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Transcutaneous Immunization with Toxin-Coregulated Pilin A Induces Protective Immunity against Vibrio cholerae O1 El Tor Challenge in Mice

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Cholera, a severe, dehydrating diarrhea in humans, is caused by the gram-negative bacterium Vibrio cholerae. Strains of V. cholerae that produce cholera belong to serogroup O1 or O139. V. cholerae O1 is comprised of two biotypes, classical and El Tor. Globally, O1-associated cholera is caused by the El Tor biotype. Cholera toxin (CT), the cause of the secretory diarrhea seen in cholera, is the major virulence factor for all toxigenic strains of V. cholerae (4). Toxin-coregulated pilus (TCP) is a second major virulence factor of V. cholerae. The major structural protein of this pilus, toxin-coregulated pilin A (TcpA), is encoded by tcpA, and its expression is regulated in V. cholerae in parallel to cholera toxin (39). TCP is essential for colonization and virulence in both animal models and human volunteers (18, 39), and recent data support its role in biofilm formation and binding to chitinous surfaces in aquatic environments (30). Although TcpA from El Tor and classical strains are approximately 80% homologous at the amino acid level, monoclonal antibodies have shown epitope differences between these proteins (19, 22, 31, 36). TcpA proteins from El Tor and O139 strains are identical (31).

A number of observations suggest that immune responses to TcpA may contribute to protection against V. cholerae infection. TcpA has been shown to be essential for V. cholerae colonization in both mice and humans (18, 39), tcpA mRNA is up-regulated during early human infection (27), and systemic and mucosal anti-TcpA immune responses occur in over 90% of individuals infected with V. cholerae O1 El Tor in Bangladesh (1, 16). Furthermore, passive administration of both polyclonal and monoclonal antibodies against TcpA in mice is fully protective against V. cholerae challenge (36, 37), and active parenteral immunization of adult female mice with a TcpA peptide along with an immunoadjuvant induces protective anti-TcpA immune responses. Anti-TcpA responses may contribute to an optimal cholera vaccine.
robust immune responses (10). TCI is thought to target antigen to Langerhans cells in the epidermis, which subsequently carry antigen to draining lymph nodes. TCI has been safe and well-tolerated in both animals and humans and has generated strong systemic immune responses in both (9, 11, 12, 44). TCI is also capable of eliciting mucosal immune responses, including inducing antigen-specific immunoglobulin A (IgA) antibody-secreting cells in circulation 1 week after topical application of antigen and inducing antigen-specific secretory IgA in a variety of mucosal sites in animals and humans (10, 12). In order to assess whether TCI would induce immune responses protective against cholera, we transcutaneously immunized mice with TcpA with or without the immunoadjuvant CT, measured anti-TcpA immune responses, and evaluated protection afforded by transcutaneous immunization with TcpA in a mouse cholera challenge model.

(The results of this study were presented in part at the 40th U.S.-Japan Cholera and Other Bacterial Enteric Infections Joint Panel Meeting, Boston, Mass., November 2005, and the 54th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Washington, D.C., December 2005.)

MATERIALS AND METHODS

Bacterial strains and media. We used an E. coli Origami strain (Novagen) for production of recombinant TcpA and wild-type El Tor Inaba V. cholerae O1 strain N16961 for the infant mouse challenge experiments. Both strains were maintained at −80°C in Luria-Bertani (LB) broth containing 15% glycerol. LB cultures contained streptomycin (100 μg/ml), ampicillin (100 μg/ml), tetracycline (12.5 μg/ml), or kanamycin (25 μg/ml), as appropriate.

Purification of TcpA. We amplifed via PCR DNA encoding TcpA (nucleotides 160 to 675) from V. cholerae strain C6706 by using oligonucleotides 5′-GTCATATGATTGGCAGAATATGACTAAGGCTGC-3′ (forward primer) and 5′-CCGCTCAGTGTTAACGTTTACCAAAGATCTGTGAAT-3′ (reverse primer) and gel purified the product using a gel extraction kit (QIAGEN, Valencia, CA). We then digested the product with NdeI and XhoI and gel purified again. We ligated the resultant fragment into pET-15b (Novagen, San Diego, CA) downstream of and in frame with the N-terminal His tag. The His tag extension contains a thrombin cleavage site and comprises a total of 21 residues; the final TcpA molecule lacks the prepropeptide cleavage site and 28 amino acids from the amino terminus of the mature TcpA molecule. We transformed the pET-15b vector into E. coli Origami cells. We diluted overnight LB broth cultures of this strain into fresh medium containing ampicillin, tetracycline, and kanamycin and grew cultures at 37°C with shaking at 220 rpm until an optical density at 600 nm of 0.6 was reached. We induced TcpA expression with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG), growing cells overnight at 30°C at 220 rpm. We then lysed cells by sonication and purified TcpA from the supernatant by using metal affinity chromatography (BD Talon metal affinity resin; BD Biosciences, Clontech, Mountain View, CA) followed by gel filtration on a HiPrep Sephacryl S-100 column (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). We concentrated the resulting product by using a Centricon concentrator (Millipore, Billerica, MA) to 1.5 mg/ml in a buffer containing 50 mM NaH2PO4 and 300 mM NaCl (pH 7.0) and assessed product purity by runing a C18 reverse-phase HPLC (Amersham Biosciences). We concentrated the final product by using metal affinity chromatography (BD Talon metal affinity resin; BD Biosciences, Clontech, Mountain View, CA) followed by gel filtration on a HiPrep Sephacryl S-100 column (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). We concentrated the resulting product by using a Centricon concentrator (Millipore, Billerica, MA) to 1.5 mg/ml in a buffer containing 50 mM NaH2PO4 and 300 mM NaCl (pH 7.0) and assessed product purity by runing a C18 reverse-phase HPLC (Amersham Biosciences).

Detection of immune responses. To detect antibody responses to TcpA, we coated microtiter plates overnight at room temperature with 50 ng of TcpA per well in 50 mM carbonate buffer (pH 9.6) and subsequently blocked the plates for 1 h at 37°C with phosphate-buffered saline (PBS)-1% ovalbumin (Sigma, St. Louis, MO). We added dilutions of sera (IgG, 1:1,000; IgA, 1:200) in PBS-0.1% ovalbumin-0.05% Tween 20 (Sigma) and incubated plates at 37°C for 1.5 h. We washed the plates and applied sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Amersham Biosciences) or goat anti-mouse IgA conjugated to HRP (Southern Biotech, Birmingham, AL) to each well. We then reincubated plates at 37°C for 1.5 h, washed them with PBS-0.05% Tween 20 (PBS-T), and developed them with a 0.55-mg/ml solution of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) with 0.05% H2O2 (Sigma). We then determined the optical density at 405 nm kinetically with a Vmax microplate reader (Molecular Devices Corp., Sunnyvale, CA) at a dilution of 5 min at 14-s intervals, reporting the maximum slope for an optical density change of 0.2 U as millioptical density units per minute, as previously described (21).

To detect antibody responses to CT, we sequentially coated microtiter plates with 1 μg of type III ganglioside (Sigma) in 50 mM carbonate buffer (pH 9.6) overnight and 100 ng CT (List Biological Laboratories, Campbell, CA) per well in PBS overnight. We then blocked these plates for 4 to 6 h at room temperature with PBS-1% ovalbumin. We added dilutions of sera (IgG, 1:5,000; IgA, 1:200) in PBS-0.1% ovalbumin-0.05% Tween 20 and incubated the plates overnight at room temperature. We then washed the plates and applied sheep anti-mouse IgG conjugated to HRP or goat anti-mouse IgA conjugated to HRP to each well. We incubated the plates at 37°C for 1 h, washed them with PBS-T, and developed them as described above.

To detect specific IgA antibody in stool, we first measured total stool IgA in samples. Duplicate serial twofold dilutions of stool samples (1:10 to 1:320) in PBS-T were added to wells previously coated with rat monoclonal anti-mouse IgA antibody C10-3 (Pharmingen, San Diego, CA) at a dilution of 1:1,000 (33). We then added a 1:1,000 dilution of goat anti-mouse IgA HRP-linked antibody to wells and developed the plates as described above. Comparisons were made to a mouse IgA standard (Kappa TEPC 15; Sigma). To detect specific anti-CT or anti-TcpA antibodies in stool, we added 75 ng of total stool IgA in PBS-T to wells in enzyme-linked immunosorbent assays identical to those used to measure serum anti-CT or anti-TcpA responses, and we processed these plates as described above.

Determination of immunization efficacy by use of the infant mouse challenge model. To establish a 50% lethal dose (LD50) for V. cholerae N16961 among offspring of immunized mice, we first inoculated 3- to 5-day-old CD-1 suckling mice born to immunized mothers with a range of V. cholerae N16961. In brief, when neonatal pups were 3 to 5 days old, we removed them from their mothers at least 3 h prior to inoculation. We then intrastrachally administered a 50-μl dose containing 2 × 106 to 2 × 107 CFU of wild-type El Tor V. cholerae N16961
(n = 5 to 35 mice/cohort). Following oral challenge, we kept neonates separate from dams at 30°C. We monitored animals every 3 to 6 h for 48 h and, after 48 h, euthanized any surviving animals. Using this protocol, we established that 10^6 CFU of *V. cholerae* N16961 resulted in 50% survival in offspring of unimmunized adult mice at 48 h (neonatal cohort size, 35 mice). Next, to determine if transcutaneous immunization with TcpA resulted in protection in neonatal mice born to dams immunized with TcpA and immunoadjuvant (CT) versus immunoadjuvant alone, we mated adult immunized CD-1 mice following the fourth transcutaneous immunization and boosted gravid mice 1 to 2 weeks prior to expected delivery (42). When neonatal pups were 3 to 5 days old, we removed them from their mothers at least 3 h prior to inoculation. We then intragastrically administered a 50-μl dose containing 2 × 10^6 CFU of wild-type El Tor *V. cholerae* N16961, representing approximately one LD50, monitored animals every 3 to 6 h for 48 h, and, after 48 h, euthanized any surviving animals.

**RESULTS**

**Systemic and mucosal anti-TcpA and anti-CT antibody responses in mice transcutaneously immunized with TcpA and/or CT.** In order to determine whether transcutaneous immunization with TcpA and an immunoadjuvant would elicit systemic anti-TcpA immune responses, we compared immune responses in sera collected on day 54 from mice given three transcutaneous immunizations of CT alone, CT and TcpA, or TcpA alone. Immunization with TcpA in the absence of CT did not induce a significant anti-TcpA IgG response. However, mice immunized with TcpA and CT had robust anti-TcpA IgG responses compared with mice immunized with TcpA alone (P < 0.01) or CT alone (P < 0.05) (Fig. 2A). We observed no significant anti-TcpA IgG in the group given TcpA and CT on day 33 (data not shown), indicating that three immunizations were necessary to produce a significant systemic response. Further immunizations on day 40 and later increased anti-TcpA IgG responses for the group immunized with TcpA and CT but not for either of the groups immunized with TcpA alone or CT alone. Interestingly, transcutaneous application of TcpA did not elicit a significant IgA response in day 54 serum either in the presence or in the absence of an immunoadjuvant (Fig. 2B). The administration of further booster immunizations did not alter this observation.

Mice that were transcutaneously immunized with CT or CT and TcpA developed significant serum IgG (P < 0.001) and IgA (P < 0.01) anti-CT responses compared to mice that were transcutaneously immunized with TcpA alone (Fig. 2C and D, respectively). Analysis of day 145 serum revealed that additional boosting, while raising serum IgG and IgA anti-CT levels overall, did not change the relative magnitudes of anti-CT responses in the three vaccine groups.

We did not observe significant anti-TcpA or anti-CT IgA responses in stool (data not shown).
colony formation of *V. cholerae* at the intestinal surface, and although TcpA apparently does not mediate direct contact of *V. cholerae* organisms to the intestinal surface, strains of *V. cholerae* deficient in TcpA are markedly attenuated and unable to colonize intestinal surfaces, suggesting that microcolony formation is required for full bacterial adherence and virulence (6, 18, 25, 35–40). TcpA is also the receptor for the filamentous bacteriophage that encodes cholera toxin (designated CTXφ) (41) and appears to be involved in *V. cholerae* biofilm production on chitinous surfaces in aquatic environments (30).

TCP is encoded on a 40-kb pathogenicity island (23), and expression of the genes in the TCP island, as well as expression of cholera toxin, is coordinately regulated by two pairs of membrane-localized proteins, ToxR/ToxS and TcpP/TcpH (3, 8), that both act via a common downstream regulatory protein within the TCP island, ToxT (8). During in vitro growth, expression of this virulence regulon requires specific environmental conditions for both the classical and the El Tor biotype of *V. cholerae* O1 (28). Recent work has demonstrated up-regulation of tcpA mRNA during infection of the upper human intestine (27), and TcpA was recently identified as an immunogenic antigen expressed in vivo during *V. cholerae* infection of humans in Bangladesh (16).

Some previous studies examining patients with cholera for immune responses to TcpA have either examined North American volunteers challenged with the classical biotype of *V. cholerae* O1 or utilized classical TcpA for immunoassays of patients infected with El Tor *V. cholerae* O1; these studies have shown low rates of seroconversion (15). Because classical and El Tor TcpA proteins share only 80% amino acid identity, however, these previous studies may have underestimated immune responses to TcpA in patients infected with El Tor *V. cholerae* O1, the current strain of pandemic cholera. Indeed, using El Tor TcpA as the antigen, we have recently documented systemic and mucosal anti-TcpA immune responses in over 90% of individuals infected with *V. cholerae* O1 El Tor in Bangladesh (1). Whether recurrent antigenic exposure and anamnestic responses contribute to the differences in immune responses observed between North American volunteers and patients who reside in areas where *V. cholerae* is endemic is currently unclear.

None of the cholera vaccines currently available or under development is yet able to reproduce the level of immunity seen following natural infection, and although not systematically assayed, none would be expected to induce anti-TcpA responses comparable to those observed to occur in individuals recovering from wild-type cholera, either because they contain minimal TcpA (killed oral vaccines) or because they are deleted for cholera toxin (live attenuated oral vaccines). This deletion removes the immunoadjuvant properties of cholera toxin compared to natural disease and may alter in vivo expression of other key proteins important to protective immunity following oral immunization.

In an effort to induce anti-TcpA responses, we thus investigated transcutaneous immunization, since this technology is simple, can be used to induce immune responses against selected important antigens, has been used to induce immune responses in humans, does not require the use of needles, and could be practical for development in the developing world, where cholera is endemic. We were able to establish that anti-
TcpA immune responses could be induced following coapplication of TcpA and cholera toxin to the skin of mice and that immune responses were protective in a mouse cholera challenge model. Whether such protection in neonatal pups related to the effects of translacental antibodies or antibodies in breast milk of immunized dams is currently unclear. Of note, Wu et al. (42) also found protection to occur in offspring of dams immunized parenterally with a TcpA pilin peptide polymer and adjuvant, although there were measurable IgA responses in vaccinated dams in this experiment. Also, a number of researchers have assayed protection in neonatal mice by using passively administered convalescent-phase sera from immunized and unrelated adult mice (the neonates were, therefore, not ingesting breast milk of immunized dams, suggesting that protection was present in the serum fraction) (2, 5). In the latter experiments, convalescent-phase sera of immunized animals were mixed with the V. cholerae challenge inoculum prior to oral administration to neonates. Previous investigators have also shown that mixing purified anti-V. cholerae IgG with the oral inoculum is protective (17) and that neonatal intestines contain Fc receptors that mediate bidirectional transport of serum IgG into the intestinal lumen (43). Since V. cholerae is a noninvasive organism, our results suggest that protection may reflect the presence of serum antibodies in the intestinal lumina of neonatal mice via the Fc receptor-mediated transportation or the presence in intestinal lumina of unassayed antibodies in ingested breast milk.

Interestingly, although transcutaneous application of CT induced serum IgA responses in addition to IgG responses in our experiment, transcutaneous application of TcpA did not induce serum IgA responses, despite induction of prominent anti-TcpA IgG responses. We found that additional immunizations with TcpA did not alter this observation. In this experiment, we were also unable to detect significant anti-TcpA or anti-CT IgA responses in stool samples of immunized animals. Despite these observations, transcutaneous immunization has been used previously to elicit mucosal immune responses, as evidenced by the detection of IgA antibody-secreting cells in circulation 1 week after topical application and subsequent secretory IgA antibody in a variety of mucosal sites in animals and humans (14, 44).

In this feasibility study, we did not evaluate various doses or immunization regimens; however, previous work has found induction of immune responses following transcutaneous immunization with as little as 1 µg of antigen and 10 µg of immunoadjuvant (13, 29). Previous work has also shown that, when antigen is used alone, three or four transcutaneous applications of antigen are usually required to induce immune responses to topically applied antigens (10, 12, 14). In the present study, we observed a similar result, i.e., three transcutaneous applications of TcpA and CT were required to induce anti-TcpA immune responses in mice. We have shown previously with mice that the combination immunization strategy of priming with an oral, live, attenuated V. cholerae vaccine followed by transcutaneous immunization with a purified antigen induces prominent systemic and mucosal immune responses (20), suggesting that the combination of oral cholera vaccine administration and transcutaneous immunization with TcpA may warrant further evaluation. In summary, our current findings suggest that transcutaneous immunization with TcpA is feasible, immunogenic, and protective in mice. The role of anti-TcpA responses in the development of an optimal cholera vaccine warrants further investigation.

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