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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) 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. Stool 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.
observed in three independent experiments. Essentially identical results were obtained for 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. 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 supplicative 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. To exclude this possibility, we compared the immune response using 100 \(\mu\)g of CT by TCI with oral gavage using 25 \(\mu\)g 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 compared with those 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. 3A). 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 immununized 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 \(\mu\)g of CT by TCI with oral gavage using 25 \(\mu\)g 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 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. 3A). 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 immununized 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.

**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. 3A).

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

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>(n)</th>
<th>Survival at 9 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune sera</td>
<td>4</td>
<td>0/4</td>
</tr>
<tr>
<td>Immune sera</td>
<td>3</td>
<td>3/3</td>
</tr>
<tr>
<td>Hyperimmune sera</td>
<td>5</td>
<td>5/5</td>
</tr>
<tr>
<td>No sera</td>
<td>7</td>
<td>1/7</td>
</tr>
</tbody>
</table>

\(a\) Sera was collected from C57BL/6 mice either before immunization (preimmune), at 6 wk following TCI with 100 \(\mu\)g of CT at 0 and 3 wk (immune), or at 9 wk following TCI with 100 \(\mu\)g of CT at 0 and 3 wk and i.n. challenge with 20 \(\mu\)g 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 \(\mu\)g of CT (1 mg/ml) at \(-1\) h after the passive transfer of sera and observed for morbidity and mortality.
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 mg 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 mg of CT resulted in an anti-CT response of 39,828 EU. Similar results were obtained using 25 mg of CT on the unshaved ear, which is less accessible than the back for grooming, as compared with 25 mg 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
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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