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CUTTING EDGE

Cutting Edge: Transcutaneous Immunization with Cholera Toxin Protects Mice Against Lethal Mucosal Toxin Challenge[1]

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We recently reported that application of cholera toxin (CT) to the skin results in transcutaneous immunization and induces a systemic Ab response to both CT and coadministered Ags. In this paper, we demonstrate antitoxin IgG and IgA Abs in sera, lung washes, and stool samples from immunized mice as well as a broad spectrum of IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3) in the sera. Mice immunized with CT by the transcutaneous route exhibited significant protection from intranasal challenge with a lethal dose of CT. Thus, clinically relevant immunity against mucosal toxin challenge can be achieved via the transcutaneous route. ***The Journal of Immunology,*** **1998, 161: 3211–3214.**

Cholera toxin (CT)[4] is an 86-kDa heterodimeric protein that is secreted by the bacterium *Vibrio cholerae* when colonizing the small intestine, where it induces massive fluid secretion by the intestinal epithelium (1, 2). When administered perorally or intranasally (i.n.), CT induces Ab responses against both itself and coadministered proteins and is thus considered a potent mucosal adjuvant (3). In both animal and human vaccine studies, CT-specific Abs have been shown to play a protective role in host immunity against cholera (4, 5). However, the toxicity of the native toxin has limited its widespread use as a vaccine component (3). Recently, we reported that topical administration of CT to the skin induces a systemic Ab response to vaccine Ags and consequently acts as an adjuvant for coadministered Ags on the skin (6). In the present study, we show that protective immunity to toxin-mediated mucosal disease can be induced transcutaneously. Moreover, the toxicity that accompanies the use of CT by other routes of administration is not apparent following transcutaneous immunization (TCI).

Materials and Methods

Immunization

C57BL/6J or BALB/c mice were shaved on the dorsum and rested for 24 h. The mice were anesthetized, immunized with 100 μg of CT (List Biologicals, Campbell, CA) in saline placed on the skin for 2 h, extensively washed, patted dry, and washed again. Neither erythema nor induration were seen at the immunization site for ≤72 h after Ag exposure. In indicated experiments, animals were given 25 μg of CT in 200 μl of PBS by oral gavage.

ELISA

Ab levels against CT were determined by ELISA as described previously (7). Briefly, anti-CT specific Abs were detected using horseradish peroxidase-linked goat anti-mouse IgG (heavy + light) (Bio-Rad, Richmond, VA), goat-anti-mouse IgA (Zymed, San Francisco, CA), goat anti-mouse IgM (μ) (Bio-Rad), or goat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 (The Binding Site, San Diego, CA) as secondary Abs. Product was revealed using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Kirkegaard and Perry, Gaithersburg, MD), and the reaction was stopped using 1% SDS. Individual Ag-specific subclass levels were computed using a standard curve generated with myeloma proteins (The Binding Site). Ag-specific IgE Ab quantitation was performed as described in PharMingen Technical Protocols (PharMingen, San Diego, CA).

Lung washes and stool collection

Lung washes were obtained from vaccinated animals on the day of, but before challenge. The trachea was transected, a 22-gauge polypropylene tube was inserted, and PBS was infused to gently inflate the lungs. After three cycles of infusion, the material was stored at −20 C. Stool pellets were collected on the day before challenge after spontaneous defecation. Pellets were weighed and homogenized in 1 ml of PBS per 100 mg of fecal material and centrifuged; supernatant was collected and stored at −20 C until assayed.

Toxin challenge

Mice were anesthetized and challenged i.n. with 20 or 30 μl of CT (Calbiochem, La Jolla, CA) (1 mg/ml) in 10 mM Tris (pH 7.5). Mice were observed daily following challenge, and both morbidity and mortality were recorded.

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[4] Abbreviations used in this paper: CT, cholera toxin; EU, ELISA units; TCI, transcutaneous immunization; i n , intranasal(ly)

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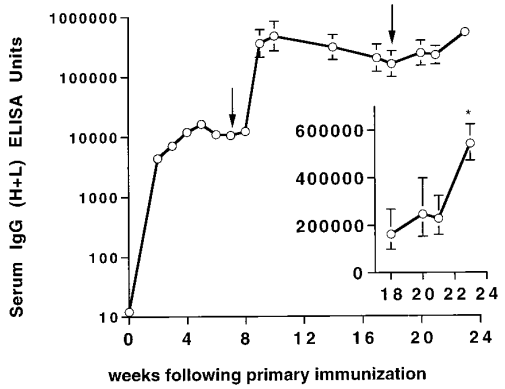

FIGURE 1. CT-specific Ab responses in BALB/c mice ($n = 5$) immunized transcutaneously with CT (100 μg) at 0, 8 (arrow), and 18 (arrow) wk. The results shown are the geometric mean and SEM of CT-specific IgG measured in sera from five individual animals and reported in EU, which is the inverse dilution at which the absorbance is equal to 1.0 at 405 nm. The inlay displays the Ab titers induced after the 18-wk boost on a linear scale. *, a statistically significant increase ($p < 0.05$) in Ab titer between the 18- and 23-wk anti-CT titers. Essentially identical results were observed in three independent experiments.

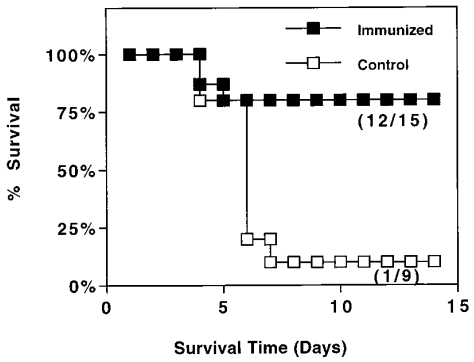

FIGURE 2. Mortality studies in C57BL/6 mice following immunization with CT by the transcutaneous route and i.n. challenge with native toxin at 3 wk after immunization. The number of mice per group is indicated in parentheses (total survivors/number of mice in study).

Statistical analysis

A comparison between Ab titers in groups was performed using the Student *t* test. For challenge studies, the groups were compared by Fisher's exact test (SigmaStat, SPSS, Chicago, IL).

Results

Kinetics of anti-CT serum IgG response induced by TCI

Placing CT on the skin induced a rise in detectable anti-CT Abs from <10 ELISA units (EU) before immunization to >10,000 EU after a single application (Fig. 1). Elevated CT titers were apparent within 2 wk of Ag exposure and persisted for ≥8 wk. Subsequent immunizations at 8 and 18 wk following the primary immunization induced ~30-fold (Fig. 1) and 3-fold (Fig. 1, inlay) increases in the CT-specific Ab titers.

Induction of protective host immunity by transcutaneous vaccination with native CT

Challenging C57BL/6J mice i n. with CT induces fatal cytotoxic pulmonary lesions that are characterized by suppurative interstitial pneumonia with marked perivascular edema, fibrin deposition, and hemorrhage (our unpublished observations). We used this challenge model to assess the significance of the antitoxin response induced by TCI. Mice were immunized on the skin with native CT and challenged i n. with a lethal dose of CT. Following a single immunization, only 11% (1 of 9) of control mice survived the challenge compared with 80% (12 of 15) of the vaccinated animals (Fig. 2, $p = 0.002$). Older mice (20 wk) immunized twice and similarly challenged were 100% protected vs 54% of control mice ($p<0.007$). The passive transfer of sera from either transcutaneously immunized or immunized and challenged mice (hyperimmune) resulted in 100% protection in this model (Table I).

Characterization of transcutaneously induced mucosal IgG and IgA responses

To characterize the nature of the immune response induced by TCI, sera, lung washes, and stool samples were collected and analyzed for CT-specific IgG and IgA. The titer of anti-CT IgG Abs increased by >3 logs following a single immunization (Fig. 3*A*); sera from mice exposed twice to CT at 0 and 3 wk exhibited significantly augmented IgG titers at 3 wk after the second transcutaneous application (Fig. 3*A*). CT-specific IgG was also detected in five of five lung wash samples and in eight of nine stool sample homogenates from the single exposure groups (Fig. 3, *B* and *C*). Further analysis of the samples revealed a potent IgA response, albeit lower than the IgG titers, in the sera, lung wash, and stool (Fig. 3, *D–F*). In contrast, lung wash samples from animals exposed to an irrelevant protein, ricin A-subunit, failed to exhibit detectable anti-CT IgG or IgA levels (Fig. 3, *B* and *E*, ●), and stool samples from unimmunized mice had <0.2 IgG OD units at a 1/2 dilution and no detectable IgA (Fig. 3, *C* and *F*, ●). Neither IgM nor IgE anti-CT Abs were detected in the sera of transcutaneously immunized mice.

CT Ab responses in the sera of orally and transcutaneously immunized mice

It was conceivable that animals vaccinated by TCI might, through normal grooming, ingest small amounts of Ag and orally expose themselves to CT. To exclude this possibility, we compared the immune response using 100 μg of CT by TCI with oral gavage using 25 μg of CT, a log greater than the amount that was estimated to be left on the skin after washing using ^{125}I-labeled CT (data not shown). As shown in Figure 4, the magnitude of the anti-CT IgG response at 4 wk after immunization was significantly higher in sera from mice in which CT was introduced by the transcutaneous route (geometric mean = 19,973 EU) compared with

Table I. *Passive transfer of protection by sera in an i.n. cholera toxin challenge model*[a]

Experimental Group	*n*	Survival at 9 Days	
		n	%
Preimmune sera	4	0/4	0
Immune sera	3	3/3	100
Hyperimmune sera	5	5/5	100
No sera	7	1/7	14

[a] Sera was collected from C57BL/6 mice either before immunization (preimmune), at 6 wk following TCI with 100 μg of CT at 0 and 3 wk (immune), or at 9 wk following TCI with 100 μg of CT at 0 and 3 wk and i n challenge with 20 μg of CT at 6 wk (hyperimmune) Individual sera were pooled within each group, and recipient mice were injected i v with 0 5 ml Mice were challenged i n with 30 μg of CT (1 mg/ml) at ~1 h after the passive transfer of sera and observed for morbidity and mortality

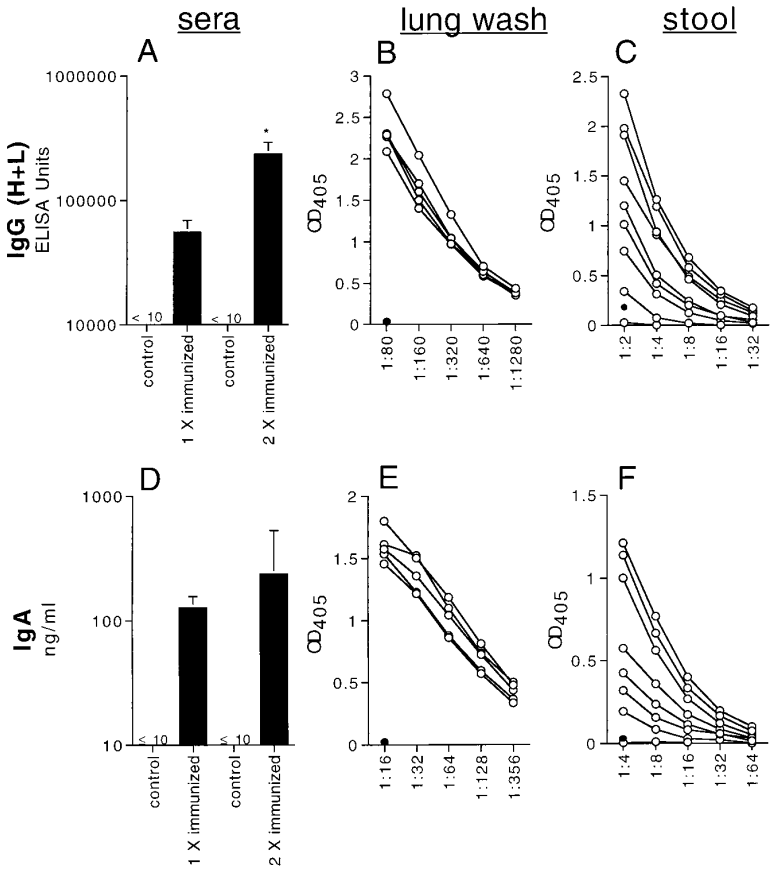

FIGURE 3. Sera (*A* and *D*), mucosal lung (*B* and *E*), and stool (*C* and *F*) Ab responses to CT after TCI. *A* and *D*, C57BL/6 mice (17–22 animals per group) were immunized transcutaneously at 0 or at 0 and 3 wk with 100 μg of CT. Sera were collected at 3 and 6 wk. Data shown are the geometric mean ± SEM for ELISA measurements from five individual animals. *, a statistically significant ($p < 0.05$) difference between the titers measured in the 1× and 2× immunization groups. *B* and *E*, C57BL/6 mice were immunized transcutaneously at 0 wk; lung washes were collected from vaccinated, unchallenged mice ($n = 5$) on the day of challenge (3 wk). ●, indicates the OD detected from control lung washes from mice immunized with an irrelevant protein. IgG and IgA levels were assessed by ELISA; the titers (OD = 405 nm) from individual animals are shown. *C* and *F*, C57BL/6 mice were immunized transcutaneously at 0 wk. Single stool pellets were collected immediately after defecation on the day before toxin challenge (6 wk). IgG and IgA levels were assessed in fecal homogenates by ELISA; the dilution curves from eight (*F*) or nine (*C*) individual animals are shown. ●, the maximal level of anti-CT Ig or anti-CT IgA Ab detected in 1/2 dilutions of stool from unimmunized mice (background).

the oral route (geometric mean = 395 EU). Moreover, while TCI induced a full complement of IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3), only IgG1 (four of five animals) and to a lesser extent IgG2b (three of five animals) were detected in the sera from the orally exposed mice. In a separate experiment, oral feeding with 10 μg of CT in saline at 0 and 3 wk induced a 6-wk mean IgG Ab response of <1,000 EU, whereas TCI with 100 μg of CT resulted in an anti-CT response of 39,828 EU. Similar results were obtained using 25 μg of CT on the unshaved ear, which is less accessible than the back for grooming, as compared with 25 μg of CT administered by oral feeding (34,426 vs 2,829 EU, respectively).

Discussion

In this study, we present data indicating that TCI with CT can induce Abs detectable both in the systemic and mucosal compartments and can confer protection against toxin-mediated disease. The model described induces a lethal toxin-mediated disease that can be prevented by passive transfer of immune sera containing antitoxin Ig (Table I). This protective anti-CT immune response to TCI may be relevant to other toxin-mediated diseases active at the mucosal level.

CT is exquisitely sensitive to degradation in the low pH of the stomach and is generally administered orally with a buffer to induce a mucosal response (8). Therefore, it is unlikely that ingestion of CT by grooming causes the dramatic rise in Ab titers that we observe following TCI. Consistent with this argument, we observed that the IgG subclass responses following oral and transcutaneous immunization differed; oral immunization induced almost exclusively IgG1 and IgG2b Abs, whereas TCI induced a broad IgG subclass response (Fig. 4). Additionally, comparative immunization by either gavage or oral feeding failed to achieve Ab responses comparable with those induced by TCI. Moreover, TCI via the ear, which is a site less accessible to oral grooming, resulted in similar Ab responses, and radiolabeled CT studies suggested that the amount of Ag remaining on the skin after washing is negligible (our unpublished observations).

Protection against toxin-mediated diseases such as pertussis is known to be mediated in large part by antitoxin Abs (9). The role

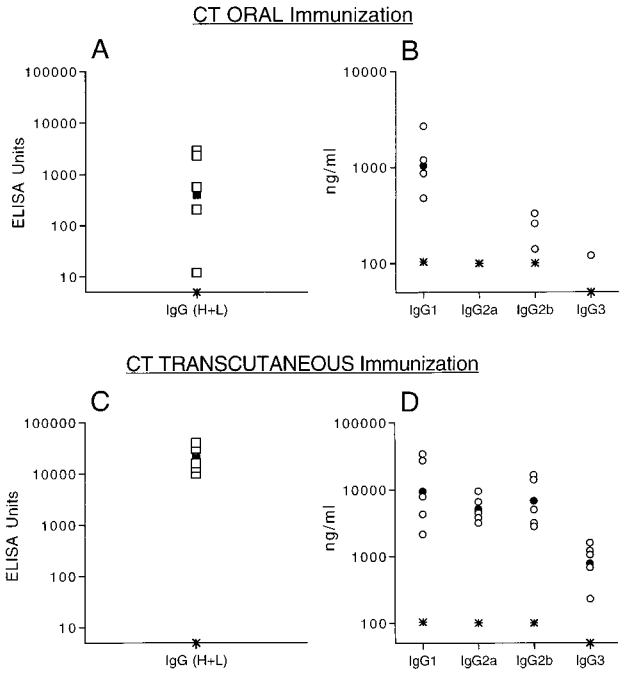

FIGURE 4. Characterization of sera Ab responses induced by oral (*A* and *B*) or transcutaneous (*C* and *D*) exposure to CT. BALB/c mice ($n = 5$) were immunized with 25 μg of CT by oral gavage or with 100 μg of CT by transcutaneous application to the back. Sera was collected after 4 wk, and the levels of CT-specific IgG, IgG1, IgG2a, IgG2b, and IgG3 were assessed by ELISA. The results shown are measurements from five individual animals (□, *A* and *C*; ○, *B* and *D*). Solid symbols indicate the geometric mean value for each cohort of animals. ∗, the mean value detected in prebleed sera of the mice.

of antitoxin immunity in protection against human cholera is not entirely clear (5, 10), but antitoxin immunity can be completely protective in animals (11–13) and clearly contributes to immunity in resistant humans (10). For example, dogs parenterally immunized with CT or cholera toxoid (14) or administered anti-CT IgG Abs parenterally (13) are protected against intragastric challenge with CT-producing strains of *V. cholerae*. Moreover, anti-CT IgA reduces rabbit ileal loop secretory responses to CT (15). Based on studies such as these, it is tempting to speculate that the Abs detected at the mucosa that are induced by TCI confer protection against toxin challenge. Consistent with this hypothesis, lung washes and stool samples from transcutaneously immunized mice exhibited elevated anti-CT IgG and IgA Ab levels (Fig. 3), and passive Ab transfer to naive mice was clearly protective (Table I).

The toxicity of CT administered via the mucosal route has limited its use as a vaccine component; consequently, studies on the protective role of anti-CT Abs have used less toxic but less immunogenic derivatives of CT such as its B subunit (CTB) (5, 16) and cholera toxoid (17). TCI may prove to be a powerful technique that elicits potent immune responses in the absence of obvious toxicity. Additional studies are warranted to assess the utility of TCI in human vaccines against infectious and toxin-mediated diseases, particularly cholera and traveler's diarrhea.

Acknowledgments

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