

Prolonged oral treatment with low doses of allergen conjugated to cholera toxin B subunit suppresses immunoglobulin E antibody responses in sensitized mice

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Abstract

Background Oral tolerance is a long recognized method for inducing systemic immunological tolerance. However, large doses of antigen and frequent administrations are often required. By linking the antigen to the nontoxic mucosa-binding B subunit of cholera toxin (CTB), the required amount can be dramatically reduced. We have previously shown that mucosal administration of small amounts of antigens coupled to CTB can suppress peripheral Th1 cell-reactivity and associated inflammatory immunopathology in both naive and systemically-immunized animals. Induction of oral tolerance by repeated feeding of relatively small doses of antigen has, in some cases been shown to involve the generation of regulatory Th2-like CD4⁺ T cells, and hence could promote rather than suppress type I immunoglobulin (Ig) E-mediated allergic responses.

Objectives We examined whether oral prophylactic or therapeutic administration of a model allergen coupled to CTB would modulate allergen-specific IgE responses in high IgE responder Balb/c mice.

Methods Ovalbumin (OVA) was used as a model allergen. Mice were treated perorally with free or CTB-coupled OVA before or after systemic priming with alum-adsorbed OVA. Allergen-specific IgE levels in serum were measured with the passive cutaneous anaphylaxis test at various time-points.

Results Oral administration of a single low dose of CTB-linked OVA, prior to systemic sensitization and challenge with OVA, suppressed allergen-specific serum IgE antibody responses. Treatment with comparable doses of free OVA was much less effective. Most importantly, oral treatment with CTB-OVA conjugate could also suppress an already initiated IgE antibody response, but to achieve such a 'therapeutic effect', administration of multiple low doses of conjugate over a long time was required. Oral treatment with CTB-OVA conjugate could also effectively suppress antigen-specific Th1-mediated delayed-type hypersensitivity. Thus treatment with a CTB-conjugated model allergen can affect a broad range of T-cell-driven immune responses, even in antigen-experienced animals.

Conclusion These results may impact on the development of therapeutic vaccines against type I allergies.

Keywords: Cholera toxin B subunit, ovalbumin, oral tolerance, immunoglobulin E, allergy, immunotherapy

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Introduction

Oral tolerance is a long recognized method for inducing systemic immunological tolerance [1,2], and has been advocated as a strategy to prevent allergic reactions [3–5] and autoimmune diseases [6–11]. However, under certain conditions and especially with relatively low doses of antigen fed repeatedly, mucosal tolerance has been associated with increased Th2 responses together with and possibly resulting in decreased Th1 reactivity [12–14]. Since IgE responses are the archetype of Th2-driven immune responses, there could be a theoretical risk that this form of tolerance induction may in fact promote rather than suppress type I allergic reactions.

We and others have earlier shown that cholera toxin B subunit (CTB), the nontoxic receptor-binding moiety of cholera toxin (CT), can be used as carrier adjuvant molecule for the induction of mucosal SIgA immune responses to orally co-administered antigens [15–19]. We have later shown that recombinant CTB, free of CT, can also serve as an extremely efficient carrier immunomodulating system for inducing suppression of peripheral T-cell tolerance in naive as well as in antigen-sensitized animals [20–22]. Although the thrust of our experimental studies has dealt with the suppression of Th1-driven immune reactions, these observations underscore the exceptional plasticity of the mucosal immune system, which had already been elegantly illustrated by Challacombe and Tomasi [23].

Since IgE antibody responses, the archetype of Th2-driven responses, have not been investigated in the above context, the purpose of this study was to compare the effect of oral administration of small doses of a prototype allergen, ovalbumin (OVA), conjugated to CTB. The conjugate was administered orally before and after the onset of systemic sensitization with allergen. We now report that such treatment can readily prevent induction of OVA-specific systemic IgE responses, and if given in multiple low doses over a prolonged period, it can also suppress an ongoing allergic response. Thus, the immunomodulating properties of the B subunit linked to various antigens appear to affect a broad range of T-cell-driven immune responses.

Materials and methods

Animals

Male Balb/c or female C57BL/6 mice, 6–8-week-old at the start of experiments were maintained on an OVA-free diet. Male Sprague-Dawley rats, 12–14-week-old and mice were purchased from BK Universal (Stockholm, Sweden).

Antigen

Ovalbumin (OVA; grade VI for feeding and grade V otherwise) was purchased from Sigma (St Louis, MO, USA).

Preparation of CTB-OVA conjugate

Highly purified CTB was prepared from the culture supernatant of a mutant strain of *Vibrio cholerae*, which had first been deleted off the CT genes and then complemented with a recombinant multicopy overexpression plasmid encoding CTB [24,25]. OVA was conjugated to CTB with N-succinimidyl (3-(2-pyridyl)dithio)-propionate (SPDP, Pharmacia, Uppsala, Sweden) as the bifunctional reagent according to the manufacturer's instructions. Briefly, CTB and OVA were derived separately with SPDP at molar ratios of 1:4 and 1:1.5, respectively. OVA-SPDP was reduced and mixed with CTB-SPDP. The conjugate retained both GM1-binding activity, CTB and OVA serological reactivity, as judged by a solid phase ELISA using GM1 as capture system [26] and antibodies to CTB and OVA as detection reagent.

Oral tolerization protocol

Balb/c mice and C57BL/6 mice were used for tolerization against reagent IgE-mediated responses and delayed-type hypersensitivity (DTH) responses, respectively. Slightly sedated mice were given free or CTB-coupled OVA (CTB-OVA) dissolved in 300 µL (3%) Na₂HCO₃ intragastrically (orally) through a plastic baby feeding catheter.

Induction of systemic IgE antibody responses

Balb/c mice were sensitized and challenged intraperitoneally (i.p) with 10 µg OVA adsorbed on 2 mg Al(OH)₃ gel (alum, Superfos Biosector, Kvistgaard, Denmark) at time-points indicated in each figure and table. Sera were prepared from tail vein blood.

Induction and measurement of delayed-type hypersensitivity (DTH) reactions

Adult C57BL/6 mice were primed by subcutaneous (s.c.) injection at the base of the tail with 100 µg OVA emulsified in Freund's complete adjuvant (FCA, Difco, Detroit, USA). Twelve days later, animals were challenged by injecting one footpad with 20 µg OVA in pyrogen-free isotonic phosphate-buffered saline (PBS).

Thickness of rear footpad was measured with a dial gauge caliper (Oditest, Essen, Germany) before and 24 h after footpad challenge. The intensity of DTH reactions was determined for each individual animal by subtracting the

value obtained before challenge from those obtained 24 h after challenge. From these, values were subtracted and the mean nonspecific footpad swelling responses determined on a group of control mice primed with FCA only, 24 h after footpad challenged with OVA. The resulting value, referred to as specific DTH footpad thickness increment, was expressed in units of $\text{cm} \times 10^{-3}$.

Passive cutaneous anaphylaxis (PCA) titrations

The levels of circulating anti-OVA IgE antibody were assayed by PCA in Sprague-Dawley rats [27,28]. In brief, rats were anaesthetized by intraperitoneal (i.p.) injections of Hypnorm® (0.032 mg fetanyl citrate and 1 mg fluanisone per rat) and Stesolid Novum® (0.5 mg/rat). Test mouse sera were serially diluted twofold in PBS, and 50 μL of each dilution was injected intradermally into the shaved dorsal skin of rats. Seventy-two hours later the rats were challenged intravenously with 5 mg OVA diluted in 1 mL pyrogen-free PBS containing 1% Evans blue (Sigma, St Louis, MO). One hour later, the rats were sacrificed and the skin areas were examined for the appearance of blue reactions. The PCA titres were defined as the reciprocal of the highest dilution of serum giving a blue spot ≥ 5 mm in diameter.

A characteristic of the IgE antibody class is that heat treatment (56°C, 4 h) destroys its skin-sensitizing ability [28]. Heat treatment of randomly-selected sera from saline-fed OVA-sensitized and OVA-challenged animals virtually abrogated the PCA activity of corresponding sera, indicating that the PCA activity measured was mediated almost exclusively by IgE antibodies (data not shown).

ELISAs

Total concentrations of serum IgE were determined by a solid phase sandwich ELISA. Briefly, 96-well high-binding polystyrene plates (Greiner, Labtechnik, Frickenhausen, Germany) were coated over night at room temperature (RT) with a rat monoclonal antibody to mouse IgE (Pharmingen, San Diego, CA), diluted to 2 $\mu\text{g}/\text{mL}$ in PBS. Serially diluted mouse sera were incubated for 2 h at RT. After washing with PBS, biotinylated rat monoclonal anti-mouse IgE (Southern Biotechnology) antibody reagent was added and incubated for 1 h at RT. Finally, peroxidase-conjugated avidin (Sigma, St Louis, MO, USA) was added for 1 h at RT. The plates were washed and developed with H_2O_2 and orthophenylene diamine (OPD) (Sigma) and the absorbance at 450 nm was measured. The concentration of total IgE was calculated by extrapolation from a standard curve generated by assaying known amounts of a purified mouse IgE (Pharmingen).

For detection of specific serum IgG and IgG1 antibody levels to OVA, 96-well low-binding polystyrene plates

(Nunc, Roskilde, Denmark) were coated with OVA (1 $\mu\text{g}/\text{mL}$ in PBS over night at RT). Serially diluted mouse sera were incubated for 1 h and the plates were washed with PBS. Solid phase-bound antibodies were detected as above by stepwise incubation with peroxidase-labelled goat antibodies to mouse IgG or IgG1 (Southern Biotechnology) and finally developed with OPD and H_2O_2 and the absorbance at 450 nm was measured. The titre of a serum was defined as the reciprocal of the highest dilution giving an absorbance of 0.4 above background.

Statistics

Data were compared by nonparametric Mann-Whitney or Fisher's exact tests and two-tailed *P*-values < 0.05 were considered significant.

Results

Oral prophylaxis with CTB-OVA conjugate suppresses IgE and IgG antibody responses

Mice received different doses of free OVA or CTB-OVA by oral administration on days 14, 11 and 8 before they were sensitized by an i.p. injection with alum-adsorbed OVA (day 0). At 2 weeks after sensitization, the mice were challenged by a second i.p. injection with alum-adsorbed OVA. Sera were collected 7 days after challenge and reagent anti-OVA antibody titres were determined by PCA in recipient rats. As shown in Table 1, oral administration of as little as 3 $\times$ 5 μg OVA coupled to CTB reduced the PCA titre significantly compared to PBS-treated control animals, and three doses of 50 μg CTB-conjugated OVA suppressed by approximately 80% the mean PCA titres seen in control animals. Oral administration of comparable amounts of unconjugated OVA had no significant effects on specific PCA titre responses, and co-administration of free CTB together with 50 μg OVA also did not reduce the PCA titre significantly (Table 1). However, as expected [29], repeated oral administration of a much higher dose of free OVA, 3 $\times$ 5 mg, suppressed the PCA titres significantly.

Based on these findings we tested the effect of single vs repeated doses of CTB conjugates as compared to free OVA. The results show that one, two or three doses of 150 μg CTB-OVA reduced the PCA titres significantly, with as much as 90% after three CTB-OVA doses, compared to PBS-treated control animals (Table 2). Again free OVA was much less, if at all, effective (Table 2).

In an attempt to maximize the effect, we tested the efficacy of giving mice CTB-coupled or free OVA orally three times a week for 6 weeks, starting 2 weeks before sensitization and continuing for 1 week after challenge given on day 21 (Fig. 1). The results show that the six

Table 1. Oral prophylactic treatment with CTB-conjugated OVA suppresses systemically induced IgE antibody responses

Oral treatment		PCA titre GM ($\pm$ 1 SD)
CTB-OVA	3 $\times$ 5 μ g	162 (32–818)*
	3 $\times$ 50 μ g	81 (31–208)*
CTB + OVA	3 $\times$ 50 μ g	446 (209–952)
	3 $\times$ 5 μ g	295 (78–1114)
OVA	3 $\times$ 50 μ g	447 (181–1101)
	3 $\times$ 5 mg	65 (21–200)*
PBS		645 (366–1136)

Balb/c mice (six animals per group) were orally treated with PBS, free OVA, CTB-coupled OVA (CTB-OVA) or CTB admixed free OVA (CTB + OVA) 14, 11 and 8 days before the animals were sensitized and then another 2 weeks later also challenged with 10- μ g alum-adsorbed OVA given as i.p. injections. OVA-specific PCA titres were determined in sera collected on day 21. Data are expressed as the geometric mean (GM). PCA titres $\pm$ one SD for each group of mice. Doses indicated in the table refer to the amount of OVA administered. *Statistically significant differences with control PBS-treated animals.

consecutive oral doses of free OVA or CTB-OVA given during the 2-week presensitization period did not *per se* evoke any detectable PCA responses. Furthermore, sera collected 3 weeks after i.p. sensitization with OVA

Table 2. Suppression of specific IgE responses after single as compared to repeated oral treatment with CTB-conjugated allergen

Oral treatment		PCA titre GM ($\pm$ 1 SD)
CTB-OVA	1 $\times$ 150 μ g	138 (68–282)*
	2 $\times$ 150 μ g	129 (79–209)*
	3 $\times$ 150 μ g	74 (32–174)*
OVA	1 $\times$ 150 μ g	457 (135–1549)
	2 $\times$ 150 μ g	550 (331–912)
	3 $\times$ 150 μ g	282 (60–1318)
PBS		794 (380–1660)

Balb/c mice (six animals per group) were orally-treated with PBS, free OVA or CTB-coupled OVA (CTB-OVA) starting 2 weeks before sensitization, and repeated every third day for multiple feeding. Animals were sensitized and challenged with 10- μ g alum-adsorbed OVA given i.p. on day 0 and 14, respectively. OVA-specific PCA titres were determined in sera collected on day 21. Data are expressed as per Table 1. *Significant ($P < 0.05$) differences between groups of animals orally treated with CTB-OVA and groups of animals orally treated with the corresponding number of doses of free OVA.

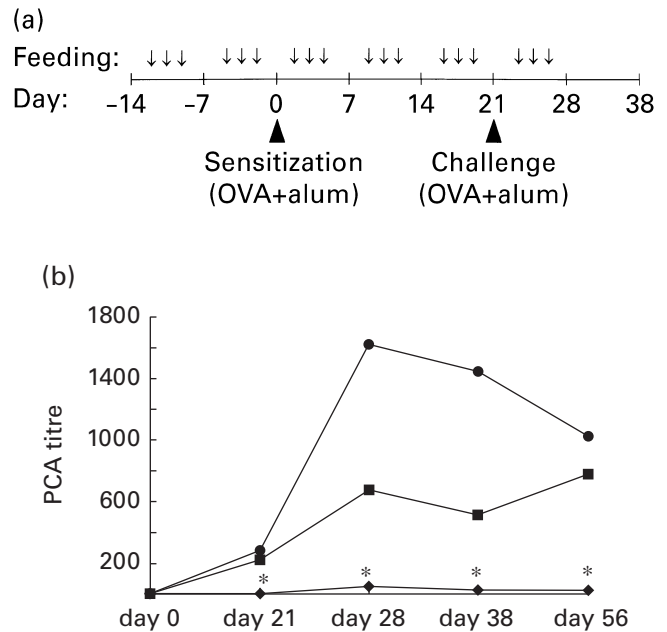


Fig. 1. Prevention of systemic IgE responses by oral administration of CTB-conjugated OVA. (a) Experimental design: groups of Balb/c mice (six animals per group) were orally treated with 50- μ g free or CTB-coupled OVA three times per week, each administration indicated with an arrow, starting 2 weeks before sensitization through an intraperitoneal (i.p.) injection of 10- μ g alum-adsorbed OVA on day 0. Treatment was continued for 3 weeks after sensitization and thereafter also for an additional week after i.p. challenge on day 21 with 10- μ g alum-adsorbed OVA. (b) Serum PCA titres in mice treated orally with free OVA (■), CTB coupled OVA (◆) or PBS (●). Sera were taken at different time-points. Results represent the geometric mean PCA titres determined at the indicated time-points. *Significant differences between the groups treated with CTB-OVA and free OVA, respectively.

showed significantly reduced PCA reactivity in the CTB-OVA-treated animals (mean PCA titre = 4) as compared to either the OVA-treated mice (mean PCA titre = 223, $P < 0.01$) or the PBS-treated controls (mean PCA titre = 287, $P < 0.01$). Indeed, sera which was also collected from CTB-OVA-fed animals after i.p. OVA challenge had significantly lower PCA activity than corresponding sera from mice fed with a comparable amount of free OVA even as late as 5 weeks after challenge, i.e. 4 weeks after the last oral treatment (Fig. 1). Also oral administration of free OVA reduced the PCA titres on day 28 and 38 as compared with the PBS-treated controls, although not at all to the same extent as the suppression achieved with the CTB-OVA conjugate.

Sera from CTB-OVA-treated animals taken on day 56 after systemic challenge with OVA also had significantly lower concentrations of total IgE compared to sera from

Oral treatment	Anti-OVA IgG titre (GM [$\pm$ 1 SD] $\times 10^{-2}$)	Anti-OVA IgG1 titre (GM [$\pm$ 1 SD] $\times 10^{-2}$)	Total IgE (ng/mL)
CTB-OVA	6.6 (1.7–26)*	11 (2.0–63)*	70 (20–290)*
OVA	56 (38–83)	110 (56–210)	340 (160–670)
PBS	44 (23–81)	93 (50–170)	370 (270–500)

Balb/c mice were orally treated with PBS, free or CTB coupled OVA (50 μ g/dose) according to Fig. 1. On day 56, sera were taken and OVA-specific IgG and IgG1 titres as well as total IgE antibody levels were determined by ELISA. *Significant *P*-values compared to both OVA- and PBS-treated animals.

OVA-treated or control animals (Table 3). In addition, OVA-specific serum IgG as well as IgG1 antibody titres were reduced in CTB-OVA-treated animals compared to OVA-treated or control-treated mice (Table 3). Serum IgG2a and IgA antibody responses to OVA were too low to result in measurable titres in any of the experimental groups using our predefined titre cut-off value. However, at the lowest dilution (1:3) tested, serum from CTB-OVA-treated group had an absorbance of 0.05 above background as compared to 0.09 and 0.10, respectively, for the OVA and PBS-treated groups (data not shown).

Oral treatment with CTB-OVA conjugate suppresses established allergen-specific IgE responses

As shown above, feeding CTB-OVA was very efficient for suppressing the development of OVA-specific IgE responses when the conjugate was administered starting before the i.p. sensitization. We therefore wished to examine whether oral treatment with CTB-OVA could also suppress an already ongoing systemic IgE response induced by i.p. sensitization with alum-adsorbed OVA. Preliminary studies had suggested that a single or a few oral doses would not be significantly efficient in reducing the PCA titre in presensitized animals (data not shown). Therefore, and taking support from the very efficient suppression achieved by the multiple-dose treatment regiment in the combined pre/postsensitization treatment experiments described above (Fig. 1), groups of mice that had been sensitized with OVA three weeks earlier were treated orally with five 50- μ g doses per week of free or CTB-conjugated OVA for 2 weeks and then challenged with alum-adsorbed OVA (Fig. 2). On day 21, i.e. immediately before the first oral treatment, all mice had developed an OVA-specific IgE response (mean PCA titre = 380). Systemic challenge with alum-adsorbed OVA raised the mean PCA titres dramatically further in mice treated with either free OVA or PBS. In contrast, the mean PCA titre in the CTB-OVA-treated animals only increased slightly (Fig. 2). Perhaps more important than the overall reduction of PCA titres in the

Table 3. Suppression of OVA-specific IgG and total IgE antibody levels after oral treatment with CTB-OVA

treated as compared to the PBS placebo-treated group may be the number of conjugate treated animals that show a significant treatment effect. We used a greater than twofold increase in serum PCA titre as an arbitrary but predefined cut-off level indicating a treatment 'failure'. By using this criterion, 60–88% of the CTB-OVA-treated group showed a

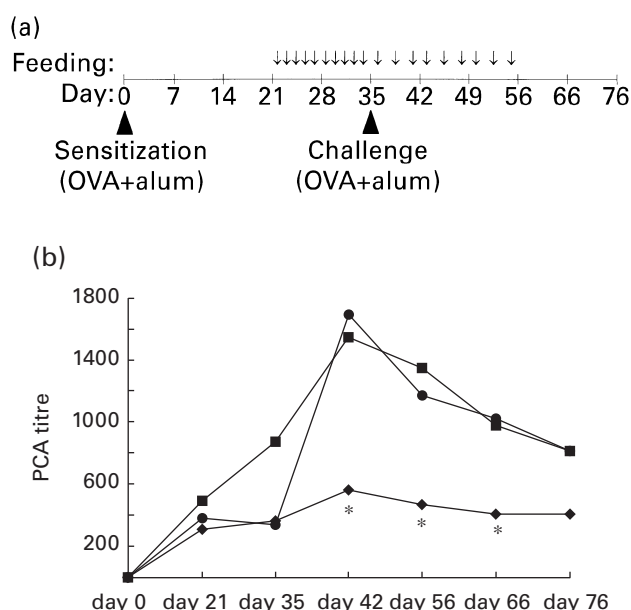


Fig. 2. Oral treatment with CTB-conjugated allergen can suppress continued IgE production in systemically sensitized mice: (a) Experimental design: groups of Balb/c mice (15 per group) were sensitized with 10- μ g alum-adsorbed OVA given i.p. on day 0. Three weeks after sensitization, groups of animals were orally treated with PBS, 50- μ g free OVA or 50- μ g CTB-coupled OVA 3–5 times per week (as indicated with arrows). (b) OVA-specific IgE antibody levels in serum as determined by PCA at various time-points after oral treatment with PBS (●), free OVA (■) or CTB-coupled OVA (◆). Results show the geometric mean PCA titres determined in serum from the different groups of mice at the indicated times: *Significant differences (*P* < 0.05) between the CTB-OVA and free OVA-treated groups.

Table 4. Efficacy of oral treatment with CTB-OVA or free OVA in suppressing further progression of the IgE response in sensitized mice

Oral treatment	Rates of treatment failures (% protective efficacy)		
	day 56	day 66	day 42
CTB-OVA	4/15 (60)*	1/15 (89)*	1/15 (88)*
OVA	8/15 (20)	9/15 (0)	6/15 (25)
PBS	10/15	9/15	8/15

Balb/c mice were treated according to Fig. 2. The table shows the number of mice per group that were not adequately protected after each treatment. Protection criterion: to have a PCA titre not exceeding more than twofold the corresponding PCA titre on the starting day of the oral treatment. *Significant difference between CTB-OVA as compared to the OVA-treated or PBS-treated control groups determined with Fisher's exact test. Protective efficacy: $100\% - 100\% \times (\text{numbers of failures in treatment groups} \div \text{number of failures in PBS group})$.

positive treatment effect as compared to 0–25% in the OVA-treated group from day 42 and forward (Table 4).

In addition, specific IgG anti-OVA antibodies on day 42 were significantly reduced in CTB-OVA-treated animals (GM titre = 160) as compared to PBS-treated animals (GM titre = 980), $P < 0.01$.

Oral administration of CTB-conjugated OVA suppresses DTH responses

In the above experiments we have shown that CTB-conjugated OVA can suppress Th2-driven IgE and IgG responses efficiently. Others [5,30] have suggested that Th2 responses can be suppressed as a result of the induction of a Th1 response against the specific antigen. To study whether this was the case in our system, we investigated the effect of oral administration of CTB-OVA on the development of DTH, which is a typical Th1-driven response. Oral administration of CTB-OVA, whether given before or after systemic priming was very efficient in reducing the DTH reaction (Fig. 3). In fact, as compared to the treatment of already sensitized animals, it required less frequent doses to reduce this Th1-mediated reaction than the Th2-mediated IgE responses described above (Fig. 2).

Discussion

We have previously shown that mucosal administration of minute quantities of CTB-conjugated antigens can suppress peripheral Th1-driven immune responses and

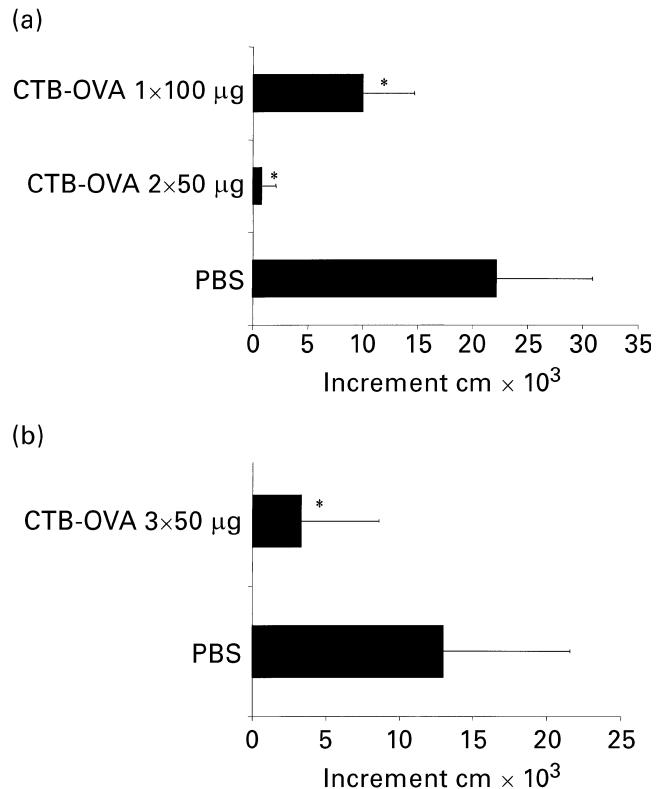


Fig. 3. Suppression of systemic DTH reactions by oral treatment with CTB-OVA conjugate. Groups of five C57BL/6 mice were fed CTB-OVA or PBS either 7 days (for single dose treatment) or 7 and 4 days (for the double-dose treatment) before (a) or 3, 5 and 7 days after (b) systemic priming with 100 µg OVA/FCA. Twelve days after priming, animals were challenged with 20 µg OVA injected into the footpad. Specific increments in footpad swelling after 24 h (average ± SD) were determined after subtracting the mean background footpad thickness determined on a group of unprimed but concurrently challenged control animals. *Significant differences between the CTB-OVA and PBS treated groups.

immunopathology in naive as well as in systemically immune animals [20–22]. The present study shows that feeding CTB-conjugated OVA before and after systemic immunization can also suppress specific reagenic IgE antibody responses, the archetype of Th2-driven immune responses in mice.

Prior feeding with microgram amounts of CTB-conjugated OVA could readily suppress systemic IgE responses whereas comparable amounts of unconjugated OVA were much less, if at all, effective. These findings confirm previous reports that mucosal administration of OVA linked to *Escherichia coli* heat-labile enterotoxin B subunit, a GM1 ganglioside-binding analogue of CTB, can suppress OVA-specific IgE responses in naive mice [31]. However, at variance with the latter report, the present study

demonstrates that suppression of IgE responses can also be achieved in already sensitized animals. However, different from the prophylactic effect, suppression of serum IgE responses after systemic sensitization required prolonged multiple-dose oral treatment with CTB-OVA.

Our findings are akin to previous reports indicating that humoral immune responses to OVA can be suppressed more easily in naive than in antigen-sensitized animals [32]. Both prophylactic and therapeutic oral immunotherapy may be an efficacious tool in eliminating or reducing the severity of allergic disorders. Treatment with glucocorticoids is widely used for immunosuppressive or anti-inflammatory control of allergic diseases in general, and allergic asthma in particular. However, this treatment is unspecific and is associated with severe side effects. In addition, administration of glucocorticoids has been reported to increase total IgE levels [33–35], which is correlated with airway hyper-responsiveness [36]. The alternative treatment, subcutaneous specific immunotherapy (SIT), was introduced over 90 years ago [37] and has been successfully used against allergic rhinitis, asthma, and anaphylactic reactions to insect stings [38,39]. Because of the inconvenience of closely spaced repeated injections, the cost, and the incidence of systemic adverse reactions [40,41] associated with subcutaneous immunotherapy, a number of investigators have attempted oral/sublingual [42–44] administration of allergens, with the hope not only for enhanced positive clinical effects, but also for increased safety and convenience. The suitability of using mucosal tolerization for allergic reactions may seem contradictory, since this route of tolerization has been reported to promote Th2 cytokines [12–14]. However, we and others [45,46] have seen that short-term mucosal administration of antigen can suppress Th1 reactivity without a concomitant increase of Th2 responses. The present study in fact indicates that prolonged feeding of a CTB-allergen conjugate does not appear to induce regulatory mechanisms similar to those described after administration of free antigens. It is, however, also clear from this study that Th2 responses appear more difficult to suppress than corresponding Th1 responses after oral treatment with CTB-conjugated antigen. Thus, especially when given to sensitized animals, it required many repeated oral doses to suppress the IgE antibody responses, three doses were sufficient to almost completely suppress DTH to OVA. This was consistent with our previous findings in the other systems where a single or a few oral or nasal administrations of various CTB-antigen conjugates efficiently suppressed DTH and autoimmune reactions in sensitized animals [20–22,47].

Repeated oral administration of free protein antigens have been shown to induce anergy [32,48,49] and/or active suppression by cells producing IL-10 and TGF- β [12,13,50]. Either or both of these mechanisms may be

responsible for the tolerance induction seen in our model. Recent data in another study have shown that intranasal administration of CTB-OVA reduces IL-4 and IL-5 production in lung and spleen, without affecting IL-10 and IFN- γ production [51]. However, TGF- β was not measured in these experiments. At this point we cannot exclude other regulatory mechanisms that may down-regulate allergen-specific T cells in our system. In this respect, mucosal CD8 + T cells have been assigned a suppressive role in this form of mucosally-induced tolerance [52–54], although other investigators have argued against this mechanisms [55].

In addition to suppression of reagent allergen-specific IgE responses, our findings demonstrate that the repeated oral treatment with CTB-OVA conjugate also decreased total IgE levels and specific IgG1 antibody responses to OVA. The reduction in total serum IgE levels seen after tolerization with CTB-OVA conjugate may be explained by the fact that most or all of the reduced IgE levels were accounted for by specific IgE. In this regard increased levels of IgE have been shown to be associated with asthma in children [33,56] and in individuals with hay fever as much as 25–50% of the total IgE is made up by IgE specific to pollen. The decrease in OVA-specific IgG1 is usually explained by immune deviation from a Th2 to a more Th1-like response. However, this does not seem to be the case in our system since typical Th1-driven responses such as DTH responses, were also and even more readily suppressed after feeding with CTB-OVA conjugate. A shift towards a Th1 response would also be expected to result in increased IgG2a antibody titres, which we did not see; rather, OVA-specific IgG2a responses were quite low in all experimental groups, and if anything they were suppressed in the CTB-OVA treated group as compared to OVA alone or PBS-treated animals. It is believed that there is a differential degree of tolerization susceptibility in different cell populations and immunological events so that Th1 cells are easier to tolerize than Th2 cells, and IgE responses are more readily tolerized than IgG responses. The fact that with orally-administered CTB-OVA we can also tolerize IgG responses supports the notion that we achieve a 'higher degree' of tolerance than noted with previously reported mucosal tolerization regimens.

We conclude that it is possible to reduce allergen-specific and total IgE levels in naive and in sensitized mice by feeding microgram amounts of OVA allergen chemically coupled to CTB and that this effect appears to be associated with suppression or anergy of both Th1 and Th2 driven immune responses. These findings may impact on the development of allergen-based oral immunotherapy. However, in this context it is of great importance to examine to which extent the results described here in mice and using OVA as model allergen hold true also in humans and for other clinically allergens. Indeed, Wiedermann *et al.* [56]

have recently shown that it is more difficult to induce tolerance against the major birch pollen allergen, Bet v1, than against OVA. Work with further allergens are in progress in our laboratory to define the limitations in the applicability of the approach described in this study to use mucosal administration of CTB-conjugated allergens for specific immunotherapy of IgE-mediated allergies.

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