered statistically significant. Each figure is presented as the mean value of individual values, and an error bar indicates the standard deviation.

Results

Simultaneous Feeding of the Antigen and IL-12 Abrogated the Induction of Oral Tolerance

We examined the influence of IL-12, a key modulator of the antiallergic effect of ALGO, upon the induction of oral tolerance when taken orally. Our experimental pro-

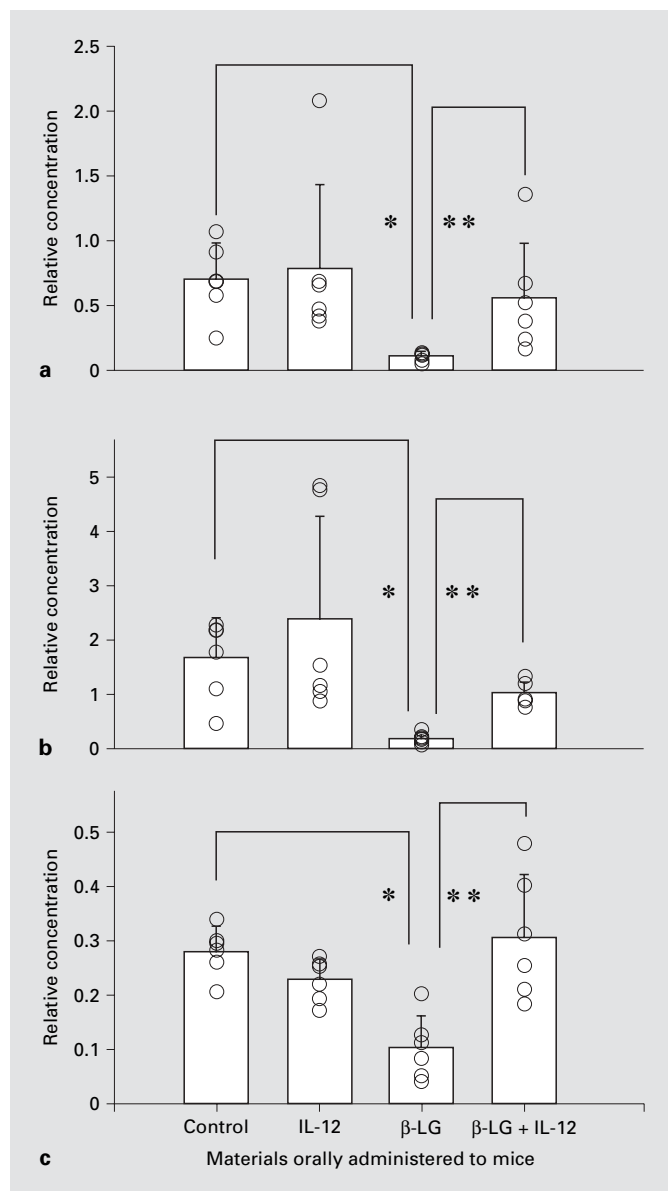


Fig. 2. Effect of oral IL-12 on the induction of oral tolerance. BALB/c mice were fed β -LG and IL-12 individually or simultaneously. These mice were then immunized and boosted, and the antigen-specific antibody titers of total Ig (a), IgG1 (b) and IgG2a (c) in their sera were measured by ELISA. The result is representative of 3 independent experiments. $n = 6$ for each group. * $p < 0.01$, significant difference compared with the mice fed PBS alone. ** $p < 0.05$, significant difference compared with the mice fed β -LG alone.

cedure is summarized in figure 1. The mice were orally administered 200 μ g of β -LG and 100 ng of IL-12 four times at an interval of 2–3 days. Although this amount of IL-12 could seem too low to have any effect via oral ad-

ministration, a previous study [34] demonstrated that such an amount of IL-4 administered orally was stable enough to affect the immune response of host mice. After immunization and boosting, the sera were collected from these mice, and the β -LG-specific antibody titer (total Ig) in each serum sample was determined (fig. 1a). As shown in figure 2a, the antibody concentration in the sera of the mice fed β -LG alone was significantly less than that of the control (PBS-fed) mice, indicating that oral tolerance had been induced in those mice. In contrast the antibody titer of the mice simultaneously fed β -LG and IL-12 was similar to that of the control mice. The antibody production of the mice fed IL-12 alone was at almost the same level as that of the control mice. Similar results were obtained when we tested the concentrations of IgG1 and IgG2a (fig. 2b, c). These findings demonstrate that simultaneous feeding of the antigen and IL-12 failed to induce oral tolerance.

Oral Administration of IL-12 Did Not Affect the Maintenance of Oral Tolerance

We next examined whether the oral administration of IL-12 would also abrogate the already established oral tolerance. The mice were fed β -LG 4 times, and after the last feeding IL-12 was similarly administered 4 times. After immunizing and boosting the β -LG-specific antibody titer (total Ig) in the sera of these mice was determined (fig. 1b). Figure 3a shows that the antibody titer of the mice fed IL-12 after the establishment of oral tolerance remained at a low level compared with that of the control mice and at a similar level to that of the mice fed β -LG alone. The oral administration of IL-12 with β -LG to mice that had been rendered tolerant similarly did not abrogate the maintenance of oral tolerance to β -LG (fig. 1c, 3b). These data demonstrate that the oral administration of IL-12 did not affect the maintenance of oral tolerance.

Oral Tolerance Was Established in the Mice That Had Had the Induction of Oral Tolerance Blocked by IL-12

Our findings revealed that the induction of oral tolerance was abrogated by simultaneously feeding IL-12 with the antigen. We next tested whether oral tolerance could be induced in those mice. β -LG was orally administered 4 times to the mice that had previously been fed β -LG and IL-12. These mice were immunized, and the antibody production in the serum of each mouse was measured (fig. 1d). As shown in figure 4, feeding with β -LG induced oral tolerance even in those mice that had failed to be rendered orally tolerant.

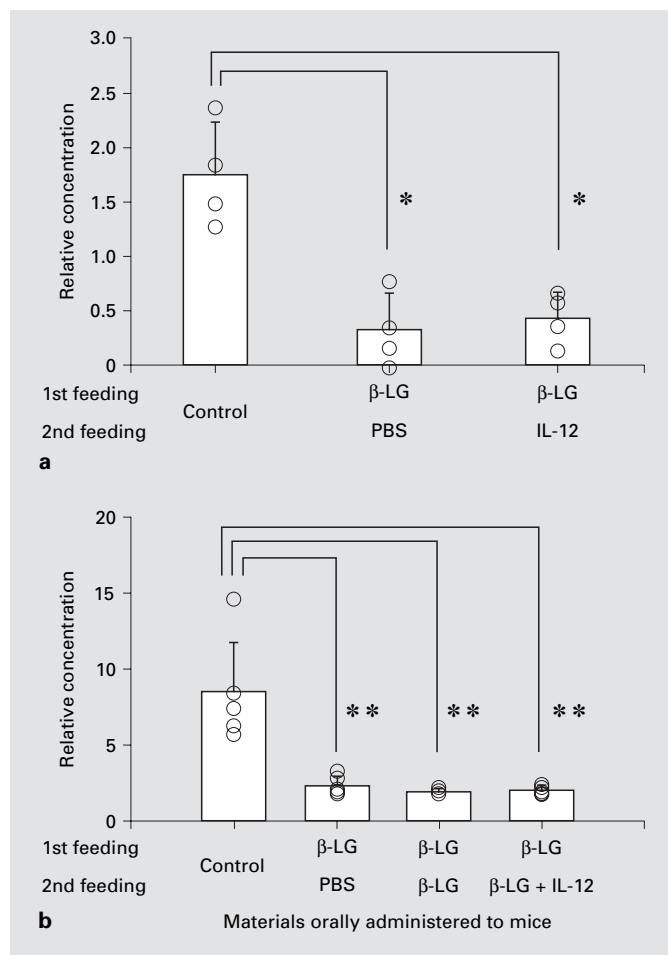


Fig. 3. Effect of oral IL-12 on the maintenance of oral tolerance. BALB/c mice were fed β -LG. These mice were further fed IL-12 (a) or β -LG and IL-12 (b). After immunization and boosting the antigen-specific antibody titers of total Ig in their sera were measured by ELISA. The result is representative of 2 (a) or 4 (b) independent experiments. $n = 4$ or 5 for each group. * $p < 0.01$, significant difference compared with the mice fed PBS alone. ** $p < 0.05$, significant difference compared with the mice fed PBS alone.

Oral Administration of IL-12 Did Not Induce the Activation of Antibody Response to Any Epitope, while It Did Not Completely Abrogate the Induction of Oral Tolerance

We next examined the profile of B cell epitopes of the mice with abrogated tolerance after their immunization with the same antigen (fig. 5). The antibody of the control mice (β -LG-immunized normal mice) recognized peptides 1–6, 8–11, 63–70, 79 and 80, and 90 and 91, while that of the mice with abrogated tolerance recognized peptides 64–69, 79 and 80, and 90 and 91. The residues cor-

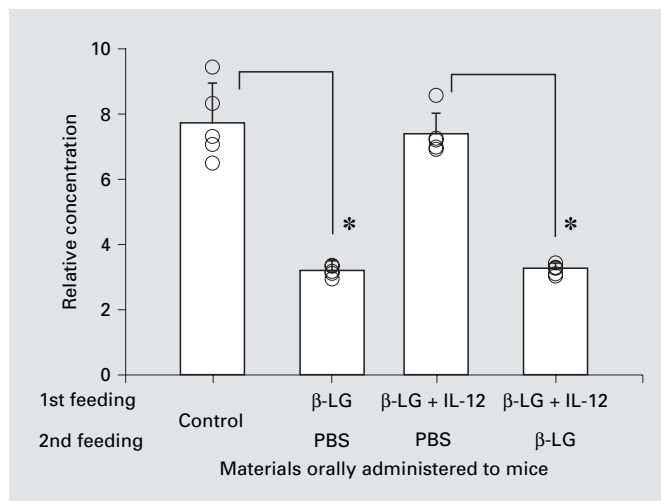


Fig. 4. Induction of oral tolerance in mice that had failed to become orally tolerant. BALB/c mice were simultaneously fed β -LG and IL-12. These mice were further fed β -LG. After immunization and boosting the antigen-specific antibody titers of total Ig in their sera were measured by ELISA. The result is representative of 3 independent experiments. $n = 5$ for each group. * $p < 0.01$, significant difference compared with the mice fed PBS.

responding to peptides 1–6 and 8–11 were only recognized by the control mice. This evidence indicates that the induction of oral tolerance was not completely abrogated by IL-12. We consider that the amount of IL-12 administered in this study was not enough to completely abrogate the induction of oral tolerance. Furthermore, our findings indicate that IL-12 did not affect each epitope equally. On the other hand, we did not observe any activation of the immune response to any epitope in the mice with abrogated tolerance when compared with the control mice. This result indicates that IL-12 did not induce activation and just abrogated the induction of oral tolerance. From these results and those in figure 4 we conclude that the intolerant epitopes retained an almost naive character sufficiently to be rendered tolerant.

Oral Administration of IL-12 Abrogated the Induction of Oral Tolerance That Consisted of Anergy or Deletion

In this study we have demonstrated the effect of IL-12 on the induction and maintenance of oral tolerance. Although the mechanisms for oral tolerance have not been clarified, several hypotheses have been proposed in previous studies [10, 35, 36]. It has been suggested that the preferential mechanism induced by an oral antigen de-

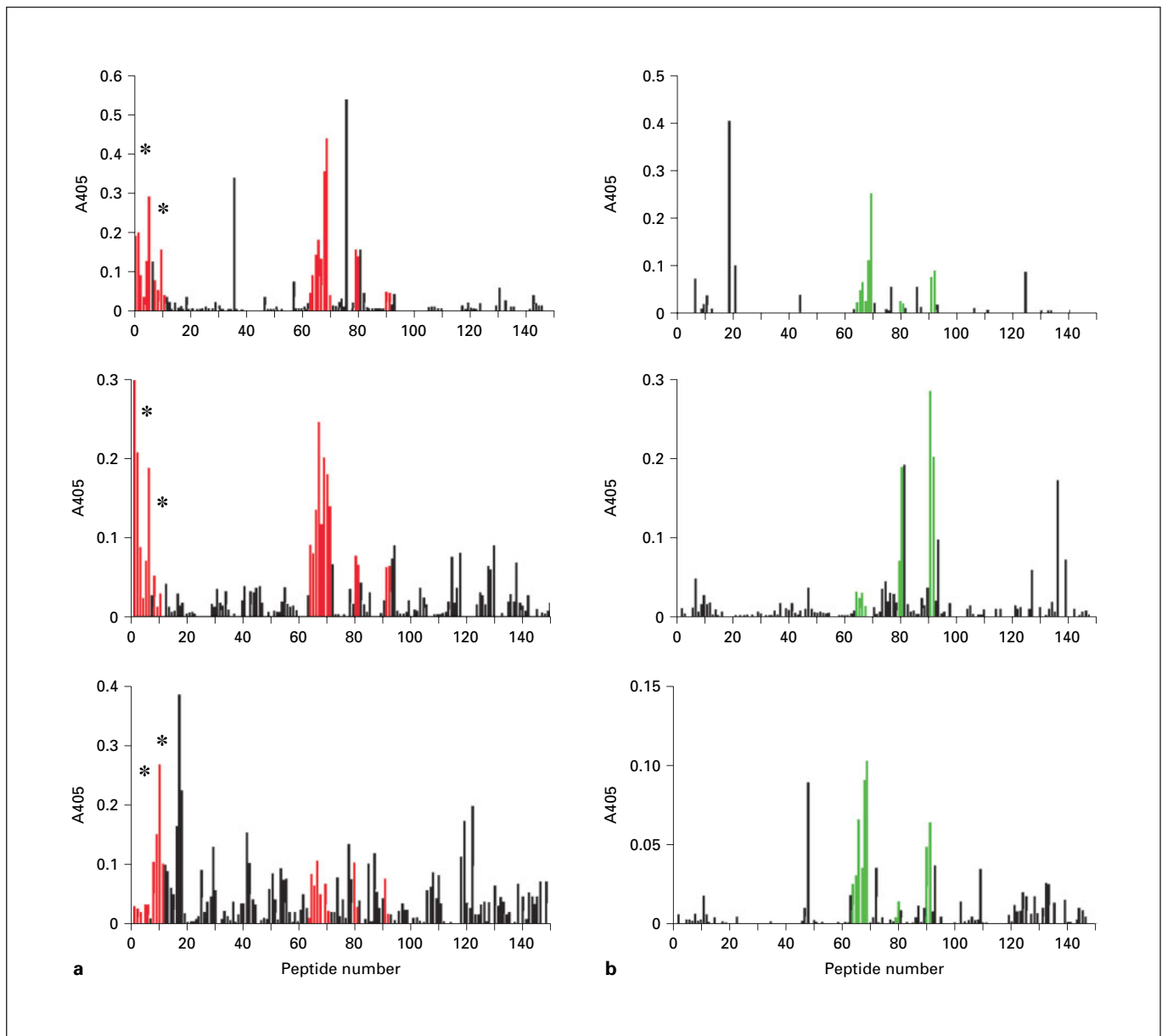


Fig. 5. Comparison of B cell epitopes between the untreated control mice (**a**) and the mice fed β -LG and IL-12 (**b**). BALB/c mice were simultaneously fed β -LG and IL-12. These mice and untreated control mice were immunized and boosted with β -LG. The epitopes of the antibody in the sera from these mice were scanned as described in the 'Methods' section. The results of 3 individual mice for each group are shown. The red and green bars indicate epitopes of the control mice and the mice fed β -LG and IL-12, respectively, as determined by the criteria described in the 'Methods' section. The epitopes observed only in the control mice are indicated by an asterisk.

depends on the dosage of the antigen [11]. We therefore examined the major mechanism for oral tolerance established with our experimental system. We analyzed the inhibitory activity of the splenocytes from mice that had been rendered orally tolerant according to a previous re-

port [11]. In brief those splenocytes were cocultured with lymph node cells obtained from β -LG-immunized mice in the presence of the antigen. However, no difference between the groups was apparent (table 1). Furthermore no suppressive effect was apparent when using Peyer's

Table 1. Proliferation of lymph node cells cocultured with splenocytes from the mice fed β -LG

Responder:modulator	A450	
	control	fed β -LG
1:0.5	1.68 ± 0.22	1.70 ± 0.22
1:1	1.46 ± 0.28	1.82 ± 0.13
1:2	1.55 ± 0.18	1.78 ± 0.06

BALB/c mice were fed β -LG, and the splenocytes were obtained from these mice 7 days after the last feed. These cells were cocultured with lymph node cells of β -LG-immunized mice in the presence of β -LG. Each culture was performed in triplicate. The proliferative response of each cultured sample was measured by a BrdU cell proliferation ELISA kit. The result is representative of 2 independent experiments.

patch cells as a modulator (data not shown). This result suggests that simultaneously feeding IL-12 with the antigen might have abrogated the induction of T cell anergy or its deletion, but not active suppression of the antigen.

Discussion

The aim of this study was to examine the influence of orally administered IL-12, which may be induced in vivo by some food factors, on the induction and maintenance of oral tolerance. Our results demonstrate that the oral administration of IL-12 blocked the induction of oral tolerance, while it did not affect its maintenance. Furthermore, oral tolerance was induced in the mice in which it had been blocked by simultaneously feeding IL-12 with the antigen. These findings will contribute to applying the function of many food factors which induce IL-12 to produce antiallergic, antitumor and antiviral foods.

IL-12 is a cytokine which is involved in Th1 induction. Many recent studies have reported that some food factors induced IL-12 and could be used for preventing and treating many diseases [1–5]. However, the full effects of such a type of food factor on the immune system have not been clarified. The results of the present study show that oral IL-12 administration blocked the induction of oral tolerance. It has been suggested in previous studies [18, 19] that IL-12 may modulate the induction of oral tolerance. However, the effect of IL-12 administered orally has not been clearly investigated. Our findings support those previously reported and provide new evidence that IL-12 induced by some food factors in the intestine would also

modulate oral tolerance. This result suggests the potential risk of using an antiallergic food such as ALGO for preventing allergic diseases. On the other hand we have also shown that IL-12 did not abrogate the maintenance of oral tolerance, indicating that the risk of an antiallergic food to oral tolerance may be relatively low after oral tolerance to daily foods has been established. We have also demonstrated that oral tolerance can be induced in mice in which it had been blocked by simultaneously feeding IL-12 with the antigen. This result suggests that the sensitivity of oral tolerance in these mice hardly changed in comparison with that in naive mice. These findings together prompt us to conclude that a daily intake of antiallergic food would be a useful method for preventing allergy without any marked risk of oral tolerance.

Our results demonstrate that the administration of IL-12 could not abrogate oral tolerance after it had already been established, indicating that IL-12 did not directly affect T cells which had been rendered tolerant. It is therefore considered that the antigen-presenting process of an oral antigen may be modified by IL-12. We hypothesize that IL-12 altered the function of the antigen-presenting cells (APC) involved in the induction of oral tolerance. A study on the effect of IL-12 on APC is now in progress at our laboratory. On the other hand the site where oral tolerance was induced remains to be identified. No IL-12 could be detected in the sera from the mice fed IL-12 (data not shown), although it cannot be excluded that an undetectable amount of IL-12 would be enough to block the induction of oral tolerance. It is therefore possible that sites other than the intestine might participate in the induction of oral tolerance; indeed, the sc administration of IL-12 has also abrogated the induction of oral tolerance [19].

The important role of antigen presentation in the induction of T cell tolerance has been demonstrated by many previous studies [37–39]. Costimulatory molecules expressed on APC play a critical role in regulating the activation or rendering tolerant of T cells [40–43]. CD80, CD86 and CD40 in particular have been well investigated, and the absence of these molecules resulted in the tolerance of T cells [41–43]. It has also been reported that the expression of these molecules was significantly up-regulated by such cytokines as IFN- γ and TNF- α [44, 45]. IL-12 is a potent inducer of such cytokines, indicating that it might block the induction of oral tolerance by modifying the function of APC through regulation of the expression of costimulatory molecules.

Grohmann et al. [46] have reported that the function of dendritic cells to induce tolerance was reduced by IL-

12, and an activating ability appeared instead. On the other hand we found that oral tolerance could be induced by orally administering more antigen to the mice that had failed to show oral tolerance. It is suggested that T cells in the mice retained the nature of naive rather than activated T cells because it has been reported that T cells could hardly be rendered tolerant if they had been activated [47]. However, further examination showed that some B cell epitopes had been inhibited in the mice, indicating that the T cells in these mice were not completely naive. Nevertheless we did not observe any epitopes that were activated in comparison with untreated mice. Taken together, the results imply that IL-12, in our system at least, weakened the ability of APC to induce oral tolerance, but did not change their function from rendering tolerant to activating. It is possible that a larger amount of IL-12 might make tolerance-inducing APC alter their function to activating.

It has been reported that the mechanisms for oral tolerance could be generally classified into 3 distinct types. Clonal anergy is defined as the unresponsive state of antigen-specific T cells [8, 10], and clonal deletion occurs by the elimination of antigen-specific T cells through apoptotic cell death [36]. On the other hand active suppression is mediated by the induction of antigen-specific regulatory T cells that secrete some inhibitory cytokines and suppress other T cell responses [11, 12]. Previous studies have reported that the oral administration of a high-dose antigen preferentially induced clonal anergy and deletion, while a low-dose antigen induced active suppression [11]. Although the antigen dose used in our experiments, administering 200 µg of the antigen 4 times, has been classified as low-dose administration in accordance with the previous reports, no induction of active

suppression was apparent in the present system. In this respect Tsuji et al. [12] have reported that a significant induction of active suppression was observed by feeding 5 times with 1 mg of β-LG, and that it was very weak at doses lower than 500 µg per feeding. We have previously observed that lower doses of the antigen, administering 10 or 100 µg 4 times, also induced oral tolerance in the absence of any suppression [29]. It has been reported that IL-12 prevented the induction of inhibitory T cells that secreted TGF-β [48, 49]. However, we found in this study that IL-12 can block the induction of oral tolerance by a mechanism other than active suppression. We consider that IL-12 might affect clonal anergy because it has been reported that clonal deletion was induced by an extremely high amount of an oral antigen [36].

In conclusion, the results of the present study clearly demonstrate that the oral administration of IL-12 abrogated the induction of oral tolerance against the antigen when simultaneously fed IL-12. However, IL-12 did not influence the maintenance of oral tolerance. In view of these results it cannot be completely excluded that antiallergic or antitumor foods that induce IL-12 production could also abrogate oral tolerance. However, oral tolerance can be induced by orally administering further antigen to the mice that have failed to become orally tolerant. This evidence would be useful for evaluating the risk and safety of IL-12-inducing foods.

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