

Suppression of Specific and Bystander IgE Responses in a Mouse Model of Oral Sensitization to β -Lactoglobulin

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Key Words

Oral tolerance · Allergy · Th2 · Cholera toxin

Abstract

Background: Mechanisms of systemic IgE suppression by oral tolerance have been extensively studied, but less is known about oral tolerance induction in mice challenged at mucosal sites. We have previously shown in systemically challenged mice that high-dose tolerance suppressed specific but not bystander IgE. In an attempt to mimic oral tolerance in food-allergic patients, we have investigated how IgE suppression could be induced in mice sensitized orally against β -lactoglobulin (BLG).

Methods: Mice were immunized orally against BLG using cholera toxin as adjuvant. Before oral sensitization, mice were administered milk whey proteins, either in the form of a single high-dose gavage, or by prolonged ad libitum administration of various doses. **Results:** Orally sensitized mice mounted a BLG-specific IgE response. In contrast to systemically challenged mice, a single high-dose gavage of whey protein given prior to the onset of oral sensitization resulted in the suppression of both specific and bystander IgE. When mice were fed moderate to low doses of milk whey proteins daily ad libitum in the drinking water during 3 weeks prior to oral sensitization, all doses effectively suppressed antigen-specific IgE.

However, bystander IgE suppression was observed only at the lowest doses. When mice were tolerized during 4 days instead of 3 weeks, IgE titers remained unchanged.

Conclusions: In orally sensitized mice, bystander IgE suppression depended on the dose of tolerogen, but also on its mode of administration. Mucosally induced IgE responses were suppressed by a mechanism that was distinct from that operating in the periphery.

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Introduction

Oral tolerance is a form of peripheral tolerance in which mature lymphocytes in the lymphoid tissues are rendered unresponsive to a specific antigen by prior oral administration of that antigen. This phenomenon has been demonstrated in many species, including mouse, rat and human [1]. The discovery that oral administration of antigen effectively prevents the onset of inflammatory pathologies such as experimental autoimmune encephalomyelitis or arthritis in animal models [2, 3] has led to the hypothesis that oral tolerance could be used as immunotherapeutic intervention against immunopathological situations in humans, including food allergy.

Cow's milk (CM) allergy is a common adverse food reaction, as it occurs in 2–3% of children under 2 years of

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age [4] and in 1% of the adult population [5]. The most sensitizing proteins are β -lactoglobulin (BLG), caseins, α -lactalbumin and serum albumin that are all recognized by serum IgE in a majority of patients [6]. Although CM allergens are well defined, the mechanisms involved in the breakdown of tolerance to these allergens are poorly understood. This is in great part due to the lack of immunologically well-characterized animal models of oral sensitization. Using cholera toxin (CT) as a mucosal adjuvant, previous studies in mice have reported that, besides IgA, several antigen-specific Th2 functions were clearly induced at the systemic and mucosal levels [7–9]. It has been shown that intestinal IgA could be effectively suppressed by prior induction of oral tolerance in mice sensitized orally with CT [10, 11], but there has so far been no report on the suppression of IgE functions in this model.

We have previously shown that oral administration of milk whey protein hydrolysates strongly suppressed serum IgE levels in Sprague-Dawley rats challenged systemically with BLG and alum [12]. Similar experiments carried out in BALB/c mice receiving a single high-dose gavage of BLG as tolerizing treatment have shown that circulating IgE suppression was accompanied by a decrease in cellular-mediated functions as determined by DTH, splenocyte proliferation and Th1 cytokine (IL-2 and IFN- γ) production in response to BLG [13]. Importantly, in both models, it was observed that suppression of humoral and cellular immunity was specific to the tolerogen, but not to bystander antigens.

In the present study, we wanted to place the concept of oral tolerance to BLG in a context that was closer to CM allergy. To do this, we have studied IgE suppression in mice sensitized orally using CT as a mucosal adjuvant. We have determined that orally induced tolerance generated different types of suppressive mechanisms depending on whether the immune challenge was triggered in the periphery or in the intestinal mucosa. We also describe an optimal tolerization regimen that induces both specific and bystander suppression of IgE responses to BLG in orally sensitized mice, which could be relevant for food allergy prevention.

Materials and Methods

Mice and Experimental Design

Animal experimentations were performed in accordance with the regulations of the Veterinary Service of the Canton de Vaud, Switzerland. Female BALB/c mice (IFFA-Cr do, L'Abresle, France) were bred and raised on a CM-free diet. Experiments were initiated at 6 weeks of age, a time at which mice were either tolerized or immu-

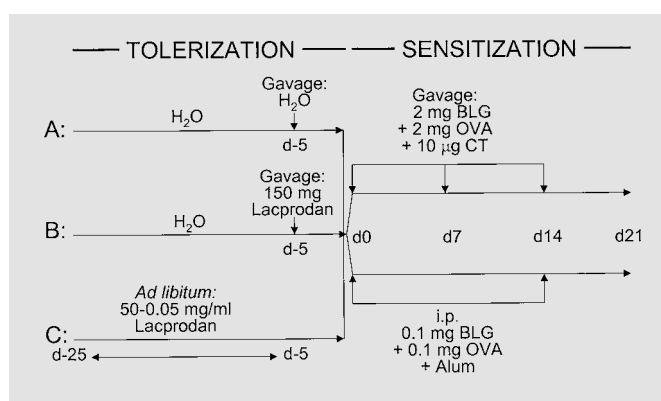


Fig. 1. Outline of the tolerizing and sensitizing regimens.

nized following the procedures described below and in figure 1. Oral tolerance was induced by feeding a milk whey preparation (Lacprodan 80, Danmark Protein AS, MD-Foods, Skanderborgvej, Denmark) produced by ultrafiltration of acid whey. The protein content of Lacprodan was 80%, of which approximately 80% was BLG. Lacprodan was given ad libitum in the drinking water during a period of 3 weeks between day –25 and day –5 (fig. 1; protocol C). The drinking water containing Lacprodan was changed daily to prevent bacterial contamination. Addition of Lacprodan to the water did not modify the daily water intake as both plain water control and test mice drank on average 3–4 ml per day. Alternatively, mice were maintained on plain water and were fed a single gavage of 150 mg Lacprodan in 0.3 ml water on day –5 (fig. 1; protocol B). Negative controls were given plain water throughout the tolerization procedure (fig. 1; protocol A). During 5 days following the ad libitum administration or the gavage of Lacprodan, a washout period with plain water was allowed before proceeding to immunization. Mice were immunized either systemically by intraperitoneal injection of 0.1 mg BLG (3 $\times$ crystallized, Sigma Chemicals, St. Louis, Mo.) and 0.1 mg ovalbumin (OVA, grade V, Sigma) emulsified in 2% alum (aluminum hydroxide, Superfos Biosector, Frederikssund, Denmark) on days 0 and 14, or orally by weekly gavages of specified amounts of BLG and OVA mixed with 10 μ g CT (List Biological, Campbell, Calif.). Mice were sacrificed 1 week after their last intraperitoneal immunization or 1 week after their last oral sensitization for the sampling of blood and mesenteric lymph nodes (MLN). Serum was separated from blood and stored at –20°C until assayed for Ig levels by ELISA and CD4+ T cells were isolated from MLN by MACS and restimulated in vitro (see below).

ELISA

BLG- and OVA-specific Ig isotypes were measured in sera by coating Maxisorp ELISA plates (Life Technologies, Basel, Switzerland) with 100 μ g/ml BLG or OVA, respectively, in PBS overnight at room temperature. After 4 washes in PBS containing 0.05% Tween 20, wells were blocked 1 h at room temperature with PBS containing 20% FCS and 0.05% Tween 20. After further washing, sera were added at a dilution of 1/10 for IgE and IgA measurements or at 1/100 for IgG, IgG1 and IgG2a measurements. The samples were serially diluted in PBS containing 20% FCS and 0.05% Tween 20 and were incubated in the presence of negative (normal mouse serum) and pos-

itive (hyperimmune serum from mice repeatedly immunized intraperitoneally) controls for 4 h at room temperature. After further washing, secondary biotinylated polyclonal anti-mouse IgA, IgG, IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, Ala.), or monoclonal anti-mouse IgE (Pharmingen, Mountain View, Calif.) were added at a concentration of 0.5–2 µg/ml and incubated for 1 h at room temperature. Wells were then washed and 1 µg/ml HRP-labeled streptavidin (KPL, Gaithersburg, Md.) was incubated for 30 min. Plates were washed one last time and incubated with TMB microwell substrate (KPL). Reactions were stopped with 1 M phosphoric acid. Optical densities were read at 450 nm and titers of specific Ig were expressed as maximum dilutions of test samples giving an OD value twice higher than the negative control (pool of sera from 10 unimmunized 8-week-old BALB/c mice). Because there was a background of circulating antigen-specific Ig in mice fed CT alone (data not shown), the data represent titers from which this background has been subtracted.

Cytokines were detected in culture supernatants by sandwich ELISA. Briefly, IFN-γ was detected using R4-6A2 and biotinylated XMG1.2 as coating and detecting mAbs, respectively. For IL-4 detection, 1D11 and biotinylated 24G2 were used (all monoclonal antibodies from Endogen, Woburn, Mass., coating at 2 µg/ml; conjugate at 0.5 µg/ml). Concentrations of cytokines were extrapolated from the standard curve calculated from dilutions of the recombinant cytokines (Pharmingen).

Restimulation of CD4+ T Cells in Vitro

CD4+ T cells were isolated from the MLN by positive selection using the MACS system and following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified CD4+ T cells were checked for homogeneity by flow cytometry using a FACScalibur (Becton Dickinson, San Jose, Calif.) and cultured at 2×10^5 cells/well in the presence of 10^6 irradiated splenocytes from naïve BALB/c mice in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 mM streptomycin (all reagents from Life Technologies) in 96-well round-bottom plates. Cultures were stimulated in the presence or absence of 1 mg/ml BLG or OVA for 3 days at 37°C and 5% CO₂ in an 80% humidity atmosphere. 150 µl supernatants were then collected for cytokine measurements by ELISA.

Statistical Analysis

In figures 2 and 4 showing immunoglobulin titers, histogram bars represent means of 5 mice per group of one representative experiment out of three. For cytokine data (fig. 3, 5), histogram bars represent means of three independent experiments in which supernatants from triplicate cultures were pooled. Error bars represent 2 times the standard error of the mean (SEM). Significant differences between means were ascertained by using the unpaired two-tailed Student's *t* test and considered significant if $p < 0.05$.

Results

Kinetic of Circulating BLG-Specific Immunoglobulin Production during Oral Sensitization

It was previously shown that serial gavages of antigens given at weekly intervals in the presence of CT induced

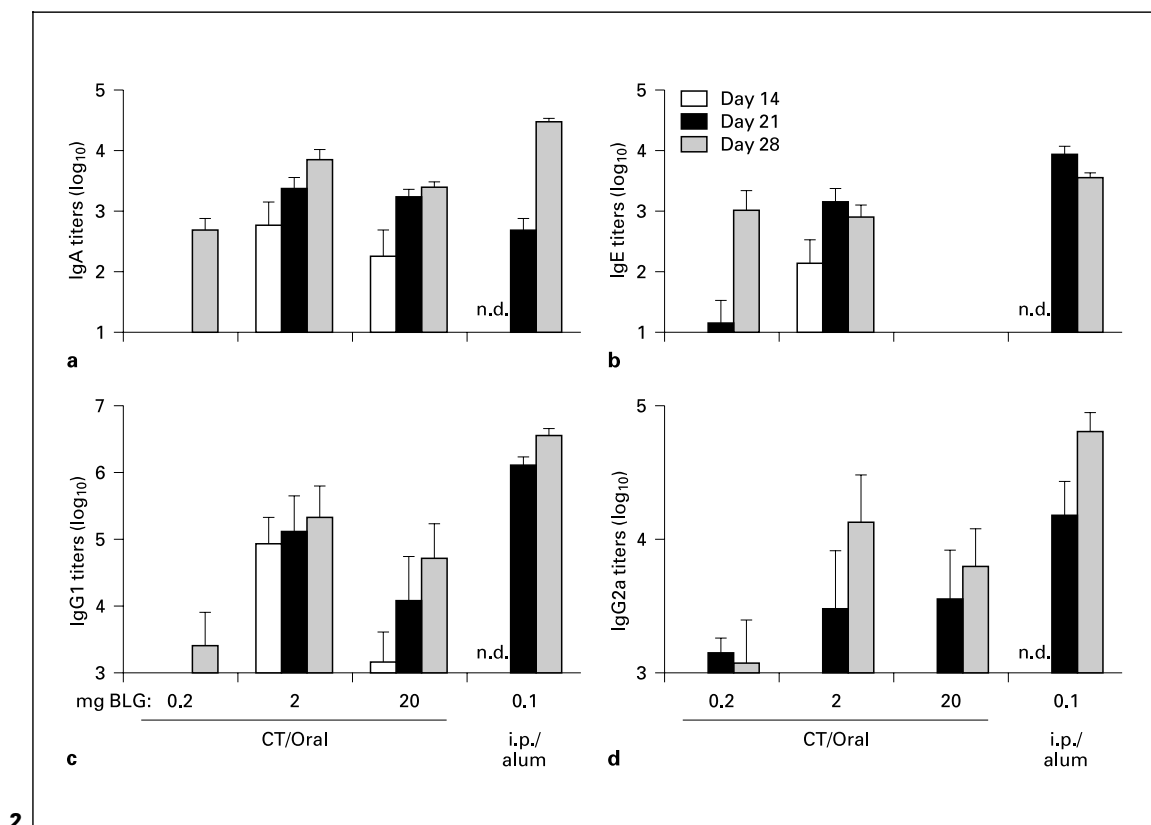
secretion of antigen-specific circulating IgA [14] and IgE [15] that could be detected in the serum. It is likely that the number of gavages and the dose of antigen needed to induce these responses varies depending on the mouse strains and on the antigen used. Here, we first wanted to confirm the experimental conditions for the optimal induction of an IgE response in BALB/c mice after weekly gavages of BLG plus 10 µg of CT. BALB/c mice were sensitized orally by giving four consecutive gavages of various doses of BLG plus CT on days 0, 7, 14 and 21. Circulating IgA was measured 1 week after each gavage, i.e. on days 7, 14, 21 and 28 (fig. 2a). In mice receiving gavages of 2 or 20 mg BLG plus CT, marked levels of BLG-specific IgA were induced above background (i.e. mice given CT alone) on days 14, 21 and 28. IgA levels were maximal 1 week after the fourth gavage of 2 mg BLG (day 28) and approached levels found in systemically challenged mice at that same time point (i.e. mice given two injections of 0.1 mg BLG i.p. in alum at days 0 and 14). Administration of 0.2 mg BLG plus CT induced detectable IgA above background only after 4 gavages (day 28). No or little BLG-specific IgA was detected above background after 1 gavage (i.e. day 7) at any of the BLG doses tested (data not shown).

BLG-specific IgE production was reproducibly highest 1 week after the third weekly gavage (day 21) of 2 mg BLG (fig. 2b). At this time and dose, there was a peak of IgE secretion which decreased thereafter despite continuation of a fourth gavage (day 28). Substantial IgE titers were also induced after 4 gavages of 0.2 mg BLG. No or little BLG-specific IgE was detected above background after 1 (data not shown) or 2 weekly gavages (day 14). Thus, although kinetics of IgE secretion differed from those of IgA in this system, the administration of 3 weekly gavages of 2 mg BLG plus CT was optimal for both IgA and IgE responses.

IgG1 and IgG2a isotypes were also monitored in these experiments. BLG-specific IgG1 were best induced after 4 gavages of 2 mg BLG plus CT, but these levels remained much lower than those observed in systemically immunized mice (fig. 2c). Although IgG2a levels remained low in all groups compared to IgG1, highest levels were also reached after four administrations of 2 mg BLG plus CT (fig. 2d).

Cytokine Production by CD4+ T Cells Isolated from the MLN of Orally Sensitized Mice

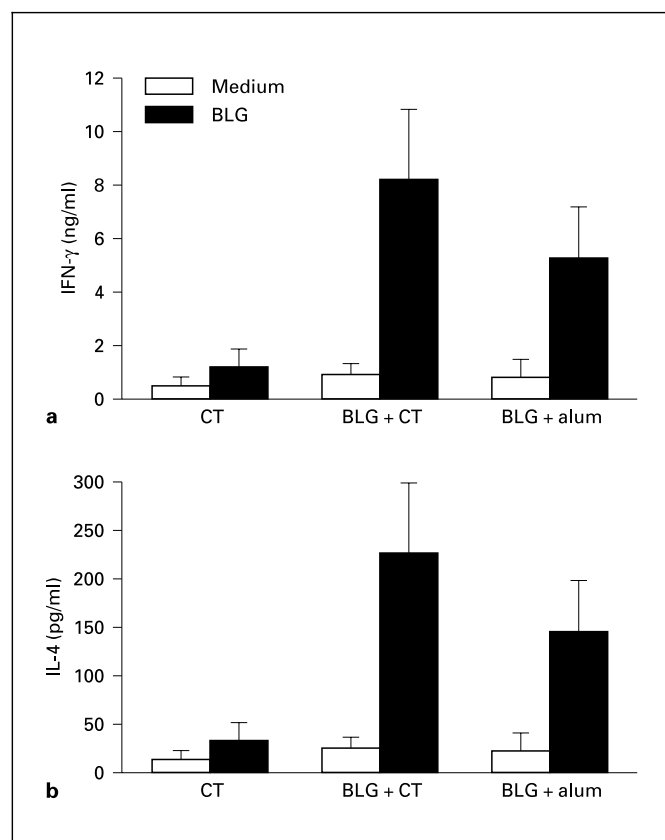
Since BALB/c mice given 3 gavages of 2 mg BLG plus CT at weekly intervals generated the strongest IgE response, this sensitization regimen was applied throughout



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Fig. 2. Kinetics of circulating BLG-specific immunoglobulin isotype titers in mice sensitized orally. Mice were sensitized orally by administration of four successive gavages containing various amounts (0.2, 2, 20 mg) of BLG plus 10 μ g CT or by two intraperitoneal injections of 0.1 mg BLG emulsified in alum. Gavages were given on days 0, 7, 14 and 21 and intraperitoneal injections were given on days 0 and 14. Mice were bled on day 14, on day 21 and on day 28 for the measurement of BLG-specific IgA (**a**), IgE (**b**), IgG1 (**c**) and IgG2a (**d**). Histogram depicts mean titers per group ($n = 5$) that were detected above background. Error bars represent $2 \times \text{SEM}$. No Ig were detected above background after one sensitizing gavage (day 7, not shown). n.d. = Not determined.

Fig. 3. Cytokine production by CD4⁺ T cells from MLN of orally sensitized mice in response to BLG. MLN were taken 1 week after the third sensitizing gavage or 3 weeks after i.p. injection of 0.1 mg BLG plus alum. IFN- γ (**a**) and IL-4 (**b**) were measured in 3 days' recall culture supernatants containing 1 mg/ml BLG or medium alone. CD4⁺ T cells from 5 mice/group were pooled and put in triplicate cultures. Histogram represents means of 3 independent experiments where the triplicate cultures were pooled. Error bars represent $2 \times \text{SEM}$.



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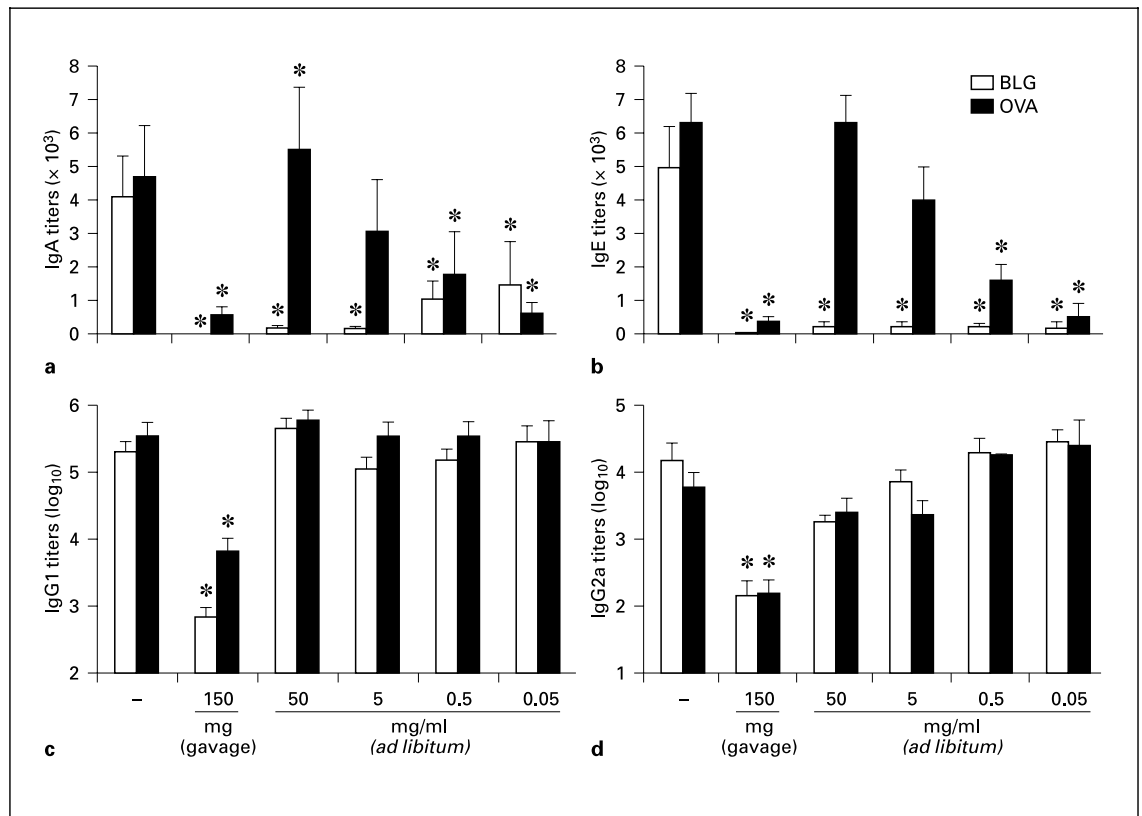


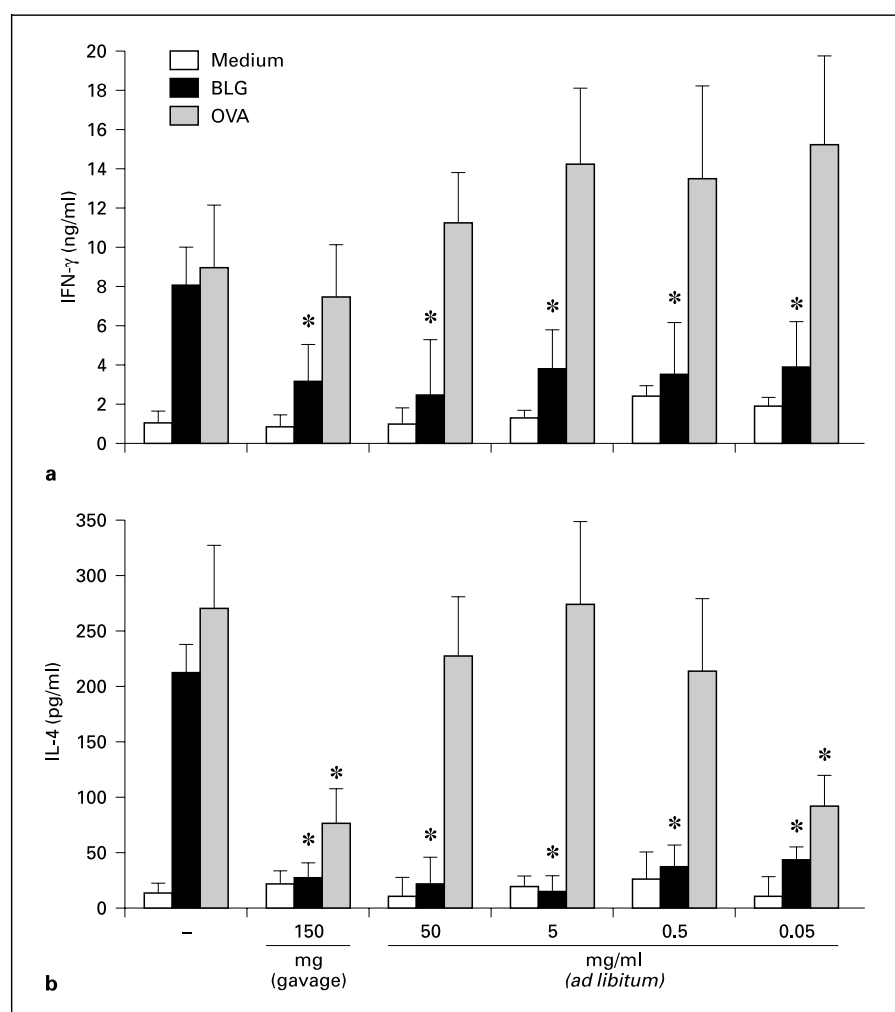
Fig. 4. Titers of circulating immunoglobulin isotypes in orally sensitized mice that received different tolerizing regimens. BLG-specific and OVA-specific isotypes were IgA (**a**), IgE (**b**), IgG1 (**c**) and IgG2a (**d**). All mice were sensitized orally by 3 gavages of 2 mg BLG and 2 mg OVA plus 10 μ g CT given at weekly intervals. Before oral sensitization, mice were given either plain water for 3 weeks (–), 1 gavage of Lacprodan (150 mg), or various doses of Lacprodan ad libitum in the drinking water for 3 weeks (50, 5, 0.5, 0.05 mg). Error bars represent $2 \times$ SEM from 5 mice per group. * $p < 0.05$, significantly suppressed vs. nontolerized controls.

the following experiments as depicted in figure 1. The T helper phenotype elicited in orally sensitized mice was determined. The pattern of cytokines produced by CD4⁺ T cells isolated from MLN was compared to that elicited in mice sensitized systemically in conditions known to favor Th2 responses in BALB/c mice [16]. CD4⁺ T cells were isolated 1 week after the third gavage or 21 days after one intraperitoneal immunization (given on day 0). MLN CD4⁺ T cells from orally sensitized mice produced both IFN- γ and IL-4 in response to BLG (fig. 3). This pattern was similar to mice injected with BLG plus alum, although the amounts were reproducibly lower than those found in orally sensitized mice. Cytokine production remained low in cultures of MLN CD4⁺ T cells from mice receiving CT alone (fig. 3). Hence, both Th1 and Th2 cytokines were produced by MLN CD4⁺ T cells from orally sensitized mice.

Circulating Immunoglobulin Titers in Tolerized Mice

Different tolerizing regimens were applied preventively before oral sensitization (fig. 1). For this purpose, we used a milk whey powder containing approximately 64% BLG (Lacprodan). Mice were either left untreated (fig. 1, protocol A), or were tolerized with one high-dose gavage of 150 mg Lacprodan (protocol B), or were fed daily ad libitum various doses (50–0.05 mg/ml) of Lacprodan in the drinking water (protocol C). After tolerization, mice were sensitized orally by administration of 3 gavages of 2 mg BLG and OVA plus CT given at weekly intervals. As shown in figure 4, mice given a single high dose gavage of Lacprodan (150 mg) 5 days before oral sensitization showed dramatically (more than 100-fold) suppressed levels of BLG-specific IgA (fig. 4a) and IgE (fig. 4b) in their serum. Bystander OVA-specific IgA and IgE were also suppressed by this treatment, although at a lesser degree

Fig. 5. Cytokine production by CD4⁺ T cells from MLN of orally sensitized mice that received different preventive tolerizing regimens as described in figure 4. MLN were taken 1 week after the third sensitizing gavage. IFN- γ (a) and IL-4 (b) were measured in 3 days' culture supernatants of CD4⁺ T cells restimulated with medium alone, 1 mg/ml BLG or 1 mg/ml OVA. CD4⁺ T cells from 5 mice/group were pooled and cultured in triplicate wells. Histogram indicates means values obtained from 3 independent experiments in which triplicate cultures were pooled. Error bars represent $2 \times$ SEM. * $p < 0.05$, significantly suppressed vs. nontolerized controls.



(approximately 10-fold). As shown previously [13], a single high-dose gavage of Lacprodan suppressed specific but not bystander IgE in mice immunized intraperitoneally (data not shown).

Mice given various doses (50–0.05 mg/ml) of Lacprodan ad libitum daily during 3 weeks before oral sensitization contained very low (approximately 10-fold reduced) BLG-specific IgA titers in their serum compared to controls (fig. 4a). This IgA suppression was partially lost, but still significantly reduced, at the lowest doses (0.5–0.05 mg/ml). In contrast, bystander suppression of OVA-specific IgA could only be observed at the lowest doses of Lacprodan (0.5–0.05 mg/ml). A similar pattern was observed for IgE (fig. 4b) where specific suppression was effective at all doses of Lacprodan, whereas bystander suppression was only observed when low doses were given. No significant changes in IgA and IgE titers were

observed in mice fed various doses (50–0.05 mg/ml) of Lacprodan ad libitum during 4 days before oral sensitization (data not shown).

We next wanted to assess whether the suppression of IgE was due to a switch towards a Th1 response or whether it was due to a general suppressive mechanism. To address this, we measured IgG1 and IgG2a titers in this experiment. Both BLG- and OVA-specific IgG1 and IgG2a titers were strongly decreased compared to controls after administration of a single high-dose gavage of Lacprodan (fig. 4c, d). However, IgG1 and IgG2a were not significantly affected in the groups receiving Lacprodan ad libitum. Thus, the suppression of IgE did not correlate with an increase in IgG2a in any of the groups, suggesting that immune deviation to a Th1 response was not involved in the process.

Cytokine Production by CD4+ T Cells Isolated from the MLN of Tolerized Mice

To address how the different tolerizing regimens would affect intestinal Th cell functions, we isolated CD4+ T cells from the MLN and restimulated them with BLG or OVA in the presence of syngeneic irradiated splenocytes. When isolated from mice receiving a single high-dose gavage of Lacprodan, CD4+ T cells were completely blocked for their secretion of IL-4 in response to BLG and partially impaired in their IL-4 response to OVA (fig. 5b). IFN- γ was only partially suppressed in BLG-specific cultures and was produced at normal levels in OVA-specific cultures from this group (fig. 5a). Thus, a high-dose gavage of Lacprodan given to orally sensitized mice induced both specific and bystander suppression of IL-4, but only partial specific and no bystander suppression of IFN- γ . As expected from our previous studies in mice immunized systemically [13], both IL-4 and IFN- γ were suppressed in BLG-specific cultures, but not in OVA-specific cultures (data not shown).

In mice receiving daily doses of Lacprodan in the drinking water, IL-4 was also strongly suppressed in cultures restimulated with BLG (fig. 5b). This was observed in all groups of mice receiving the various doses of Lacprodan. In contrast, a partial bystander suppression of IL-4 in cultures restimulated with OVA was only observed in the group of mice receiving the lowest dose of Lacprodan (0.05 mg/ml). IFN- γ in response to BLG was only partially decreased in all groups fed Lacprodan ad libitum and was normal or even increased (not significantly; $p > 0.05$) in cultures restimulated with OVA (fig. 5a). Altogether, these data show that IL-4 is strongly modulated in this system and that bystander IL-4 suppression follows at pattern similar to IgE, i.e. a suppression observed only at the lowest doses of tolerogen.

Discussion

This study was aimed at demonstrating that suppression of IgE by induction of oral tolerance to a CM protein, BLG, could be preventively induced in mice challenged orally against this antigen using CT as an adjuvant. It also evaluated the impact of different types of tolerizing regimens on specific and bystander suppression of orally induced IgE. We found that IgE unresponsiveness could be induced by preventive oral administration of milk whey proteins containing BLG, and that the specificity of this suppression depended greatly on the dose of tolerogen and its mode of administration. We also observed in this

model that Th2 parameters (IL-4 and IgE) were very sensitive to oral tolerance induction. This was especially the case when addressing bystander suppression, as certain regimens that effectively suppressed bystander IgE and IL-4 had no measurable impact on IFN- γ . Lastly, the IgE suppression was not associated with a corresponding increase in IgG2a, suggesting that the suppressive mechanism did not involve immune deviation towards a Th1 response.

A recent study made in C3H mice has shown that 6 serial gavages of CM proteins mixed with 10 μ g CT given at weekly intervals gave rise to increased serum IgE, anaphylaxis, mast cell degranulation, PCA reactions and histamine levels [15]. This highlighted the validity of CT as a mucosal adjuvant to induce intestinal hypersensitivity in the mouse and as a model of CM allergy. It should be noted, however, that oral immunization with 2 mg BLG plus CT also induced detectable levels of specific IgG2a and substantial IFN- γ secretion in MLN CD4+ T cells in this study. In addition, experiments using RT-PCR performed on Peyer's patches and on lamina propria from duodenum of orally sensitized mice have shown that not only IL-4 and IL-5, but also IFN- γ , were induced in vivo (unpubl. data). Thus, although Th2 parameters were clearly induced in this model, we cannot rule out the induction of discrete concomitant Th1 effector functions, as already described by others [17, 18].

One main finding of this paper is that suppression of orally induced IgE can be achieved by preventive oral administration of milk whey proteins. Our data thus demonstrate that, although CT has been shown to abrogate the induction of oral tolerance in a number of systems [14, 19–21], local and systemic tolerance to previously orally administered BLG was not reversed by CT. This is in line with previous studies where IgA was effectively suppressed by oral tolerance induction in mice sensitized orally with CT [10, 11] and extends the concept to IgE suppression. Altogether, these studies suggest that preventive induction of oral tolerance is very difficult to reverse once established, even when both tolerization and immunization are given via the oral route. We are currently determining the time limit until which oral tolerance can be broken in our IgE model.

In the periphery, mechanisms of oral tolerance to systemic challenges may vary greatly depending on the mode and dose of tolerogen administration [1]. It is generally believed that gavages of high doses of tolerogen preferentially result in anergy of reactive T cells, whereas successive gavages or continuous administration of low doses of tolerogen result in the generation of suppressor T cells

producing inhibitory cytokines such as TGF- β [1, 22, 23]. In this case, only low-dose tolerance should have suppressive bystander effects, while high-dose tolerance should remain specific to the tolerogen. This has major implications in the design of antigen- or T-cell-specific therapy for inflammatory autoimmune diseases, in which the autoantigen is unknown or where there are reactivities to multiple autoantigens in the target tissue. Because TGF- β has also been shown to inhibit Th2 differentiation [24], this notion can be applied to allergy prevention as well. Indeed, many food components, such as CM proteins, contain a large number of allergens. Hence, active suppression of one major CM allergen (i.e. BLG) should render the hosts tolerant to all bystander CM allergens. To our knowledge, no study has addressed induction of bystander suppression of IgE at the mucosal level using a model of oral sensitization.

We, therefore, addressed the effects of different doses of Lacprodan on bystander suppression of IgE in orally sensitized mice. Because high-dose gavages of tolerogen represent a tolerizing regimen that is somewhat difficult to envision for potential clinical application, we administered moderate to low doses of Lacprodan given daily ad libitum in the drinking water over prolonged periods of time. In mice fed continuously with Lacprodan, all doses significantly decreased BLG-specific IgE, but we observed an inverse correlation between the dose of Lacprodan and the suppression of bystander IgE. This correlated to some extent with the lower IL-4 levels found in cultures responding to OVA in this group, although the IL-4 decrease was not as dramatic as that of IgE. One explanation for this apparent discrepancy could be that other cytokines that are involved in IgE isotype switching as well (i.e. IL-13) may also be regulated in this system. Interestingly, it should be underlined that, in contrast to IgE, IgG1 was not decreased in any of the groups receiving Lacprodan ad libitum. This suggests that, although IgE and IgG1 class switching are both regulated by the same cytokines [25], their suppression by oral tolerance may involve distinct regulatory pathways, as already shown in previous studies carried out in systemically challenged mice [26]. Altogether, the data indicate that mice receiving the highest doses of Lacprodan ad libitum over a 3-week period prior to oral sensitization suppressed their IgE by a mechanism probably involving anergy or clonal deletion of reactive T cells, whereas mice fed with the lowest doses became unresponsive by an active mechanism probably involving regulatory cells.

To test the possibility that the suppression of IgE could correlate with a switch towards a Th1 response, we also

measured IgG2a levels in this experiment. We observed that this isotype remained unaffected in all groups of mice fed the various doses of Lacprodan ad libitum, but was significantly reduced in the group receiving a single high dose gavage. The same result was obtained for IgG1. This indicates that the suppression of IgE was not due to a switch to Th1 in any of the tolerizing conditions tested, but rather to suppression of reactive Th2 cells. It also illustrates that a single high-dose gavage of Lacprodan had a more profound suppressive effect, as all isotypes tested were decreased after this treatment in both a specific and bystander fashion. This implies that active suppressive mechanisms may mediate differential inhibition of distinct isotype class switching, depending on the mode of tolerogen administration.

One intriguing aspect was that the pattern of IgG2a secretion did not correlate with that of IFN- γ in recall cultures from tolerized mice. Indeed, IFN- γ levels were partially but significantly ($p < 0.05$) decreased in BLG-specific recall cultures from all groups, whereas BLG-specific IgG2a titers were unchanged. Conceivably, the partial suppression of IFN- γ was not sufficient to affect IgG2a isotype switching. Furthermore, it is possible that a cell population not present in the mesenteric lymph nodes could produce IFN- γ and compensate for the normal production of IgG2a in the periphery.

One interesting finding was that orally sensitized mice that were preventively tolerized with a single high-dose gavage of Lacprodan effectively suppressed both specific and bystander circulating IgE, suggesting the induction of an active suppressive mechanism. This contrasted with our previous studies in systemically challenged animals, where high-dose tolerance by gavage suppressed antigen-specific, but not bystander IgE [12, 13]. Furthermore, high-dose tolerance was reported to induce clonal deletion or anergy in other systems involving systemic challenge [1]. These opposed findings found in the two types of sensitization models reveal a striking difference between systemic and mucosal tolerance. Interestingly, previous experiments made by Grdic et al. [11] have highlighted a critical role for mucosal CD8⁺ T lymphocytes in the induction of local, but not systemic, suppression of IgA. Whether mucosally induced IgE is suppressed by the same mechanism is likely and deserves to be investigated.

Another feature worth noting was that mice fed high doses (50 mg/ml) of Lacprodan ad libitum (that did not show bystander IgE unresponsiveness) consumed on average 200 ± 50 mg milk whey protein daily. This dose was in the same range as that given in the mice receiving the single high-dose gavage (150 mg) that did generate an

effective bystander IgE suppression. Hence, in orally sensitized mice, similar doses of tolerogen may or may not give rise to bystander suppression, depending on whether the tolerogen is given by gavage or by prolonged ad libitum feeding. The fact that a given dose of tolerogen may give rise to different mechanisms of immune suppression depending on the mode of administration has important implications for the possible use of oral tolerance in the management of food allergy.

In conclusion, our data imply that an effective and realistic approach to induce IgE unresponsiveness to an oral challenge would be continuous administration of a

tolerogen given at a low concentration in order to induce specific and bystander modulation. We are currently testing the possibility of further decreasing this dose by encapsulating the tolerogen into synthetic microspheres [27].

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