

Suppression of delayed-type hypersensitivity and IgE antibody responses to ovalbumin by intranasal administration of *Escherichia coli* heat-labile enterotoxin B subunit-conjugated ovalbumin

Shin-ichi Tamura*§, Emiko Hatori*, Takashi Tsuruhara†, Chikara Aizawa‡ and Takeshi Kurata*

Oral administration of a small amount of antigen conjugated cholera toxin B subunit is known to induce tolerance to the antigen. In the present experiments, whether nasal administration of allergen conjugated to Escherichia coli heat-labile toxin B subunit (LTB) induced tolerance was examined in BALB/c mice. A single administration of a small amount of LTB-coupled ovalbumin (OVA) suppressed the induction of delayed-type hypersensitivity and IgE antibody responses to OVA which was administered parenterally after nasal administration of LTB-coupled OVA. The antigen-specific suppression was abrogated by the addition of the holotoxin to LTB-coupled OVA. The suppression, induced by nasal administration with a small amount of allergen conjugated to a mucosa-binding molecule, may be applicable for preventing the development of allergy.
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It is well known that oral administration with dietary protein antigens results in the induction of a state of specific immunological unresponsiveness, “oral tolerance”^{1–3}. Inhalation of antigens also induces the state of specific immunological unresponsiveness, “nasal tolerance”⁴. Thus, antigen can induce systemic immunological tolerance, when administered orally or nasally, by mucosal route. The oral or nasal tolerance to some antigen is abrogated by the oral or nasal administration of antigen coupled to or together with cholera toxin/ cholera toxin B subunit (CT/CTB), containing a trace amount of CT and that results in the induction of IgA, IgG, and IgE antibody responses to the antigen^{5–8}. *Escherichia coli* heat-labile enterotoxin (LT), which has the same subunit structure and works in the same mechanism as CT^{9,10}, also abrogate the tolerance to the unrelated antigens¹¹. This strategy to induce local and systemic immune responses is useful for the development

of mucosal vaccine. In this regard, we have demonstrated that CTB (or LTB) containing a trace amount of the holotoxin administered intranasally (i.n.) together with the inactivated influenza vaccine enhanced IgA and IgG antibody responses to the vaccine^{12–15}. On the other hand, Sun *et al.* reported that a minute amount of soluble or particulate antigen coupled to CTB can markedly suppress systemic immune responses when administered orally⁵. They showed that either delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC) or human γ -globulin (HGG), or serum antibody (IgM and IgG) responses to SRBC were suppressed after feeding a single dose of the CTB-conjugated antigen. This finding seems to provide clinically useful means for preventing or abrogating untoward immune responses. This is because unless tolerogens are administered repeatedly in large doses, tolerance is usually modest and of short duration. It is rather difficult to induce in an already-immune host.

In the present experiments, we examined the effects of i.n. administration of ovalbumin (OVA) coupled to LTB on the development of DTH and IgE antibody responses to OVA administered parenterally to mice. The results showed that the previous nasal administration with a small amount of OVA coupled LTB suppressed the induction of both DTH and IgE antibody responses.

*Department of Pathology, National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162, Japan. †Department of Education, Tokyo Gakugei University, Tokyo, Japan. ‡Department of Technology, Kitasato Institute, Tokyo, Japan. §To whom correspondence should be addressed. (Received 15 March 1996; revised 3 June 1996; accepted 4 June 1996)

This finding suggests that allergens conjugated to LTB is effective for preventing the development of allergy.

MATERIALS AND METHODS

Mice

BALB/c female mice, 6–8 weeks old (Japan SLC Inc., Hamamatsu-shi, Japan) were used.

Antigens

Ovalbumin (OVA; Grade V) and human serum albumin (HSA) were purchased from Sigma (St Louis, Mo, USA).

LT and LTB

LT and LTB were prepared from *E. coli* strain having the plasmid constructed to produce LT or LTB, as described previously¹⁵. Briefly, a 65 kb enterotoxin plasmid (pMY1900) encoding LT type h(LTh) and heat-stable enterotoxin type Ia was obtained from enterotoxigenic *E. coli* strain 1032, isolated from an individual with traveller's diarrhea. The plasmid was partially digested and ligated to pBR322 and used to transform *E. coli* C600. The recombinant that was resistant to ampicillin and susceptible to tetracycline and that carried a 6.75 kb plasmid (1032H-19) was found to produce LT. The LT gene was amplified from 1032H-19 plasmid DNA by the polymerase chain reaction (PCR). The amplified gene was cloned into pTrc 99A plasmid (Pharmacia LKB), which was used for transformation of *E. coli* JM105 strain. *E. coli* having the LT gene containing pTrc 99A plasmid was cultured. Similarly, LTB gene was amplified from the whole LT gene cloned into 1032H-19 plasmid by PCR. The amplified gene was cloned into pTrc 99A plasmid, which was transferred to *E. coli* JM105 strain. *E. coli* having LTB gene-containing pTrc 99A plasmid was cultured. LT and LTB were purified according to the method used by Clements and Finkelstein⁹ with slight modification. Briefly, the bacteria were harvested and lysed with a French pressure cell. The crude lysate was then applied to columns of Biogel A-5m (batch No. 44191A, Bio-Rad, CA) and eluted with galactose. The purified LT and LTB contained 0.5 ng μg^{-1} and 3 ng mg^{-1} of LPS in the *Limulus* test, respectively.

Preparation of LTB- and LT-conjugated antigen

OVA was covalently conjugated to LTB/LT by using *N*-succinimidyl[3-(2-pyridyl)dithio]propionate (SPDP) as bifunctional coupling reagent¹⁶, essentially according to the supplier's instructions (Pharmacia). In brief, LTB/LT and OVA were separately derivatized with SPDP at molar ratios of 1:5 and 1:10, respectively. After reduction and purification by Sephadex G-25 chromatography, the SPDP-derivatized OVA was incubated with SPDP-derivatized LTB/LT (at ratios of 1:1, 3:1 and 9:1) for 16 h at 23 °C. The resulting LTB-OVA/LT-OVA conjugates were purified by gel filtration through Sephacryl S-300 and shown to contain GM1-binding capacity and to retain both LTB/LT and OVA and OVA serological reactivities by means of a solid-phase

ELISA using GM1 as capture system and enzyme-labeled antibodies to LTB, LT, and OVA as detection reagents.

Immunization

Mice were anesthetized by an intraperitoneal (i.p.) injection of amobarbital sodium (0.25 ml of a solution of 1 $\mu\text{g ml}^{-1}$). Then they were immunized i.n. by dropping 10 μl PBS containing a required dose of antigen.

Mice were also immunized systemically with 10 μg OVA included in 100 μg alum¹⁷ or 20 μg HSA included in 200 μg alum by injecting the antigen subcutaneously into the lumbar and nuchal regions of each mouse in a volume of 25 μl .

DTH assay

The DTH reaction was elicited with 15 μg OVA included in 30 μg alum or 20 μg HSA included in 50 μg alum by injecting the antigen in a 25 μl volume into the right hind footpad of each mouse. Increase of thickness of the footpad (footpad swelling) was determined 24 h later as a measure of the DTH reaction with a dial thickness gauge (Peacock, G, Ozaki Seisakusho, Tokyo) as described previously¹⁷.

IgE antibody assay

IgE antibodies were titrated by passive cutaneous anaphylaxis (PCA) reaction in Sprague Dawley rats (SLC Inc., Hamamatsu-shi, Japan)¹⁸. Twofold dilutions of an antiserum were injected intracutaneously for passive sensitization. Dilutions of serum samples to be compared were injected into the same rat. The PCA reaction was elicited with a 2-h sensitization period by an intravenous injection of 5 mg of OVA in 1 ml of 1% Evans blue.

IgE antibodies were also titrated by a fluorometric reverse (IgE-capture) ELISA with the method of Sakaguchi *et al.*¹⁹ Briefly, ELISA was performed sequentially from the solid phase (Dynatech, Immulon 4 plates, Dynatech, Chantilly, VA) with a ladder of reagents consisting of: anti-mouse IgE monoclonal antibody; PBS–1%BSA; twofold serial dilutions of serum; biotinylated OVA; β -D-galactosidase-conjugated streptavidin (GIBCO, BRL, Paisley, Scotland); and 4-methylumbelliferyl (MU)- β -D-galactoside (Sigma) in respective order. The enzyme reaction was stopped with 0.1 M glycine–NaOH (pH 10.3) and the fluorescence intensity was read on a microplate fluorescence reader. Antibody-positive cut-off values were set as twofold mean fluorescence units of preimmune sera. ELISA antibody titer was expressed as the highest serum dilution giving the positive reaction.

Pooled sera from each group of five mice were tested in duplicate and the titer was expressed as the geometric mean. Variations of the titer for the same sample were less than twofold.

IgG antibody assay

IgG antibodies were titrated by ELISA as described previously²⁰. The pooled sera from mice having received i.n. administration of LTB–OVA (10 g)+LT (1 μg) 28 days previously, OVA (10 μg) included in alum (100 μg) 21 days previously and footpad infection of OVA

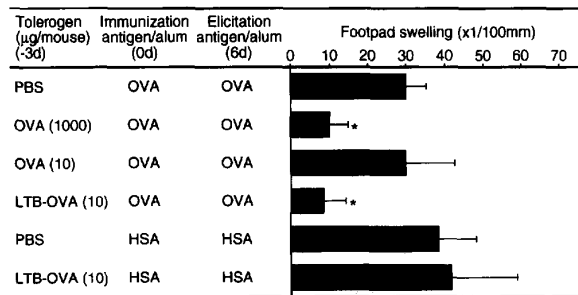


Figure 1 Suppression of systemic DTH reaction after i.n. administration of LTB-conjugated OVA. Groups of BALB/c mice (five per group) were given i.n. unconjugated or LTB-conjugated OVA. Seven days later, the mice were primed s.c. with alum-adsorbed OVA or HSA. Six days after priming, DTH reaction was elicited by injecting alum-adsorbed OVA into the footpad and was determined by the footpad thickness 24 h after elicitation. Each bar represents the mean footpad swelling $\pm$ S.D. of five mice. The asterisk (*) denotes statistically significant differences with the footpad swelling in mice given PBS i.n.

(15 μ g) included in alum (30 μ g) 15 days previously were used as a 160 unit standard for anti-OVA IgG.

Statistics

DTH results were expressed as the arithmetic mean $\pm$ S.D. Probability (*P*) values were calculated by Student's *t*-test; *P* values <0.05 were considered significant.

RESULTS

Suppression of DTH to OVA by i.n. administration of LTB-conjugated OVA (LTB-OVA)

Effects of i.n. administration of free OVA or LTB-OVA on the induction of the systemic DTH responses were examined. Mice were administered i.n. OVA, LTB-OVA or saline 7 days before systemic immunization with alum-adsorbed OVA. Six days after systemic immunization, DTH responses were elicited by challenging with alum-adsorbed OVA into the footpad. *Figure 1* shows that in mice given 1 mg OVA, DTH response was suppressed significantly, compared with that of mice given saline.

Comparable significant DTH suppression was obtained in mice given 10 μ g LTB-OVA, although mice given 10 μ g OVA i.n. had no appreciable effect on subsequent DTH reactivity. Moreover, mice given LTB-OVA did not suppress the development of DTH response to HSA. These results demonstrate that systemic DTH responses against OVA is suppressed antigen-specifically by i.n. administration of a small amount of LTB-OVA.

Suppression of IgE antibody responses to OVA by i.n. administration of LTB-OVA

Effects of i.n. administration of free OVA or LTB-OVA on the induction of systemic IgE antibody responses to OVA were examined. Mice were administered a high dose of OVA, a low dose of OVA, LTB-OVA or saline i.n. 7 days before primary systemic immunization with alum-adsorbed OVA. Six days after systemic immunization, they received a secondary systemic

Table 1 Suppression of systemic IgE antibody responses in mice i.n. given LTB-conjugated OVA

Tolerogen (mg per mouse) (-7 days)	Immunization antigen/alum (0 days)	Second antigen/alum (6 days)	Anti-OVA IgE response PCA (ELISA) titer: 2 ⁿ (21 days)	
			Exp. 1	Exp. 2
OVA (1000)	OVA	OVA	1 (1)	N.D. (5)
OVA (10)	OVA	OVA	2 (1)	3 (2)
LTB-OVA (10)	OVA	OVA	4.5 (5)	6.5 (6)
PBS	OVA	OVA	N.D. (2)	N.D. (1)
LTB-OVA (10)	HSA	HSA	N.D. (2)	N.D. (1)
PBS	HSA	HSA	N.D. (2)	N.D. (1)

Three weeks after primary immunization (0 days), mice were killed and their sera were analyzed for IgE antibody responses by PCA or ELISA. For other details, see the legend for *Figure 1*. N.D.; not done

immunization with alum-adsorbed OVA in the footpad, as described above. Sera were collected from the immunized mice 21 days after primary immunization and anti-OVA IgE antibody responses in the sera were determined by both PCA and ELISA assays. As shown on *Table 1*, the sensitivity of PCA was equal to that of ELISA. In mice given 1 mg free OVA or 10 μ g LTB-OVA, IgE antibody responses were suppressed, compared with that of mice given saline. In mice which received both primary and secondary systemic immunization with alum-adsorbed HSA, IgE responses against OVA. These observations demonstrate that systemic IgE antibody responses, as well as DTH responses, can be suppressed antigen-specifically by i.n. administration of LTB-OVA.

Anti-OVA IgG antibody responses in the sera were also determined by ELISA. The IgG titer from mice having received PBS, OVA (1000 μ g), OVA (10 μ g), or LTB-OVA (10 μ g) was <1 unit, when the pooled sera from mice having received LTB-OVA+LT were used as a 160 unit standard for anti-OVA IgG antibodies. Thus, under the immunization conditions used here, we could not detect clear difference of the IgG responses between the groups pretreated with LTB-OVA and that with PBS.

Abrogation of LTB-induced nasal suppression by LT

Effects of i.n. administration of either LTB-OVA together with free LT or LT-coupled OVA on the induction of both DTH and IgE responses to OVA were examined. Mice were i.n. administered with saline, 10 μ g LTB-OVA, 10 μ g LTB-OVA together with 1 μ g LT, 10 μ g LTB-OVA together with 1 μ g LTB, 10 μ g OVA mixed with 2 μ g LTB or 10 μ g LT-OVA 3 days before systemic immunization. Six days after systemic immunization, DTH reaction was elicited. *Figure 2* shows that mice given free LT together with LTB-OVA not only abrogated the suppression by LTB-OVA, but also enhanced DTH and IgE responses significantly, compared with that of mice given saline. Mice given either LTB-OVA+LT or LT-OVA showed 2⁸ or 2¹⁰ ELISA IgE titer 3 weeks after priming, whereas saline or LTB+OVA showed 2⁵ or 2⁴ ELISA IgE titer. Moreover, LTB-OVA and LTB-OVA+LTB showed 2² and 2³, respectively. Thus, free LT abrogated the suppression of both DTH and IgE responses induced by i.n.

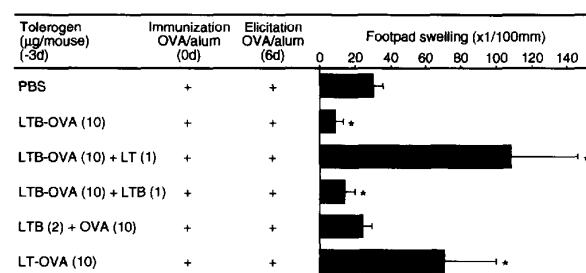


Figure 2 Abrogation of LTB-induced i.n. tolerance with LT. Mice were i.n. given LTB-OVA alone, LTB-OVA with LT, LTB-OVA with LTB, mixture of LTB and OVA, and LT-conjugated OVA. Three days later, the mice were primed s.c. with alum-adsorbed OVA. DTH responses were elicited 6 days later by footpad injection of alum-adsorbed OVA. For other details, see the legend for Figure 1

administration of LTB-OVA and resulted in enhancement of both DTH and IgE response. In addition, LT-coupled OVA also enhanced both DTH and IgE responses.

DISCUSSION

In this study, i.n. administration of a small amount of LTB-OVA suppressed the induction of both DTH and IgE antibody responses to OVA (Figure 1 and Table 1). On the other hand, i.n. administration of OVA alone required a large amount to suppress both DTH and IgE responses (Figure 1 and Table 1). Since a small amount of free LTB, when administered i.n. together with a small amount of free OVA, had no effect on systemic DTH and IgE responses (Figure 2), the physical association of LTB and OVA is required to mediate such suppression. Moreover, free LT but not LTB abrogated the suppression when coadministered with LTB-OVA (Figure 2). The LT-coupled OVA also abrogated the suppression. These results consistent with those of Sun *et al.* who showed that a single oral administration of a small dose of HGG or SRBC coupled to CTB suppressed the induction of both DTH and serum antibody (IgM and IgG) responses to HGG or SRBC and that CT abrogated the suppression when coadministered with CTB-HGG⁵. We extended their observation on IgM and IgG antibody responses against orally-administered prototype antigens to IgE antibody responses against the i.n.-administered dietary antigen, OVA. The results also suggest that LTB, as well as CTB, can serve as a powerful carrier for induction of immunological tolerance.

It is known that DTH and IgE antibody response are mediated by Th1 and Th2 cells, respectively²¹⁻²³. In this regard, Marinaro *et al.* showed that mucosal adjuvant effect of CT in mice results from induction of Th2 cells and IL-4, involved in IgE responses, as well as IgG and IgA responses.²⁴ Sun *et al.* showed that an oral administrations of CTB-coupled HGG (or SRBC) suppressed the induction of both DTH and serum IgM and IgG antibody responses⁵. In the present experiments, we showed that an i.n. administration of LTB-coupled OVA suppressed the induction of both DTH and serum IgE antibody responses. These results suggest that mucosal (oral or i.n.) pretreatment with CTB- or LTB-coupled antigen suppress the induction of both Th1 and Th2 cells which are specific for the antigen.

This study has shown that the suppressive effect of OVA-LTB on the induction of IgE antibody responses was usually modest (Table 1). In addition, preliminary experiment showed that i.n. administration of OVA-LTB failed to suppress ongoing IgE antibody responses in an already-immune mice (unpublished data). In order to suppress the ongoing IgE antibody responses, further works will be required to prepare effective OVA-LTB products and to elucidate the conditions of LTB-OVA administration.

In this study, OVA-LTB, prepared by conjugating OVA to LTB using SPDP¹⁶ was used as a tolerogen. On the other hand, preliminary experiments showed that OVA-LTB, prepared by conjugating OVA to LTB with glutaraldehyde, failed to suppress the induction of DTH (unpublished data). The result is consistent with that of Stock *et al.*²⁵ in which oral administration of OVA-CTB, prepared using glutaraldehyde, could stimulate the intestinal immune response against OVA induced by an i.p. immunization followed by an oral booster immunization was shown. The noticeable discrepancy between OVA-SPDP-LTB and OVA-glutaraldehyde-LTB may be the difference of structure.

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