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Th2 polarization enhanced by oral administration of higher doses of antigen

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Abstract

Although oral administration of a soluble protein antigen can induce various immune responses, the effect of the dosage of oral antigen on the predominance of Th2-type cytokine and antibody responses has not been well clarified yet. In the present study, we fed T cell receptor (TCR) transgenic (tg) mice various amounts of chicken ovalbumin (0.1, 5, and 250 mg) and examined the resulting immune responses to this antigen. In these TCR tg mice, the responses of antigen-specific T cells were greatly amplified concomitantly with significant antigen-specific cytokine secretion. We found that a high dose (250 mg) of antigen significantly upregulated the serum antigen-specific IgG1 and IgA antibody responses in mice later intraperitoneally injected with antigen plus adjuvant. The mice administered the same oral dose but not immunized showed upregulation of Th2-type IL-4 and IL-5 secretion and downregulation of Th1-type IL-2 and IFN-γ. This enhancement of Th2-type cytokine and antibody responses was more marked when larger doses of antigen orally administered. These results demonstrated that antigen feeding induces the development of T cells secreting Th2-type cytokines in a dose-dependent manner and that these T cells have a helper function for the production of antibodies of the Th2-type isotypes. This experimental system should be useful to screen foods and other substances that can modulate Th2-type responses relating to allergy.

Abbreviations: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; OVA, ovalbumin; PBS, phosphate-buffered saline; tg, transgenic; TCR, T cell receptor.

Introduction

Oral administration of soluble protein antigens induces various immune responses, depending on the dosage of the antigen. High doses of orally administered antigen cause antigen-specific clonal deletion and/or clonal unresponsiveness, whereas low doses of antigen generate T cells secreting TGF-β, a regulatory cytokine (Friedman and Weiner, 1994). These responses can be responsible for immune unresponsiveness, referred to as oral tolerance. On the other hand, orally ingested antigens can also induce particular immune responses. It has been demonstrated that antigen feeding mainly induces T cells secreting Th2-type cytokines, such as IL-4, IL-5, IL-6 and IL-10 (Xu-Amano et al., 1992; Melamed and Friedman, 1994). Those T cells secreting Th2-type cytokines promote the antibody response. Studies of the mechanisms underlying the induction of the antibody response to orally administered protein antigens are likely to be important in order to understand the pathogenesis of food allergy in which antibody responses specific for oral antigens are involved.

Although T cell responses induced by oral antigen have been shown to be dependent on the antigen dosage, the pattern of cytokine secretion and antibody

production induced by different doses of orally administered antigen remains to be clarified. In the present study, we fed T cell receptor (TCR) transgenic (tg) mice various doses of an antigen and examined the effect of the dosage of the antigen fed on the secretion of cytokines from antigen-specific T cells and the serum antibody responses. These tg mice have an abundant number of T cells expressing transgenic TCR specific for chicken ovalbumin (OVA), enabling us to monitor both Th1- and Th2-types of T cells and antibody responses to orally administered OVA, although it is difficult to use conventional mice for such analyses. We show that oral administration of this soluble antigen enhanced the levels of secretion of Th2-type cytokines by splenic T cells and enhanced the levels of IgG1 and IgA antibody production *in vivo*, in a dose-dependent manner. This experimental system should be useful for analysis of the effects of various foods and environmental substances on the *in vivo* immune response to orally ingested food allergen proteins.

Materials and methods

Oral administration of antigen to mice

Mice transgenic for the $\alpha\beta$ TCR genes of T cell clone 7-3-7 which recognizes the 323-339 peptide fragment of chicken OVA and I-A^d (Sato et al., 1994) were kindly provided by S. Habu (Tokai Univ. School of Med.) and both males and females were used throughout the experiments. The mice were maintained on a sterile diet (CE-2, Clea Japan, Tokyo, Japan) and water. For oral administration of antigen, the mice were intragastrically intubated under light anesthesia and each was administered 0.1, 5, or 250 mg of OVA (Seikagaku Corp., Tokyo, Japan) in 0.5 ml of phosphate-buffered saline (PBS). In the case of the control groups, mice were intubated in the same manner and administered 0.5 ml of PBS.

Cell culture

Seven days after oral administration of the antigen, erythrocytes-depleted splenocytes were prepared and cultured in 48- or 96-well plates (Costar Corp., Cambridge, MA and Becton Dickinson, Lincoln Park, NJ, respectively) at a cell concentration of 2.5×10^6 ml^{-1} with graded doses of OVA. For the assay of cytokine levels, culture supernatants were collected after 24, 48 and 72 h of incubation. For assay of the proliferative response, cells were cultured for 24, 48, 72, or 96 h and during the last 24 h, cells were incubated in the presence of [^{3}H] thymidine (ICN Pharmaceuticals, Costa Mesa, CA) at 0.5 μCi/well. The cells were harvested on glass fiber filters and the incorporated radioactivity was measured by liquid scintillation counting.

Cytokine enzyme-linked immunosorbent assay (ELISA)

Collected supernatants were stored at –20 or –80 °C until use and cytokine levels were assayed by a sandwich ELISA method. In brief, capture antibodies (JSE6-1A12, BVD4-1D11, and TRFK5 for IL-2, IL-4, and IL-5, respectively, from PharMingen, San Diego, CA, and R4-6A2, purified in our laboratory for IFN-γ) were used to coat the wells of 96-well immunoplates (Nunc, Roskilde, Denmark). The plates were washed and blocked with 1–3% bovine serum albumin (BSA)/PBS. After incubation of the culture supernatants and standard samples for the appropriate period, biotinylated antibodies (JES6-5H4, BVD6-24G2, and TRFK4 for detection of IL-2, IL-4, and IL-5, respectively, from PharMingen, and XMG1.2, purified and biotinylated in our laboratory for detection of IFN-γ) were added. Bound antibody was detected by incubation with streptavidin-conjugated alkaline phosphatase (Zymed, South San Francisco, CA), followed by incubation with disodium 4-nitrophenylphosphate. The coloring reactions were monitored by determining the absorbance at 405 nm.

Antibodies in sera

Seven days after oral administration of the antigen, the mice were immunized intraperitoneally with 0.1 mg of OVA emulsified in 0.1 ml of complete Freund's adjuvant (CFA) and PBS. Sera were prepared from tail blood by a centrifugation method at 10 days post-immunization. Antibody levels in the sera were examined by ELISA as described below. A solution of OVA was used to coat the wells of 96-well immunoplates. The plates were washed and blocked with 1–3% BSA/PBS. After the sera serially diluted with PBS containing 0.05% Tween 20 were added, alkaline phosphatase-conjugated anti-mouse immunoglobulin isotype-specific antibodies [anti-Ig(G+M+A), Cappel, Durham, NC; anti-IgG1, -IgG2a, -IgM, and -IgA, Zymed Laboratories, South San Francisco, CA] were added and the plates were incubated for an appropriate period. After the addition of disodium 4-

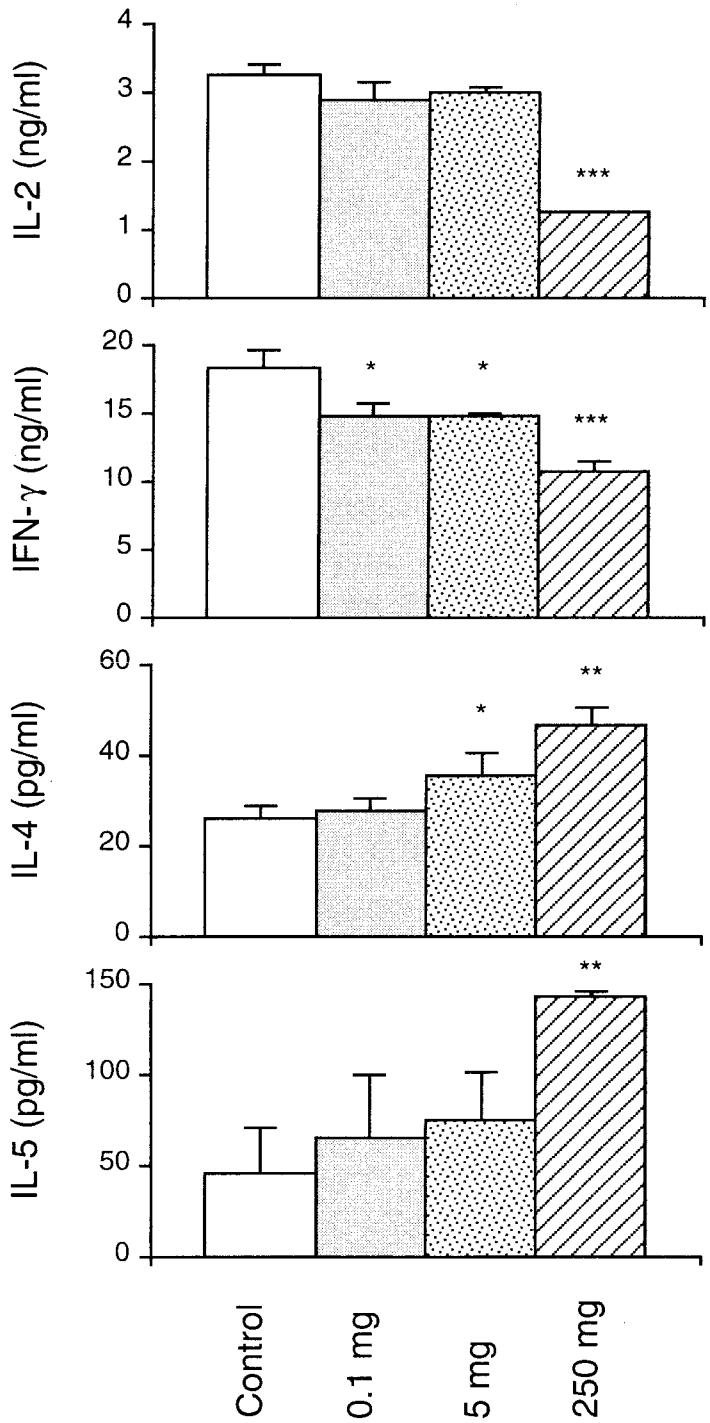

Figure 1. The cytokine responses of splenocytes derived from TCR tg mice orally administered various doses of OVA. TCR tg mice (3–4 mice/group) were orally administered various doses (0.1, 5 and 250 mg) of OVA and, one week later, splenocytes from these mice were isolated and cultured with or without 1000 μg ml^{-1} OVA. The amounts of secreted IL-2 and IL-4 in the supernatant of the 48-h culture and the amounts of secreted IL-5 and IFN-γ in the supernatant of the 72-h culture were measured by ELISA and are expressed as the mean values $\pm$ SD of triplicate cultures. The results shown are representative of at least three independent experiments. $*$, $**$, and $***$ are $p < 0.05$, < 0.005, and < 0.001 vs. the control group (administered with PBS), respectively. The levels of the responses in the medium without antigen were all below the detection limit. The lower limit of measurement was 0.06 ng ml^{-1}, 0.6 ng ml^{-1}, 4 pg ml^{-1}, and 30 pg ml^{-1} for IL-2, IFN-γ, IL-4, and IL-5, respectively.

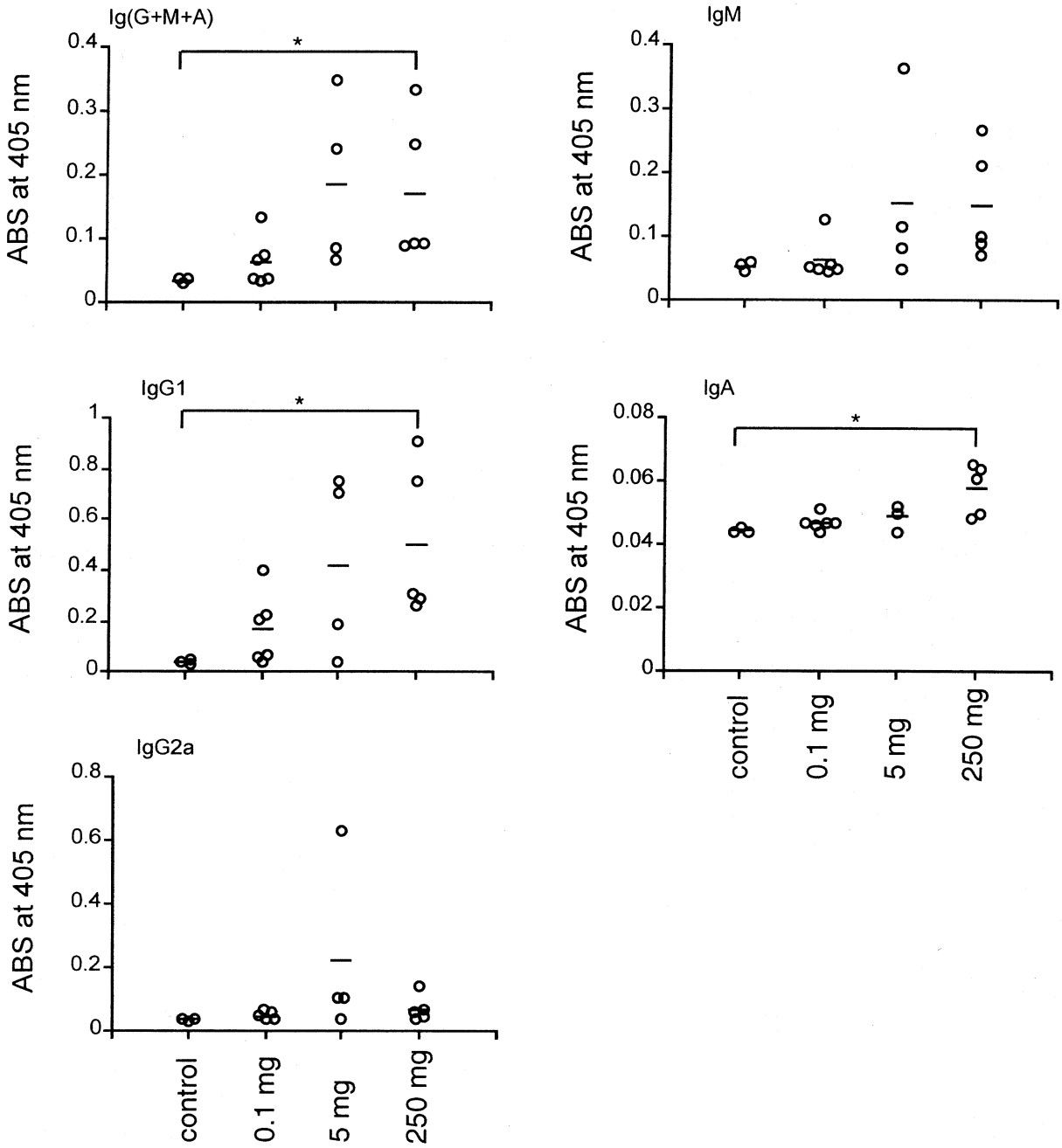

Figure 2. Antigen-specific antibody production enhanced by oral administration of OVA. TCR tg mice were orally administered OVA as indicated in Figure 1, and, one week later, immunized intraperitoneally with 0.1 mg of OVA plus CFA. Ten days after the immunization, sera were prepared from tail blood. Titers of OVA-specific antibodies were determined by ELISA. The means are shown with bars. The results are representative of mainly four, at least two, similar experiments. $*$: $p<0.05$ vs. the control group administered PBS. ABS, absorbance.

nitrophenylphosphate, the coloring reaction was monitored by determining the absorbance at 405 nm.

Delayed-type hypersensitivity (DTH) test

Alum was prepared by the standard protocol and OVA was conjugated with alum in saline. Seven days after oral administration of the antigen, the mice were administered 25 μl of alum-conjugated OVA (0.5 mg of OVA, 2.5 mg of alum ml^{-1} saline) and alum alone by injection into the left and right hind footpads, respectively. Footpad thickness was measured before and 24 h after the injection using a dial thickness gauge (Ozaki MFG, Tokyo, Japan). Footpad swelling was expressed as (change in left footpad thickness) – (change in right footpad thickness).

Statistical analysis

Antibody titers were statistically compared using the rank sum Mann-Whitney U-test, and cytokine secretion and proliferative responses in the antigen-treated and control groups were compared using Student's *t*-test.

Results

Oral feeding of higher doses of antigen upregulated the secretion of Th2-type cytokines (IL-4 and IL-5 secretion)

We investigated the effect of the dosage of orally administered antigen on the systemic T cell cytokine responses using the TCR tg mice. The TCR tg mice were orally administered various doses of OVA and the pattern of cytokine secretion by splenocytes from these mice was examined by ELISA. The responses are shown in Figure 1. Splenocytes from mice in the control group secreted IL-2, IFN-γ, IL-4 and IL-5. Antigen feeding resulted in downregulation of IFN-γ secretion in a dose-dependent manner ($p<0.05$ compared to the control group for the groups receiving 0.1 mg and 5 mg, and $p<0.001$ for the group receiving 250 mg). IL-2 secretion was also downregulated in the high dose (250 mg) group ($p<0.001$). In contrast, IL-4 secretion was enhanced in a dose-dependent manner ($p<0.05$ compared to the control group for the 5 mg group, and $p<0.005$ for the 250 mg group). Both IL-4 and IL-5 secretion were augmented in the 250 mg group ($p<0.005$). Four-time repeated feedings resulted in similar results (data not shown). These results indicated that feeding higher doses of antigen favors the induction of a Th2-type cytokine response in splenocytes.

Oral feeding of antigen upregulated antigen-specific antibody production after immunization

We further investigated the effect of the dosage of orally administered antigen on *in vivo* antibody production induced by immunization. Almost no OVA-specific antibodies were detected in the sera of mice fed the antigen but not immunized under these particular experimental conditions (data not shown). Mice fed the antigen were immunized with OVA emulsified in CFA. Ten days after immunization, sera were prepared and the OVA-specific antibody titer was determined by ELISA (Figure 2). The results indicated that OVA-specific Ig(G+M+A) antibodies were produced in mice fed any of the doses of OVA. Mice fed 5 or 250 mg of OVA had a high serum titer of anti-OVA IgG1 ($p<0.05$ for the 250 mg group vs. the control) and IgM. On the other hand, specific IgG2a was not produced in any of the mice tested, except for the 5 mg group including a mouse showing a high titer. Enhanced production of serum IgA specific for OVA was slightly but significantly ($p<0.05$) observed in 250 mg-fed mice. These results demonstrated that oral administration of soluble antigen enhanced IgG1 and IgA antibody production *in vivo*, in a dose-dependent manner.

Effect of orally administered antigen on the proliferative response of splenocytes and the DTH response

We investigated the effect of the dosage of orally administered antigen on other systemic T cell responses. Firstly, the proliferative response was examined (Figure 3). TCR tg mice were orally administered various doses (0.1, 5, and 250 mg) of antigen, and the time-course of the proliferative response of splenocytes was examined (Figure 3A). The splenocytes from mice in the control group administered PBS showed an intensive proliferative response in the presence of 1000 μg ml^{-1} OVA. The extent of the proliferative response of splenocytes from all mice fed the antigen, regardless of the dosage, was the same at any time point tested except for the 96-h culture in the case of splenocytes from the mice fed 5 mg of OVA. Although splenocytes from mice fed 250 mg of OVA showed a slight increase in proliferation when cultured *in vitro* in the

presence of 10 μg ml^{-1} OVA, the extent of proliferation was similar at different concentrations of antigen for all splenocytes (Figure 3B). These results indicated that oral administration of a single dose of 0.1, 5, or 250 mg did not significantly affect the proliferative response.

Next, we examined the DTH response, which is a typical means of assessment of Th1-type associated responses *in vivo*. The mice were orally administered various doses of the antigen and, 7 days later, injected into the left hind footpads with alum-precipitated OVA. The right footpad received alum alone. The footpad thickness was measured 24 h later, and antigen-specific swelling was observed as the difference in thickness comparing the left and right footpads (Figure 4). The results showed that some mice fed the intermediate dose (5 mg) showed an enhanced DTH response, although this was not statistically significant. No difference in DTH response was observed comparing mice fed low or high doses of the antigen.

Discussion

In the present study, we fed OVA-specific TCR tg mice various doses of an antigen (OVA) and examined the effect of the dosage of antigen on the cytokine and antibody responses. Cytokine responses of antigen-specific T cells to *in vitro* antigenic stimuli were examined by measuring the cytokine levels in the supernatants of cultures of splenocytes obtained from mice orally administered the antigen without further immunization. The *in vivo* antibody production response was tested using serum from mice fed the antigen followed by parenteral immunization with the antigen plus CFA. Our results demonstrated that oral administration of a high dose (250 mg) of the antigen significantly upregulated the serum IgG1 and IgA antibody responses to antigen injected later intraperitoneally and this was correlated with significant upregulation of IL-4 and IL-5 secretion by splenocytes. This enhancement of secretion of Th2-type cytokines and the antibody titers were dose-dependent in the range of 0 to 250 mg of OVA fed. Normal mice have antigen-specific T cells present at a low frequency, whereas TCR tg mice have antigen-specific T cells present at high frequency, and the changes in T cells are easily detected. These mice enabled us to detect the upregulated secretion of IL-4 and IL-5 in response to higher oral doses of OVA.

It has been assumed that when an antigen is administered by a route involving its passage through the mucosal immune system, a Th2-type response is favored as the systemic immune response (Weiner et al., 1994). Our results are consistent with this assumption. However, previous studies have focused on inhibitory functions of Th2-type cells on Th1-type responses (Chen et al., 1994, 1996), but not on helper functions of these cells. In our present study, a dose-dependent enhancement of the secretion of Th2-type cytokines by splenocytes was found to be correlated with enhancement of an antigen-specific serum antibody response. These results suggest that antigen feeding could prime T cells to develop into functional helper T cells secreting Th2 cytokines in a dose-dependent manner. We have also recently found that feeding these TCR tg mice a large amount of antigen as a constituent of the diet (approximately 200 mg/day) for a period of more than 2 weeks resulted in a strong Th2-type serum antibody response (Shida et al., 2000), supporting the above demonstration of the induction of T cells having a helper function for the Th2-type antibody response upon administration at high doses.

Orally administered antigens are internalized through the intestine, and immune responses may be mounted in some intestinal lymphoid tissues such as the Peyer's patches and the lamina propria (McGhee, 1991). It has been predicted that oral antigen may induce the production of antigen-specific IgA antibody in the intestinal mucosa, promoted by intestinal lymphocytes (McGhee et al., 1989). Our results demonstrate that orally administered antigen can elicit an IgA response not only in the mucosa but also in the serum. However, we do not have evidence as to whether IgA-producing B cells in the intestinal immune system or at systemic sites are sensitized under these conditions. Mucosal and systemic IgA responses to orally administered antigens should be further studied.

It has been shown that Th2-type responses are dominantly induced in the intestinal lymphoid tissues (Chen et al., 1994). Our results indicated that oral antigen can enhance Th2-type cytokine responses. Although not statistically significant, our results showed that the orally administered antigen at the intermediate dose (5 mg) induced IgG2a production after immunization. We also found that some animals given the dose of 5 mg displayed enhanced DTH. Although oral administration favors Th2-type responses, as mentioned above, Th1-type responses can be induced. We

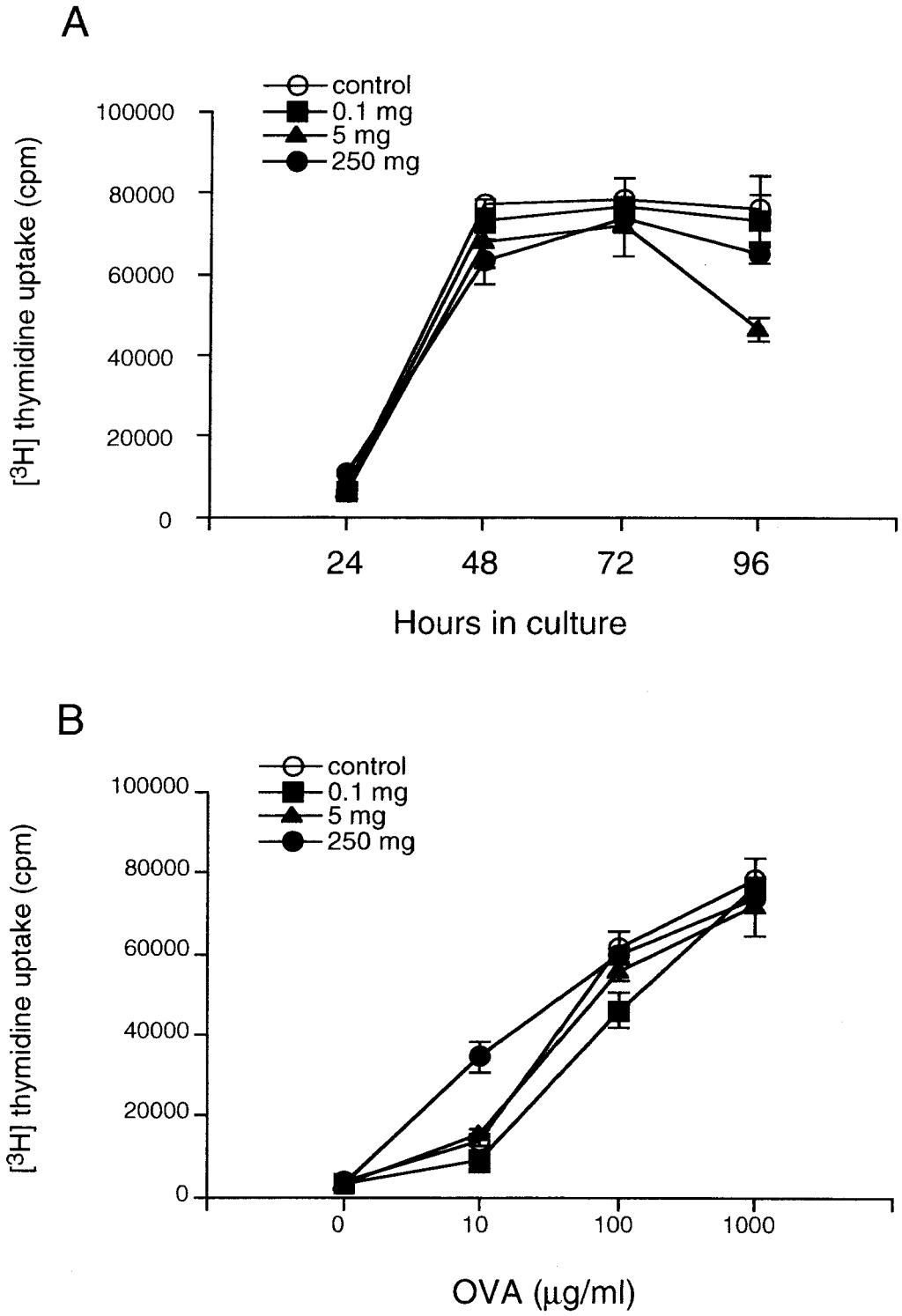

Figure 3. Proliferative responses of splenocytes from OVA-fed TCR tg mice. TCR tg mice were orally administered OVA as indicated in Figure 1 and splenocytes were prepared and cultured with OVA for assay of the proliferation response. A: the proliferative response in the presence of 1000 μg ml^{-1} OVA. B: the proliferative response of splenocytes after 72 h of culture in the presence of various amounts of OVA. The proliferative response was determined by measuring the incorporation of [^{3}H]-thymidine by means of a liquid scintillation counter. Data are expressed as the mean values $\pm$ SD of triplicate cultures. The results are representative of two independent experiments. The levels of the responses in the medium without OVA were all under 5000 cpm.

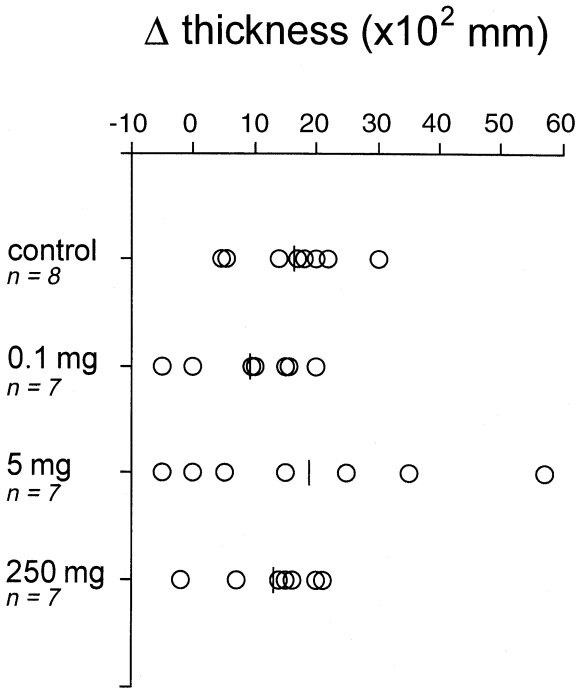

Figure 4. DTH responses of OVA-fed TCR tg mice. Administration was performed as in Figure 1, and, one week after administration, the mice were injected at hind footpads with 12.5 μg OVA plus alum. The footpad thickness was measured as described in the Section Materials and Methods. The data shown are representative of at least two independent experiments.

have previously shown that low doses of orally administered αs1-casein, a major milk allergen, provoke IFN-γ secretion by splenocytes and IgG2a production in C3H/He mice (Yoshida et al., 1997). Some other investigators have also reported that an orally administered antigen induced IFN-γ secretion (Hoyne and Thomas, 1995; Jain et al., 1996; Sun et al., 1999), although in these reports the dose of antigen fed was not consistent. Although our results could not demonstrate the upregulation of IFN-γ, it is possible that small amounts of IFN-γ secreting cells with effector functions were induced in mice fed the intermediate (5 mg) dose, resulting in Th1-type responses.

It has been shown that the differentiation of naive CD4$^+$ T cells to Th1- or Th2-type cells depends on the amount of antigen present at the time of sensitization (Constant et al., 1995; Hosken et al., 1995). The affinity of antigenic peptides to MHC molecules (Chaturvedi et al., 1996; Kumar et al., 1995; Pfeiffer et al., 1995), and probably the affinity of antigen/MHC complexes to TCR, is a definitive factor for differentiation. However, the effect of the dosage of antigen is not consistent in different experimental systems (Bancroft et al., 1994; Bretscher et al., 1992; Constant et al., 1995; Guéry et al., 1996; Hosken et al., 1995; Parish and Liew, 1972; Sarzotti et al., 1996; Wang et al., 1996). *In vitro* experiments using splenic T cells from the same TCR tg mice as those used in the present study showed that low concentrations of antigen tend to induce Th2-type cytokine responses, whereas high doses tend to induce Th1-type cytokine responses (W. Ise, M. Totsuka et al., unpublished observations). However, our results in the present study indicated that higher doses favored induction of the development of Th2 cells and a Th2-type response *in vivo*. These discrepancies may be due to differences between the *in vitro* and *in vivo* systems. For example, it may not be possible to achieve the high concentration of antigen attained in *in vitro* systems upon *in vivo* administration. *In vivo* administration of high doses of antigen may result in only a low concentration of antigen in the microenvironment concerned.

It has been documented that oral administration of antigen also leads to antigen-specific unresponsiveness, known as oral tolerance. Our results showed the upregulation of *in vivo* antibody responses, and oral administration of antigen failed to induce profound tolerance in our OVA-specific TCR tg mice. These results are profoundly different from the data obtained with conventional mice. It has been reported that an orally administered antigen could easily tolerize normal mice, whereas it was difficult to induce tolerance in TCR tg mice (Kearney et al., 1994; Lanoue et al., 1997) because of a large population of transgenic TCR-carrying T cells specific for the same antigen in these mice. A massive amount of antigen may be needed to tolerize a large number of antigen-specific T cells in these mice, whereas activation is manifested by priming only a small percentage of these specific T cells. Splenocytes from the mice fed 250 mg of OVA showed a greater proliferative response than that in the other groups in response to low (10 μg ml^{-1}) concentration of antigen (Figure 3B). A high dose (250 mg) may have primed CD4$^+$ T cells resulting in a lowering of the threshold of sensitivity for triggering proliferation.

In this study we clearly showed that feeding different doses of antigen induces different responses as shown by the cytokine secretion and antibody isotype profiles using TCR tg mice. Antigen feeding enhanced Th2-type responses in a dose-dependent manner. Recently, various food components and environmental substances have been shown to have effects on im-

mune responses. This system should be useful in analysis of the effects of those molecules on immune responses induced by oral administration of antigen, and should be useful in screening substances which can modulate food allergy.

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