

Transcutaneous Immunization with Cholera Toxin B Subunit Adjuvant Suppresses IgE Antibody Responses Via Selective Induction of Th1 Immune Responses¹

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Topical application of cholera toxin (CT) onto mouse skin can induce a humoral immune response to CT as well as to coadministered Ags. In this study, we examined the nontoxic cell-binding B subunit of CT (CTB) as a potential adjuvant for cutaneous immune responses when coadministered with the prototype protein Ag, OVA. CTB applied onto skin induced serum Ab responses to itself with magnitudes comparable to those evoked by CT but was poorly efficient at promoting systemic Ab responses to coadministered OVA. However, transcutaneous immunization (TCI) with either CT or CTB and OVA led to vigorous OVA-specific T cell proliferative responses. Furthermore, CTB potentiated Th1-driven responses (IFN- γ production) whereas CT induced both Th1 and Th2 cytokine production. Coadministration of the toxic subunit CTA, together with CTB and OVA Ag, led to enhanced Th1 and Th2 responses. Moreover, whereas TCI with CT enhanced serum IgE responses to coadministered OVA, CTB suppressed these responses. TCI with either CT or CTB led to an increased accumulation of dendritic cells in the exposed epidermis and the underlying dermis. Thus, in contrast to CT, CTB appears to behave very differently when given by the transcutaneous as opposed to a mucosal route and the results suggest that the adjuvant activity of CT on Th1- and Th2-dependent responses induced by TCI involves two distinct moieties, the B and the A subunits, respectively. *The Journal of Immunology*, 2003, 170: 1586–1592.

Cholera toxin (CT)³ and the related heat-labile enterotoxin (LT) from *Escherichia coli* are exceptionally potent immunoadjuvants when coadministered with Ags by various mucosal routes (1–3). Recently, Glenn et al. (4–6) reported that application of either CT or LT along with heterologous protein Ags induced Ab responses against both the toxin and the coadministered Ags, in both rodents and humans, and without causing the systemic toxicities attributable to the use of CT by other application routes. These findings suggest that transcutaneous immunization (TCI) with CT or related molecules is a safe and effective approach to deliver vaccines. TCI with a prototype protein Ag (tetanus toxoid) and CT or LT also induced systemic Ag-specific T cell responses with a clear Th2 bias (7). In this respect, oral and parenteral administration of CT has also been reported to induce

Th2 responses such as IgE Ab responses in rodents and humans (8–11). This bias for a Th2-type cytokine pattern could be a problem for the widespread use of TCI, because vaccine delivery approaches which favor Th2 responses such as TCI have been reported to induce IgE responses and atopic dermatitis (12, 13).

Both CT and LT are AB toxins composed of two distinct structural and functional subunits: a single toxic A subunit and a nontoxic B subunit with strong affinity for GM1 ganglioside receptors (14). Such receptors are present on all mammalian nucleated cells including skin and mucosal epithelial cells, lymphocytes, and dendritic cells (DCs). Although CT subunit B (CTB) and LT subunit B have been used as mucosal or parenteral carrier molecules for various Ags physically linked to these proteins, they have, in contrast to their holotoxin counterparts, been rather inefficient (oral immunization) or at best partly efficient (nasal immunization) as immunoadjuvants for unlinked coadministered Ags (15, 16). Furthermore, the coupling of Ag to CTB (or LT subunit B) for mucosal immunization often promotes the mucosal IgA immune response to the conjugated Ag, but instead of abrogating systemic tolerance to the conjugated Ag which CT and LT holotoxins readily do (1), CTB has been found to enhance the induction of systemic tolerance.

The markedly different and sometimes opposite immunomodulating effects of CT and CTB in many systems have prompted us to compare the effects of CT and CTB on the immune response to a coadministered prototype Ag, OVA, in TCI. In this study, we show that both CT and CTB can attract DCs in the exposed epidermis. However, and at variance with CT which induces both Th1 and Th2 responses and with CTA which enhanced Th2 responses, CTB induced a polarization of Th1 responses. Taken together, our results suggest that the adjuvant activity of CT for Th1- and Th2-driven responses in TCI involves the B and A subunits, respectively.

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³ Abbreviations used in this paper: CT, cholera toxin; LT, heat-labile enterotoxin; TCI, transcutaneous immunization; DC, dendritic cell; CTB, CT subunit B; LACK, *L. major* homolog of mammalian RACK1; CTA, CT subunit A; LN, lymph node; DLN, draining LN; RT, room temperature; PCA, passive cutaneous anaphylaxis; LC, Langhans cell.

Materials and Methods

Animals

Female BALB/c mice were purchased from Charles River Breeding Laboratories (L'Arbresle, France) and maintained on an OVA-free diet. In all experiments, 6- to 8-wk-old female mice were used. Male Sprague Dawley rats, 12- to 14-wk-old, were purchased from BK Universal (Stockholm, Sweden).

Ags and adjuvants

OVA, grade V, was obtained from Sigma-Aldrich (St. Louis, MO). *Leishmania major* homolog of mammalian RACK1 (LACK) Ag (17) was a gift from Dr. N. Glaichenhaus (IPMC, Sophia-Antipolis, France). Purified CT subunit A (CTA) was obtained from List Biological Laboratories (Campbell, CA) and contained negligible amounts of CTB (<0.3%) as determined by GM1-ELISA. Recombinant CTB was produced in a mutant strain of *Vibrio cholerae* 01 deleted of its CT genes and transfected with a multicopy plasmid encoding CTB. The CTB used was purified from the culture medium by a combination of salt precipitation and chromatographic methods, as described (18). OVA was conjugated to CTB using N-succinimidyl (3-(2-pyridyl)dithio)-propionate (Pharmacia, Uppsala, Sweden) as described earlier (19). The resulting conjugate retained both GM1-binding activity and CTB and OVA serological reactivity, as judged by a solid-phase ELISA using GM1 as the capture system (20) and Abs to CTB and OVA as detection reagents.

Immunizations

BALB/c mice were shaved on the abdomen 24 h before skin immunization. The animals were then anesthetized for 1 h by i.p. injection of sodium pentobarbital (60 μ g/g mouse) (Sanofi-Synthelabo, Paris, France), and 200 μ l of the immunizing solution, containing 50 μ g of CT or CTB and 300 μ g of OVA or LACK Ag in PBS, was placed on the shaved abdominal skin over a 4-cm² surface area and left for 1 h. In some experiments, the unshaved ears were immersed in an Eppendorf tube containing the immunizing solution. Finally, the mice were extensively washed with tap water. Separate groups of mice received three consecutive epicutaneous immunizations on days 1, 14, and 28. Mice were also immunized by s.c. injection in one footpad with 100 μ g of OVA emulsified in CFA. In some experiments, BALB/c mice were sensitized and challenged i.p. with 100 μ g of OVA adsorbed on 2 mg of Al(OH)₃ (alum) (Sigma-Aldrich) given on days 1 and 14.

Lymphocyte proliferation

OVA-specific proliferative responses were determined on triplicate cultures of lymph node (LN) or spleen cell suspensions (Falcon; BD Biosciences, Franklin Lakes, NJ). Cells were seeded at 4×10^5 cells per flat-bottom well of 96-well culture plates. After incubation at 37°C for 72 h in the presence or absence of OVA (2 mg/ml) or LACK Ag (5 μ M), cultures were pulsed for another 18-h period with 1 μ Ci [³H]thymidine. In some experiments, CD4⁺ T cells (2×10^5 cells) were stimulated with OVA (2 mg/ml) in the presence of autologous APCs (2×10^5 cells). CD4⁺ T cells (>90% pure) were obtained from either draining LNs (DLNs) and spleen cells of immunized mice by immunodepletion of CD8⁺ T lymphocytes and MHC-II⁺ cells with biotinylated monoclonal anti-CD8 α (clone 53-6-72) and anti-MHC-II (clone 2G9; BD PharMingen, San Diego, CA) and streptavidin-conjugated Dynabeads (Dyna, Oslo, Norway) according to the manufacturer's instructions. T cell-depleted splenocytes were prepared by immunomagnetic depletion with biotinylated anti-CD3 (clone KT3), anti-CD4 (clone GK1-5), and anti-CD8 α (clone 53-6-72) and streptavidin-conjugated Dynabeads and served as APCs. Cultures were harvested onto glass filters and the extent of radioactive thymidine incorporated was measured with a beta scintillation counter.

Cytokine assays

Culture supernatants from in vitro OVA-stimulated lymphocytes from DLN or from spleens were assayed for IL-2, IFN- γ , IL-4, and IL-5 contents using ELISA kits (Duosets; R&D Systems, Abingdon, U.K.) according to manufacturer's instructions. Briefly, high-binding 96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with 100 μ l of either anti-IL-2, IFN- γ , IL-4, and IL-5 coating Abs (2 μ g/ml) in 0.1 M phosphate buffer overnight at room temperature (RT) followed by a step of saturation of 60 min in 1% nonfat milk powder with 0.12% Triton X-100. All steps were performed at RT and followed by three washes with PBS containing 0.05% Tween 20 (PBST). Supernatants were diluted 2- to 5-fold in PBST with 1% nonfat milk and incubated for 2 h at RT. After washing, the wells were exposed for 1 h to biotinylated Abs to IL-2, IFN- γ , IL-4, or IL-5 (0.1

μ g/ml) in PBST, and developed by stepwise addition of streptavidin-biotinylated HRP complex (ABC complex; Amersham, Arlington Heights, IL) and 3,3',5,5'-tetramethyl benzidine/H₂O₂ (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Measurements of serum Ab levels

Serum Abs against CTB and OVA were determined by means of solid-phase ELISA. Briefly, high binding 96-well polystyrene plates (Nunc) were coated with either CTB (0.1 μ g in 100 μ l) or OVA (1 μ g in 100 μ l) overnight at RT and blocked for 90 min with 20 mg/ml BSA. After three washes with PBST, serial 3-fold dilutions of test sera in PBST with 2 mg/ml BSA were incubated in coated wells for 3 h. After washing, the wells were incubated for 1 h with HRP-conjugated goat Abs to mouse IgG1, IgG2a, and IgG2b (Southern Biotechnology Associates, Birmingham, AL) applied alone or as a mixture. Solid phase-bound HRP activity was monitored spectrophotometrically after addition of enzyme substrate. The titer of a serum was defined as the reverse of the highest dilution yielding an absorbance value at least equal to 3-fold that of background. The nonparametric Wilcoxon Mann-Whitney's rank test was used to compare experimental groups for statistical differences.

Passive cutaneous anaphylaxis (PCA)

Serum titers of IgE Abs to OVA were determined by PCA (21). In brief, serial 2-fold dilutions of test mouse sera were injected intradermally into the shaved dorsal skin of Sprague Dawley rats. Seventy-two hours later, the rats were challenged i.v. with 5 mg of OVA diluted in 1 ml of pyrogen-free PBS containing 1% Evans blue (Sigma-Aldrich). One hour later, the rats were sacrificed and the skin was examined for the appearance of blue spots. The PCA titers were defined as the reciprocal of the highest dilution of serum giving a spot larger than 5 mm in diameter. Protective efficacy was defined as the percentage of animals whose PCA titer did not increase by >2-fold after a booster injection with OVA-alum.

Preparation of epidermal Langerhans cells (LCs) and LN DCs

Ears from mice were rinsed with 70% ethanol, split into dorsal and ventral halves, and incubated for 45 min at 37°C in PBS containing 5% FCS and 20 mM EDTA, to allow separation of the epidermis from the dermis. Epidermal sheets were then dried onto glass slides. Alternatively, epidermal cell suspensions were obtained by mechanical extraction of cells from the separated epidermis, and LCs represented 0.5–3% of total cells. DCs were purified from DLNs, following an isolation protocol previously described (22, 23). Briefly, DLNs were digested with collagenase A (0.5 mg/ml; Boehringer Mannheim, Mannheim, Germany) and DNase I (0.4 mg/ml; Boehringer Mannheim) in RPMI 1640 medium supplemented with 5% FCS for 10 min at 37°C with continuous agitation. Digested fragments were filtered through a stainless steel sieve, and the resulting cell suspensions were washed and resuspended in cold iso-osmotic Optiprep solution (Nyegaard, Oslo, Norway), pH 7.2, density 1.061 g/cm³. A low density fraction, accounting for ~1% of the starting cell population, was obtained by centrifugation at $1700 \times g$ for 10 min. Low density cells were then treated for 40 min at 4°C with a mixture of mAb to CD3 (clone KT3-1.1), to B220 (clone RA3-6B2), macrophage Ag F4/80 (clone C1.A3-1), and granulocyte Ag Gr1 (clone RB6-8C5). T cells, B cells, macrophages, and granulocytes were then depleted by magnetic removal after incubation for 30 min at 4°C with a 1:1 mixture of anti-mouse Ig- and anti-rat Ig-coated magnetic beads, (Dynabeads; Dynal) at a 7:1 bead-to-cell ratio. DC preparations contained >85% CD11c⁺ DCs as analyzed by flow cytometry. In some experiments, highly enriched (>95%) DCs were obtained from low density cells by immunomagnetic selection with MACS beads coated with mAb to CD11c according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).

Depletion of LCs by UV-C irradiation

Mice were anesthetized with pentobarbital and the dorsal side of both ears was exposed for 45 min to a UV-C source (G40T10 UV lamp (Faster, Ferrara, Italy); total irradiation, 80 mJ/cm²). LC depletion (70%) was confirmed by MHC-II staining of epidermal sheets and epidermal cell suspensions prepared 48 h after UV-C irradiation (24, 25).

Immunohistochemistry

Mice were sacrificed at various times after epicutaneous treatment with CT or CTB and their ears were excised. The ears were fixed overnight at 4°C in a Tris-calcium acetate buffer containing zinc acetate (0.5%) and zinc chloride (0.5%) and dehydrated in solutions of acetone (70% and 100%). Specimens were then embedded in low melting point (37–39°C) paraffin wax (BDH Laboratories, Poole, U.K.) (26). Serial 10- μ m sections were

prepared and dried overnight at RT onto glass microscope slides (Superfrost Plus; Kindler, Freiburg, Germany). Before staining, slides were dewaxed in acetone for 3 min, washed with PBS, and exposed to 10% FCS diluted in PBS for 30 min at room temperature. Sections were then incubated with biotin-conjugated anti-MHC-II mAb (clone 2G9; BD Pharmingen, San Diego, CA) (1 μ g/ml) or with isotype-matched control Ab (clone B39-4; BD Pharmingen) at the same concentration for 1 h at room temperature. Specific binding was revealed after incubation during 30 min with ABC-HRP complex (Amersham) followed by incubation with chromogen substrate (3-amino-9-ethylcarbazole and H_2O_2). Slides were counterstained with H&E.

Flow cytometry

Phenotypic analysis of DC subpopulations was performed by triple staining with fluorescein (FITC)-conjugated anti-CD11c (clone N418), PE-conjugated anti-CD8 α (clone 53-6-72; BD Pharmingen), and biotin-conjugated anti-CD11b (clone M1/70; BD Pharmingen), anti-B7-1 (clone 16-10A1; BD Pharmingen), anti-B7-2 (clone GL1; BD Pharmingen), anti-CD40 (clone 3/23; BD Pharmingen) followed by streptavidin-tricolor (Caltag Laboratories, San Francisco, CA). Analysis of LCs was performed after double-staining with PE-conjugated anti-MHC-II (clone 2G9; BD Pharmingen) and fluorescein-conjugated anti-CD11c, biotin-conjugated anti-B7-1, biotin-conjugated anti-B7-2, biotin-conjugated anti-CD40, followed by streptavidin-tricolor. All stainings were performed at 0–4°C in PBS containing 5 mM EDTA and 3% FCS. Analysis was performed on a FACScan flow cytometer (BD Biosciences) using Cell Quest software (BD Biosciences).

Results

CTB is a potent transcutaneous immunogen

TCI with CTB was as efficient as CT to induce homologous-specific Ab responses in serum (Fig. 1A). These responses were readily detected in half of the animals after one TCI and were enlarged by additional TCIs. After a third TCI, all mice had responded with high (>10,000) anti-CTB Ab titers.

CTB is a weak adjuvant for systemic Ab responses to a coadministered protein Ag

We then evaluated the ability of CTB to promote systemic Ab responses to a prototype soluble protein, OVA, coapplied onto skin. Preliminary dose-response experiments were conducted and based on the results of such experiments, a standard dose of 50 μ g was selected for CTB and CT. Such doses were yielding maximal and most consistent serum Ab responses to CTB. TCI with OVA and CTB (50 μ g) failed to induce consistent anti-OVA IgG responses even after three consecutive applications. After a fourth

TCI, most (66%) animals had developed detectable serum IgG anti-OVA Ab responses but these responses were relatively modest (geometric mean titer 720) when compared with corresponding responses induced with CT (geometric mean titer 3,500; $p < 0.05$) (Fig. 1B). Coadministration of OVA with doses as high as 450 μ g of CTB yielded similar results (not shown). TCI with OVA covalently coupled to CTB and administered in comparable amounts on four consecutive occasions induced marginally higher anti-OVA Ab responses when compared with TCI with free OVA and CTB (data not shown). The isotype distribution of IgG anti-OVA Ab responses did not differ between animals immunized with CT and OVA and animals given CTB plus OVA being mainly accounted for by IgG1 and to a much lesser extent by IgG2b and were almost devoid of IgG2a (mean titer < 50, $n = 15$ in each group). Thus, IgG1 anti-OVA responses were more than 10-fold higher in CT + OVA immunized mice (mean titer 25,000, range 4,050–110,000; $n = 15$) as compared with animals immunized with CTB + OVA (mean titer 2,000, range 150–12,000; $n = 15$). IgG2b anti-OVA responses were considerably weaker in both groups of animals (mean titer < 200). Furthermore, TCI with a mixture of free CTA, CTB, and OVA induced serum anti-OVA IgG Ab responses comparable to those induced by CT and OVA with respect to magnitude and isotype distribution (data not shown). Administration of OVA alone failed to induce detectable serum IgG anti-OVA responses (Fig. 1B).

CT and CTB are potent adjuvants of systemic T cell responses to skin-applied OVA

Three weeks after the last of three consecutive TCIs, the proliferative responses of cells from DLNs and from the spleen were examined after *in vitro* restimulation with OVA. TCI with OVA and CT or CTB led to the generation of similarly strong anti-OVA proliferative responses in DLN cells and spleen (Fig. 2, A and B). These responses were specific for OVA as indicated by the lack of response to an unrelated protein Ag, LACK (not shown). Interestingly, TCI with CT or CTB and OVA was as efficient as a s.c. injection of OVA in CFA for inducing OVA-specific proliferative responses (Fig. 2). Comparable responses were also seen after TCI with the CTB-OVA conjugate, confirming that the transcutaneous transport of OVA did not require physical association of OVA to CTB (data not shown). Finally, TCI with OVA and CT or a mix of

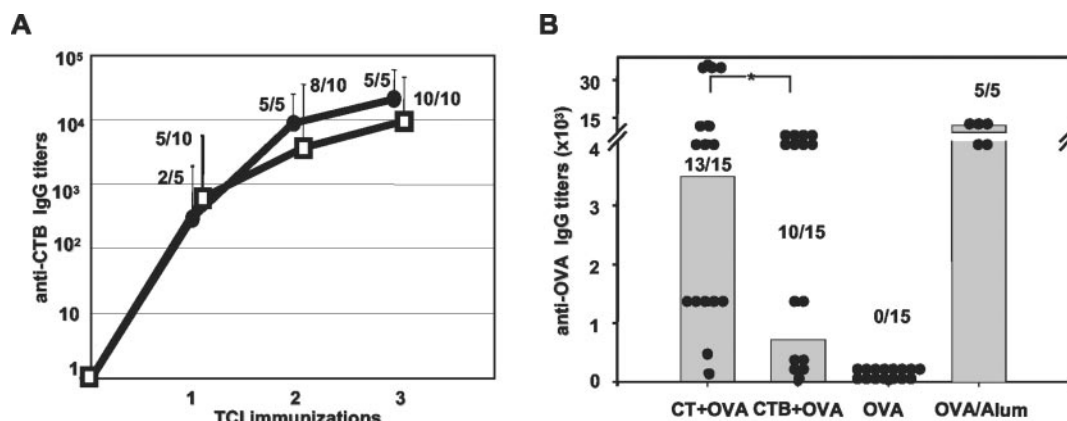


FIGURE 1. Serum Ab responses to OVA after TCI with CT or CTB. **A**, Groups of mice received three consecutive topical applications of CT (●) or CTB (□) given 2 wk apart. Serum IgG anti-Ab titers to CTB were determined 2 wk after each immunization. Data are expressed as geometric mean IgG Ab titers + SD (vertical bars). The number of responder mice is indicated. **B**, Groups of mice received four consecutive TCI with OVA alone or coadministered with CT or CTB given 2 wk apart. An additional group of mice received one i.p. injection of alum-adsorbed OVA. Levels of IgG anti-OVA were determined 3 wk after the last immunization and are expressed as geometric mean IgG Ab titers. Anti-OVA IgG titers from individual mice are displayed (●) and the frequency of responder animals is indicated in each group. *, Significant difference ($p < 0.05$; Wilcoxon's rank test).

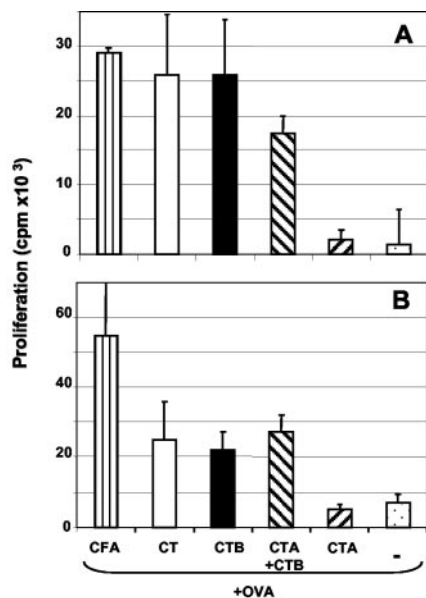


FIGURE 2. TCI with CTB enhances T cell proliferative responses to a protein Ag. BALB/c mice received three TCI with OVA alone or coadministered with either CT, CTB, CTA, or a mixture of both CTB and CTA. A separate group of mice received one s.c. injection of OVA in CFA. Proliferating responses of LN cells (A) or spleen cells (B) stimulated with OVA were assessed 3 wk after the last immunization. Data are expressed as arithmetic mean cpm $\pm$ SD (vertical bars) determined on groups of 15 individual mice.

CTA and CTB led to the generation of OVA-specific proliferative responses whereas CTA alone was inefficient.

CTB selectively potentiates Th1-driven responses

Because subclass analyses of serum IgG Abs to OVA did not enable us to indicate the type(s) of Th subset involved, we tested cytokine production by lymphocytes from TCI immunized mice. We analyzed the cytokines produced by DLN or spleen cells after three consecutive TCIs with either OVA alone or OVA together with CT or CTB, after *in vitro* restimulation with OVA. As shown in Fig. 3A, CTB enhanced the production of IFN- γ by spleen cells from immunized mice as compared with control mice. Such treatment, in contrast, did not increase *in vitro* OVA-induced IL-4 and IL-5 responses (Fig. 3B and Table I). In contrast, CT potentiated the production of both Th1 (IFN- γ) and Th2 (IL-4, IL-5) cytokines

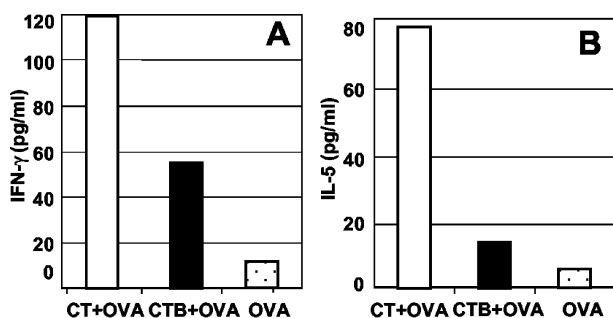


FIGURE 3. TCI with CT and CTB differentially affects Th1 and Th2 cytokine responses to coadministered OVA. Mice were given three TCI with OVA alone or coadministered with CT or CTB as described in Fig. 2. Three weeks after the last of three TCI with OVA alone or coadministered with CT or CTB (as described in Fig. 2) spleen cells were stimulated with OVA and cytokine levels were determined in supernatants of 48-h (IL-5, IFN- γ) cultures. Results are representative of three independent experiments.

Table I. Differential effects of TCI with CT subunits on cytokine responses to coadministered OVA^a

TCI	IFN- γ (pg/ml)	IL-4 (pg/ml)	IL-5 (pg/ml)
CT 50 μ g + OVA $\times$ 3	236 $\pm$ 30	27 $\pm$ 1	54 $\pm$ 40
CT 2 μ g + OVA $\times$ 3	300 $\pm$ 90	80 $\pm$ 27	80 $\pm$ 30
CTB 50 μ g + OVA $\times$ 3	120 $\pm$ 60	12 $\pm$ 1	<10
CTB 450 μ g + OVA $\times$ 3	90 $\pm$ 30	25 $\pm$ 10	<10
CTA 10 μ g + CTB 50 μ g + OVA	396 $\pm$ 200	28 $\pm$ 4	102 $\pm$ 106
CTA 10 μ g + OVA $\times$ 3	51 $\pm$ 8	29 $\pm$ 2	109 $\pm$ 2
CFA + OVA $\times$ 1	256 $\pm$ 30	<10	19 $\pm$ 15

^a Mice were immunized with OVA (300 μ g) coadministered with CT, CTA, or CTB. Spleen cells were collected 3 wk after the last of three TCI or after a s.c. injection of OVA in CFA. Data are expressed as arithmetic mean levels of cytokines ($\pm$ SD) determined in three separate experiments involving triplicate cultures of spleen cells exposed to OVA.

by spleen cells (Fig. 3 and Table I). Furthermore, as shown in Table I, doses of CT as low as 2 μ g or doses of CTB as high as 450 μ g had similar effects. Moreover, TCI with a mix of CTA and CTB induced levels of Th1 (IFN- γ) and Th2 (IL-4 and IL-5) cytokines comparable to those induced with CT. Interestingly, TCI with OVA and CTA, a treatment that failed to induce detectable Ab responses and proliferative responses to OVA, selectively enhanced the production of Th2 cytokines (IL-4 and IL-5) (Table I), indicating that CTA may penetrate and/or facilitate the passage of free OVA through the skin. To more precisely characterize the pattern of Th cell cytokine responses, cytokine production by purified CD4⁺ T cells from DLN and spleen cells was determined. Restimulated DLN and spleen CD4⁺ T cells from mice immunized with CTB and OVA produced negligible levels of IL-4 and IL-5 but did produce IL-2 and IFN- γ levels comparable to those seen in mice immunized with CT and OVA (Table II).

TCI with CTB prevents systemic induction of IgE Ab responses to OVA. Mice were immunized transcutaneously with OVA alone or coadministered with either CT or CTB every other day for 2 wk. Three weeks after the sixth and last TCI, animals were given an i.p. injection of alum-adsorbed OVA. Sera were collected 3 wk later and anti-OVA reagenic Ab titers were determined by PCA in rat recipients. As shown in Fig. 4A, whereas TCI with CT enhanced anti-OVA IgE Ab responses, TCI with CTB reduced these responses.

TCI with CTB suppresses allergen-specific IgE Ab responses in sensitized mice

Mice were sensitized and challenged by i.p. injection of alum-adsorbed OVA on day 0 and week 14. Animals were treated by TCI with OVA alone or coadministered with either CT or CTB three times a week for four consecutive weeks starting on week 12 and until week 16, i.e., 2 wk before and after booster injection. PCA titers were determined immediately before and 3 wk after the OVA booster injection. As shown in Fig. 4B, TCI with OVA and CTB significantly suppressed IgE responses in most animals (6/7) in comparison with control mice ($p < 0.02$) whereas CT failed to do it.

TCI with CTB increases LC numbers in the epidermis

We next examined whether topical application of CT and CTB onto ear skin affected the density of LC in the epidermis. Because the turnover of LCs in the epidermis is known to be slow, we induced a partial egress of resident LCs before TCI with CT or CTB by exposing the skin for 45 min to UV-C irradiation which has been shown to be an effective treatment for inducing LC depletion (24, 25). Thus, 48 h after UV-C irradiation, LC numbers

Table II. Differential effects of CT and CTB on cytokine responses to coadministered OVA in cultures from restimulated CD4⁺ T cells^a

TCI	DLN CD4 ⁺ T Cells				Spleen CD4 ⁺ T Cells			
	IL-2	IFN- γ	IL-4	IL-5	IL-2	IFN- γ	IL-4	IL-5
CT + OVA	41 $\pm$ 20	230 $\pm$ 135	83 $\pm$ 18	100 $\pm$ 30	25 $\pm$ 10	30 $\pm$ 10	40 $\pm$ 20	22 ^b
CTB + OVA	31 $\pm$ 10	90 $\pm$ 8	<5	15 $\pm$ 3	12 $\pm$ 4	30 $\pm$ 10	<5	<5

^a Mice were immunized by TCI with OVA and CT or CTB. Two weeks after the last of three TCI, CD4⁺ T cells from DLN and spleen were cultured for 72 h with OVA in the presence of syngeneic APCs. Data are expressed as arithmetic mean levels of cytokines ($\pm$ SD) determined in triplicate cultures and are representative of two experiments.

^b Determined in one experiment.

had decreased by almost 70%. At that time, mice were treated for 1 h with CT or CTB applied onto UV-C irradiated ear skin. Epidermal LC numbers in treated mice were compared with LC numbers in control irradiated mice. We determined MHC-II⁺ LC frequencies in epidermal cell suspensions after staining with fluorescent Abs specific for MHC-II and CD11c molecules, followed by flow cytometry analysis. As shown in Fig. 5A, cell suspensions from epidermal sheets of animals treated with CT and CTB displayed >2-fold higher numbers of LCs as compared with corresponding specimens from control (sham-immunized) mice. Doses of CT as low as 2 μ g and doses of CTB as high as 450 μ g induced similar increases in LC numbers (not shown).

To evaluate the kinetics of recruitment of DCs into skin after TCI with CT or CTB, we performed skin transverse sections of mouse ears 20, 40, 90, and 180 min after treatment. We analyzed LC frequency and localization by staining with a mAb specific for MHC-II followed by immunoenzymatic detection. As shown in Fig. 5B, in a control mouse we observed scattered staining with anti-MHC-II Ab both in the dermis (control, continuous arrow) and epidermis (control, discontinuous arrow). As early as 40 min after treatment with either CT or CTB, clusters of MHC-II⁺ cells were disclosed at the dermo-epidermal junction, and increased numbers of MHC-II⁺ cells were observed in the epidermis. This accumulation was maximal 90 min after epicutaneous application of CT or CTB and persisted for at least 5 h (data not shown).

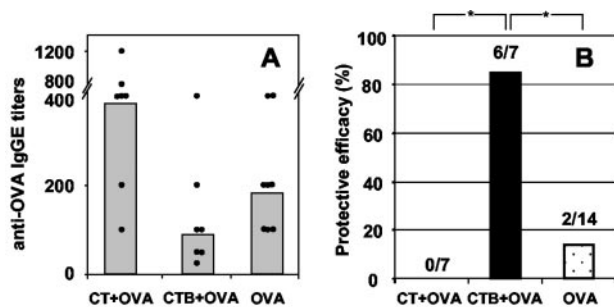


FIGURE 4. TCI with OVA and CTB suppresses allergen-specific IgE Ab responses. **A**, Groups of eight mice were immunized by TCI with OVA alone or coadministered with either CT or CTB given every other day for 2 wk. Mice were challenged 3 wk after the last of six TCI by an i.p. injection of alum-adsorbed OVA. Sera were collected 3 wk after sensitization and analyzed for OVA-specific IgE Ab levels by PCA. Results are expressed as geometric mean IgE Ab titers, determined on groups of 8–12 animals (●) and are representative of two independent experiments. **B**, Animals were sensitized by an i.p. injection of alum-adsorbed OVA on day 0 and given a booster injection on week 14. Animals were treated by TCI with OVA alone or coadministered with either CT or CTB three times a week for four consecutive weeks starting on week 12 and until week 16, i.e., 2 wk before and after a booster injection with alum-adsorbed OVA. PCA titers were determined immediately before (titer 1) and 3 wk after the OVA booster injection (titer 2). Protective efficacy was defined as the percentage of animals whose PCA titer 2 did not increase by >2-fold from titer 1. *, Significant difference ($p < 0.02$; Fisher's exact test).

CT and CTB differentially affect the expression of B7-1 and B7-2 costimulatory molecules by DLN DCs

At variance with the effects observed on epidermal DCs, there were no appreciable changes in the frequency of DCs in DLNs after TCI with either CT or CTB at all time points (2–24 h) examined (data not illustrated). However, changes in the expression of several costimulatory molecules by DLN DCs were observed after TCI with CT- and CTB. In LNs, three distinct subsets of CD11c⁺ DCs referred to as CD8⁺CD11b[−], CD8^{int}CD11b[−], and CD8[−]CD11b⁺ DCs were identified on the basis of their differential expression of CD8 α and CD11b. As shown in Fig. 6, after TCI with CT, all DC subsets expressed consistently higher levels of B7-1 and B7-2 costimulatory molecules as compared with corresponding DC subsets from control animals. In contrast, CTB induced a 2-fold increase in B7-1 expression by CD8⁺ DCs but had no appreciable effect on the expression of B7-2 by any of the three DLN DC subsets. CD40 and MHC-II expression by DLN DC subsets appeared unaffected by TCI with CT and CTB (not shown). In most cases, the levels of expression of B7-1, B7-2, CD40, and MHC-II were higher than those expressed by LCs for which we did not observe any significant modification of costimulatory molecules after TCI with CT or CTB (data not shown).

Discussion

This work demonstrates that both CT holotoxin and CTB, the non-toxic receptor-binding moiety of CT, when applied onto the skin surface, can penetrate the skin barrier and, importantly, can also facilitate the passage of heterologous and/or by other means enhance the immune response to a coadministered heterologous Ag. Hence, when applied onto skin both CT and CTB induce an immune response against themselves and also against a coadministered heterologous Ag. However, and unlike CT which promotes both strong humoral and cellular responses to the heterologous Ags, TCI with CTB seems to favor the development of vigorous systemic Th1 cellular responses, but weak systemic Ab responses to the coadministered Ag.

This study confirms recent findings indicating that CT can serve as an effective adjuvant for inducing humoral and cellular immune responses to various soluble protein Ags when coadministered onto the skin (4–6). This work also indicates that the ADP-ribosylating activity of the holotoxin does not appear to be critical for allowing the transfer of either itself or coadministered protein Ags through the epidermis. The ability of the toxin, and hence of its B subunit, to bind to GM1 receptors seems to be critical for facilitating the transport of these molecules through the epidermis.

The observation that systemic IgG Ab responses generated after topical application of CT onto the skin were superior in magnitude to those seen with CTB, although of comparable isotype distribution, is not surprising. Indeed, CT is a powerful adjuvant when

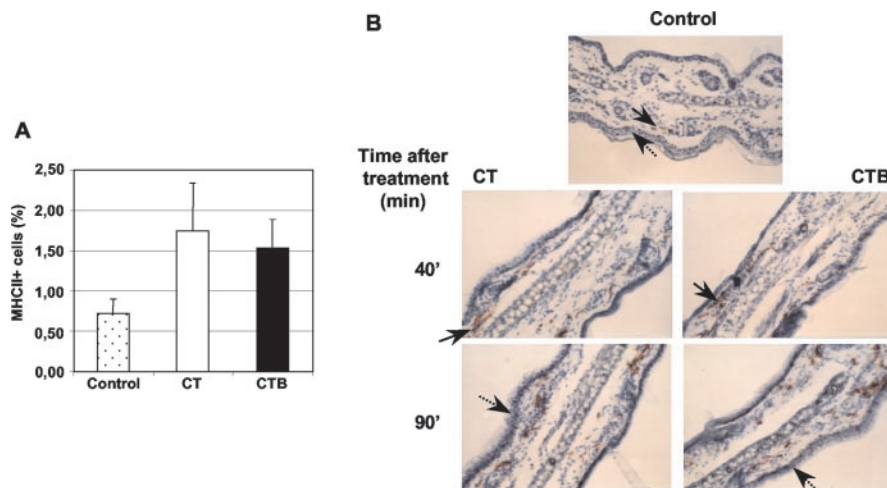


FIGURE 5. TCI with CT or CTB increases epidermal LC numbers. Groups of mice were irradiated on the ears with UV-C and immunized 2 days later by a single TCI with different doses of CT or CTB applied onto the irradiated ear. **A**, Cell suspensions from ear epidermal sheets were prepared 90 min after TCI and LC frequencies were determined by flow cytometry after double-staining with anti-CD11c and anti-MHC-II Abs and compared with LC numbers from PBS-exposed mice. Results are expressed as percentages of MHC-II⁺CD11c⁺ LCs in epidermal cell suspensions from CT-, CTB-treated or control animals, and represent arithmetic means + SD (vertical bars) of three separate experiments. **B**, Transverse sections of ear specimens collected 40 and 90 min after a single TCI with CT or CTB and stained for MHC-II show increased numbers of MHC-II⁺ cells (arrows). Note the typical cluster distribution (filled arrows) of MHC-II⁺ cells in the dermis of CT- and CTB-treated mice. Original magnification, $\times 20$.

administered by a mucosal (1, 3) or a systemic (27) route while CTB appears to be devoid or at best marginally effective when given by these routes. These observations support the notion that the adjuvant properties of CT or LT for humoral immune responses appear to be mainly associated with its enzymatic activity (7, 28). The finding that CTA admixed with CTB could potentiate serum Ab responses to coadministered OVA provides further support to this notion.

Although CTB, when coadministered onto the skin could promote a modest systemic Ab response to a coadministered Ag, and then requiring repeated coapplications, it behaved as a surprisingly powerful and hitherto unknown adjuvant for cell-mediated immune responses. In this respect, the magnitude of proliferative responses induced by skin exposure to CTB and a prototype Ag was

comparable to that induced by cutaneous exposure of the same Ag in the presence of CT, and even to that seen after systemic injection of the same Ag in CFA, one of the most potent adjuvants known for inducing cellular immune responses. The divergent activities of CTB and CT on systemic humoral vs cell-mediated immune responses to Ags coadministered through the skin are consistent with the observation that CTB, like CT, enhanced the production of Th1 cytokines but, in contrast to CT and its toxic subunit (CTA), did not affect Th2 cytokine production. This observation may well explain why TCI with CT and a prototype allergen could sensitize mice for systemic IgE Ab responses (the archetype of Th2 responses) whereas CTB coapplied by the same route failed to induce such responses and could in fact suppress these.

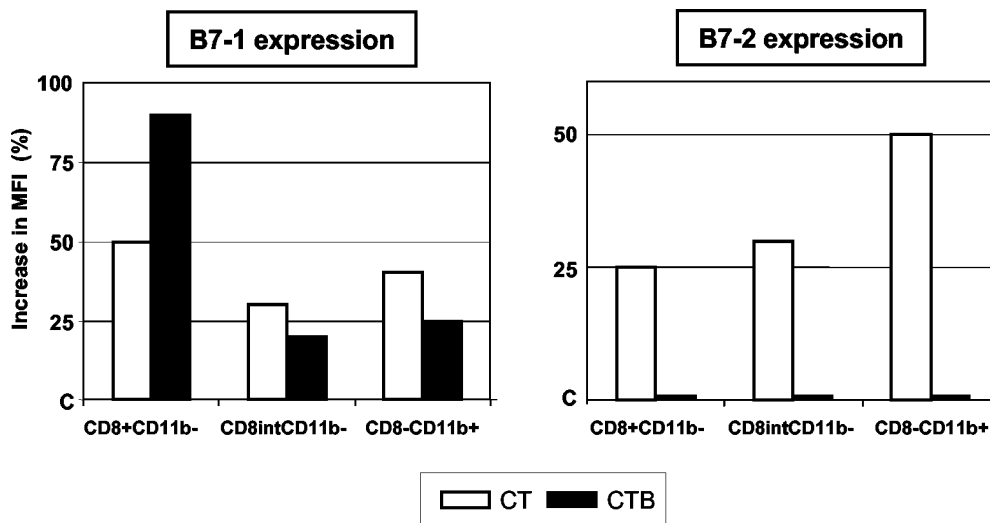


FIGURE 6. TCI with CT and CTB differentially affects the expression of B7-1 and B7-2 costimulatory molecules by DCs from DLNs. Mice were given a single TCI with CT or CTB. DLN DCs were prepared 24 h after TCI and analyzed by flow cytometry after triple-staining with anti-CD11c, anti-CD8 α , and anti-B7-1 or anti-B7-2 mAbs. Three distinct DC subsets corresponding to gated CD8⁺CD11b⁻, CD8^{int}CD11b⁻, and CD8⁻CD11b⁺ DCs were identified. The levels of expression of B7-1 and B7-2 on each DC subset is expressed as the mean fluorescence of the marker. Bars represent the percentage increases in mean fluorescence intensity (MFI) on DC subsets from CT- (□) and CTB- (■) treated mice as compared with control (PBS-exposed) mice.

The delivery of soluble proteins onto skin has earlier been reported to favor Th2 responses (12, 13). These data suggest that the adjuvant properties of CT on Th1- and Th2-dependent responses involve two distinct moieties, the GM1-binding CTB and the ADP-ribosylating CTA subunits, respectively. The finding that TCI with CTA coadministered with OVA alone led to Th2 cytokine production without affecting Th1 cytokine responses supports this interpretation. Although we cannot rule out that the CTA preparation used in this study contained trace amounts of contaminating CTB, the latter observation is intriguing in view of the failure of CTA to induce systemic Ab responses as well as proliferative responses to coadministered OVA and suggests that CTA may in fact also cross the corneo-epidermal barrier.

The exceptional adjuvant activity of CT and CTB on cell-mediated immune responses to Ags coapplied onto the skin appears to be in part associated with their capacity to induce the rapid recruitment of DC into the exposed epidermis and their subsequent exit from the epithelium to the DLNs. In this respect, exposure of the skin to CT or CTB was associated with a rapid increase in DC density in the epidermis and particularly in the underlying dermis.

The relationship between expression of B7-1 and B7-2 and preferential activation of Th subsets is still being debated. However, the observation that TCI with CT induced increased expression of B7-1, but different from CTB (at least at the dose of 50 μ g examined), led to increased expression of B7-2 on DCs, and enhanced production of IL-4 and IL-5 is compatible with the notion that up-regulation of B7-2 is associated with preferential stimulation of CD4⁺ Th2 cells (29–31).

Overall, this study suggests that the adjuvant properties of CT for Th1- and Th2-driven responses appear to involve distinct structural determinants associated with the B and A subunits, respectively. The fact that CTB suppresses systemic Th1 responses when given by a mucosal route, but potentiates these responses when given onto skin, underscores the role of the epithelial microenvironment in the regulation of immune responses. Such contrasting effects could also be reflecting DC heterogeneity between mucosal and nonmucosal tissues. From a clinical point of view, this study suggests that TCI with CTB and pertinent allergens may constitute a useful therapeutic approach for diseases associated with untoward Th2 responses such as type I allergies.

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