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Short communication

Evaluation of transcutaneous immunization as a delivery route for an enterotoxigenic *E. coli* adhesin-based vaccine with CfaE, the colonization factor antigen 1 (CFA/I) tip adhesin

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ABSTRACT

dscCfaE is a recombinant form of the CFA/I tip adhesin CfaE, expressed by a large proportion of enterotoxigenic *E. coli* (ETEC). It is highly immunogenic by the intranasal route in mice and *Aotus nancymaae*, protective against challenge with CFA/I+ ETEC in an *A. nancymaae* challenge model, and antibodies to dscCfaE passively protect against CFA/I+ ETEC challenge in human volunteers. Here, we show that transcutaneous immunization (TCI) with dscCfaE in mice resulted in strong anti-CfaE IgG serum responses, with a clear dose-response effect. Co-administration with heat-labile enterotoxin (LT) resulted in enhanced immune responses over those elicited by dscCfaE alone and strong anti-LT antibody responses. The highest dose of dscCfaE administered transcutaneously with LT elicited strong HAI titers, a surrogate for the neutralization of intestinal adhesion. Fecal anti-adhesin IgG and IgA antibody responses were also induced. These findings support the feasibility of TCI for the application of an adhesin-toxin based ETEC vaccine.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is one of the leading causes of travelers' diarrhea and endemic childhood diarrhea in developing countries [1,2]. ETEC adhere to intestinal epithelial cells via one or more colonization factors (CFs), and produce either or both heat-labile (LT) and heat-stabile (ST) enterotoxins, leading to fluid and electrolyte secretion. The CFs and enterotoxins are thus targets of current vaccine development efforts.

CFA/I is one of the most prevalent CFs in endemic regions [3,4], and is thought to promote colonization through its tip adhesin subunit, CfaE. We previously developed a stable, monomeric variant of CfaE (dscCfaE) using *in cis* donor strand complementation [5]. When applied intranasally (IN) with an adjuvant, dscCfaE is highly immunogenic in mice and the non-human primate, *Aotus nancymaae* [6,7]. Antibodies against dscCfaE inhibit agglutination of bovine erythrocytes by CFA/I+ ETEC, a surrogate for inhibition of intestinal adhesion by ETEC fimbriae [6,7]. IN immunization with dscCfaE protects *A. nancymaae* against oral challenge with CFA/I+ ETEC strain H10407 [7] and orally administered bovine colostrum containing anti-CfaE IgG protects human volunteers against H10407 ETEC challenge [8], supporting the further evaluation of this fimbrial adhesin-based vaccine.

Identifying a vaccination route that elicits strong, protective mucosal immune responses while being safe and dose-sparing, is critical to the development of an effective ETEC vaccine. Safety concerns associated with IN immunization [9] and the inefficiencies of orogastric administration both in terms of eliciting mucosal responses in a dose-sparing manner and practical administration in the field has motivated the evaluation of alternative vaccination routes for enteropathogens. Transcutaneous immunization (TCI), whereby antigens are applied directly to the intact skin [10], has been shown to induce strong systemic and mucosal immune responses in animals [10–12] and humans [13–15], particularly

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when co-administered with LT [16]. TCI with a variety of antigens can elicit protective responses, is safe, and dose-sparing [11,17–19]. Here, we demonstrate that transcutaneous immunization with dscCfaE is highly effective at inducing serum and fecal antibody responses against an ETEC fimbrial adhesin, supporting further evaluation in a human challenge model.

2. Materials and methods

2.1. Reagents

Cloning, expression and purification of dscCfaE was carried out as previously described [5]. The lot (>95% purity) used was low in endotoxin content (9.4 EU per mg). The non-toxic mutant of heat-labile enterotoxin, LT(R192G), was cloned, expressed and purified from *E. coli* strain JM83(pLC326) at the Walter Reed Army Medical Research Center Bioproduction Facility (Forest Glen Annex, Silver Spring MD). Wildtype LT from enterotoxigenic *E. coli* was produced by Iomai Corporation.

2.2. Immunization of mice

Groups of 10 female Balb/c mice, ages 6–8 weeks (Charles River Laboratories, Wilmington, MA), were immunized by the TCI route on days 1 and 15. Animal experiments were done under contract with Gene Logic, Inc., under the oversight of their IACUC and in accordance with all applicable regulations. As a bridge to previous studies, one group was immunized IN with 25 μg dscCfaE + 1.5 μg of LT(R192G) as previously described [6]. Seven groups were immunized by TCI with 1, 5 or 25 μg dscCfaE ± 5 μg LT as previously described [16] with the exception that the immunization site was pretreated with emery paper (10-strokes) to disrupt the stratum corneum prior to hydration, and patches were left on for 18–24 h.

2.3. Immunological sampling

Blood was collected via tail vein on days −2, 14, 29 and cardiac puncture on day 30, separated by centrifugation and serum was stored at −20 °C. Fecal samples were collected on day 30 and 5–6 pellets added to a 15 ml tube containing 900 μl PBS with 5 μg/ml of phenylmethylsulphonyl fluoride. Samples were maintained on ice with vortexing every 15 min over 30 min, then centrifuged and supernatant was collected and stored at −20 °C.

2.4. Detection of immune responses

Anti-dscCfaE IgG and IgA serum antibodieswere detected by coating microtiter plates at 37 °C for 1 h and subsequently overnight at 4 °C (IgG) or 37 °C (IgA) with 200 ng of dscCfaE per well. Plates were blocked and sera were added in three-fold serial dilutions, beginning with a dilution of 1:50. Plates were incubated at RT for 90 min, washed and goat anti-mouse IgG or IgA HRP conjugate was applied to each well at a 1:1000 dilution. Plates were incubated at 37 °C (IgG) or at RT (IgA) for 90 min, washed and developed with orthophenylenediamine (Sigma-Aldrich) (IgG) or 1-Step Ultra TMB (Thermo Fisher Scientific, Waltham, MA) (IgA) as per manufacturer's instructions. The optical density at 450 nm was determined and endpoint titers were calculated as the reciprocal of the interpolated dilution giving an A450 of 0.4 optical density (O.D.) units above background. Antibody titers ascribed to each sample represents the mean of duplicate samples, run on consecutive days. The reciprocal of the lowest dilution of sera was 50, and samples with a titer of <50 were assigned a value of 25 for computational purposes.

To detect serum anti-LT IgG and IgA and fecal anti-dscCfaE IgG and IgA antibodies, microtiter plates were coated overnight at 4 °C with 100 ng/well of LT or dscCfaE. Plates were washed, blocked for 2 h at RT, washed, and diluted samples (1:100 sera, 1:2 fecal IgA, or 1:10 fecal IgG) were added in two-fold serial dilutions. Plates were incubated at 4 °C overnight, washed and goat anti-mouse IgG or IgA HRP conjugate (1:1000 dilution) was added to each well. Plates were incubated at RT for 2 h, washed, developed with ABTS 2-component substrate (KPL, Seracare, Milford, MA) and read at 405 nm. Data from anti-LT ELISAs were reported as ELISA Units (EU), defined as the inverse dilution of serum that yielded an optical density of 1.0. An animal was designated a fecal antibody responder if the OD from the initial dilution of fecal extract was greater than the calculated baseline activity from the PBS control animals (calculated by the mean + two times the SD of the ODs).

Hemagglutination Inhibition (HAI) assays were performed on day 30 sera as previously described [3]. The HAI titer was expressed as the reciprocal of the highest dilution of sera that completely inhibited mannose-resistant hemagglutination. The reciprocal of the lowest dilution of sera tested was 32, and samples with a titer of <32 were assigned a value of 16 for computational purposes.

2.5. Statistical and graphical analyses

Serum ELISA and HAI titers were normalized by a $\log_{10}$ transformation prior to statistical analyses. Data from the PBS control group and six test groups were compared using an ANOVA followed by Tukey's *post hoc* tests and $P < 0.05$ was regarded as significant. The number of fecal antibody responders/non-responders were compared between specific groups using Fisher's exact tests with Bonferroni alpha corrections (alpha = 0.007). The IN group was excluded from all analyses as it served as a positive control and bridge to previous experiments. All statistical analyses and graphing were performed using GraphPad Prism Version 6.0e for Mac OS X (Graphpad Software Inc., San Diego, CA).

3. Results

3.1. Transcutaneous immunization with dscCfaE results in systemic and functional serum antibody responses

Immunization with dscCfaE by the TCI route at all dose levels resulted in maximal anti-CfaE antibody titers two weeks after the second vaccination (day 30) (Fig. 1A). On day 30, strong serum anti-adhesin IgG antibody titers were observed in all TCI test groups, with titers significantly greater than those in the PBS control group (all $P < 0.001$) (Fig. 1B). There was a dose-response effect in the three unadjuvanted groups; however, only the 1 and 25 μg group titers were significantly different ($P < 0.05$). In groups receiving 1, 5, and 25 μg of dscCfaE admixed with 5 μg LT, anti-CfaE IgG titers were not significantly different from each other, suggesting responses had reached maximal levels. Addition of LT resulted in an increase in responses at all dose levels, however only responses in the two groups receiving 1 μg of dscCfaE ± LT were significantly different ($P < 0.001$). TCI with dscCfaE ± LT generated serum anti-CfaE IgA antibody levels that were significantly greater than those in the PBS group at all dose levels (all $P < 0.001$) (Fig. 1C), and titers were significantly greater in groups receiving 5 μg of antigen than groups receiving 1 μg of antigen, for both adjuvanted and unadjuvanted conditions ($P < 0.05$ and $P < 0.001$, respectively).

The group receiving 25 μg of dscCfaE + LT by TCI mounted strong HAI responses two weeks after the second dose (Fig. 1D). While HAI titers in the three adjuvanted groups displayed a clear dose response, only titers in the 25 μg dscCfaE + LT group were significantly greater than those in the PBS control group ($P < 0.001$).

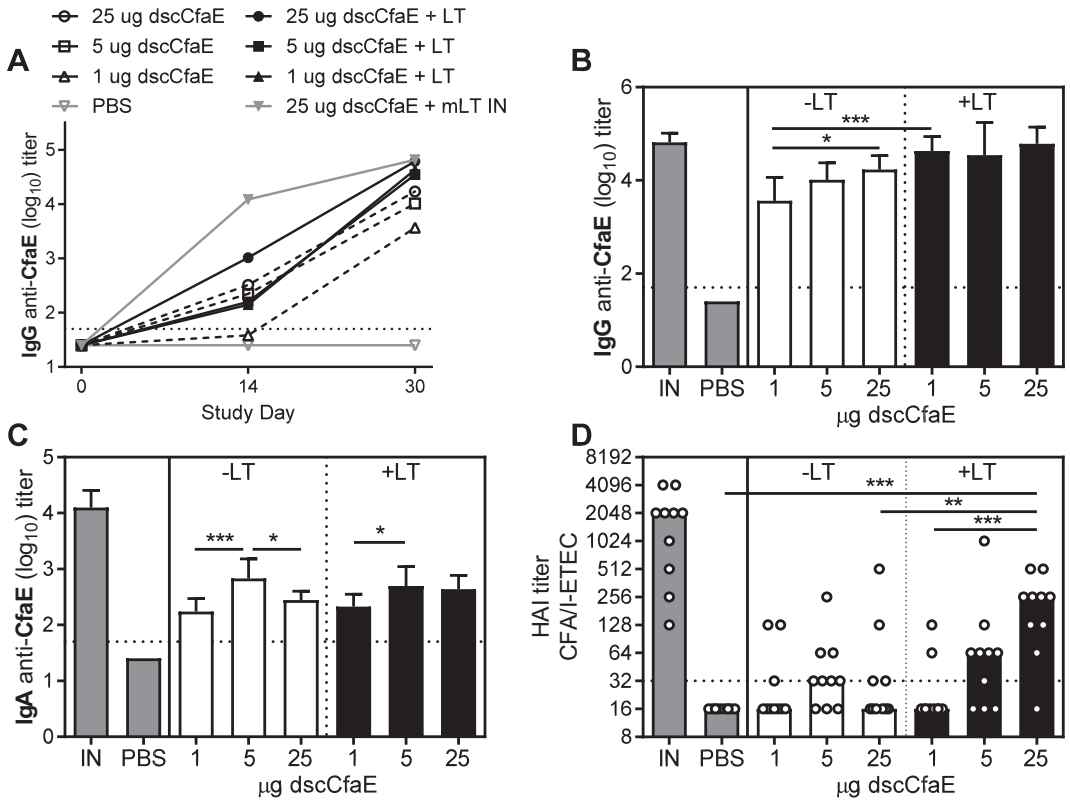

Fig. 1. Serum anti-CfaE antibody responses in mice immunized with dscCfaE and LT. Ten mice per group were immunized with two doses (days 1, 15) by the TCI route with dscCfaE (25, 5 or 1 μg) ± LT (5 μg), or PBS on wet patches, or by the IN route with dscCfaE (25 μg) + LT(R192G) (1.5 μg). White or black bars or lines represent dscCfaE given alone or with LT, respectively. Intranasal and PBS controls are represented by gray bars or lines. (A) Anti-CfaE IgG end point ELISA titers are shown for sera taken at two-week intervals from day −2 through 30. (B) Anti-CfaE IgG and (C) anti-CfaE IgA end point ELISA titers are shown for sera taken two weeks after the second dose (day 30). For A–C, values are the mean $\log_{10}$ end point titers + SD and the horizontal dotted lines (1.7) denote the lowest dilution tested. SD lines are not shown in (A) for purposes of clarity. (D) Functional antibody titers are shown, as measured by the HAI assay using CFA/I-ETEC (H10407) and bovine red blood cells. Values are the medians with individual data points shown as closed circles. The dotted line (1:32) denotes the lowest dilution of serum tested. Titers shown are from sera taken two weeks after the second dose (day 30). For B-D, statistical significance was determined by using one-way ANOVA and Tukey's multiple comparisons test, for comparisons between antigens given with and without adjuvants and the PBS group. Asterisks denote a significant difference between responses in the indicated groups (*p < 0.05; **p < 0.01, ***p < 0.001).

3.2. Transcutaneous immunization with LT results in strong anti-LT serum antibody responses

Immunization with 5 μg LT resulted in strong anti-LT IgG antibody responses two weeks following the second vaccination (day 30) (Fig. 2A). Anti-LT IgG antibody titers in all adjuvanted groups were significantly greater than those of the PBS group and corresponding unadjuvanted group ($P < 0.001$). The 5 μg dscCfaE + LT group had a significantly greater anti-LT IgG antibody response than the other two adjuvanted groups. TCI with LT led to anti-LT serum IgA antibody titers that were significantly greater than those of the PBS group and corresponding unadjuvanted group (Fig. 2B) ($P < 0.001$).

3.3. Transcutaneous immunization with dscCfaE results in fecal anti-CfaE antibody responses

Positive anti-CfaE IgG fecal responses were observed in all TCI groups except the 1 μg dscCfaE alone group (Fig. 3A, Suppl. Fig. 1A–H). In the unadjuvanted groups, increases in the number of responders were observed with increasing dose level, with the increase from 1 μg to 5 μg being significant ($P < 0.007$). Co-administration of LT resulted in an increase in the number of positive fecal IgG responders. Animals given 1 μg dscCfaE in the absence of LT, failed to develop any detectable IgA fecal response, while groups given 5 and 25 μg dscCfaE had positive anti-CfaE IgA mucosal responses in 10 and 30% of animals, respectively (Fig. 3B, Suppl. Fig. 2A–H). Co-administration of LT increased the number of animals with positive anti-CfaE IgA antibody responses in all groups, and the group given 5 μg dscCfaE with adjuvant had a significantly greater number of responders compared to both the unadjuvanted 5 μg dose group and the adjuvanted 1 μg group ($P < 0.007$ for both).

4. Discussion

Current efforts by our group to develop an ETEC vaccine are focused on the incorporation of fimbrial adhesins in a multivalent vaccine platform. Key to this is the identification of a route of immunization that provides maximal protection, while being dose-sparing and easily administered. Here, we demonstrate that dscCfaE, a recombinant, monomeric form of the CFA/I tip adhesin, is highly immunogenic by the transcutaneous route in mice, eliciting anti-CfaE antibodies in both serum and feces. Importantly, dscCfaE administered TCI led to systemic functional anti-adhesive antibody responses as inferred by the inhibition of H10407 ETEC mediated erythrocyte hemagglutination, suggesting that the elicited antibodies recognize native CfaE.

Development of an ETEC vaccine has been complicated by the considerable heterogeneity of pathogenic strains and the CFs and toxins they express. Previous studies showed LT alone applied via TCI was not protective against diarrhea in volunteers challenged with an LT+/ST+ ETEC strain [15] and in travelers infected with LT+/ST+ or ST+ only strains [20]; however, it was protective in travelers against LT+ only strains. Broad protection against this enter-

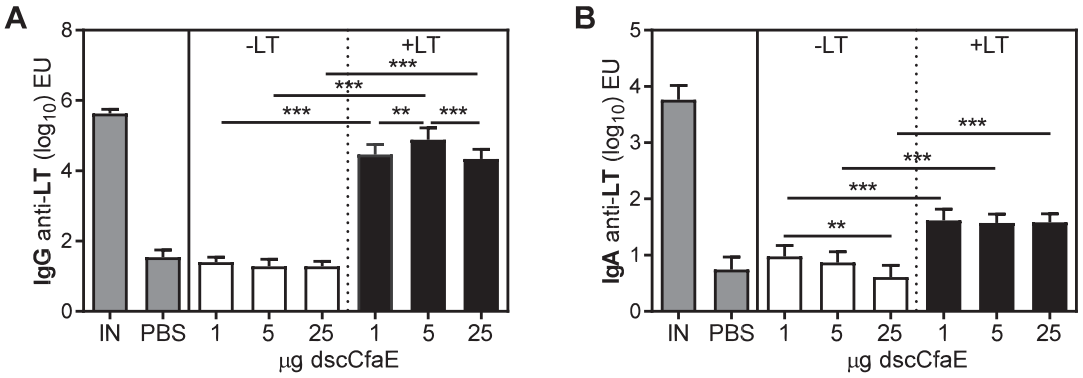

Fig. 2. Serum anti-LT antibody responses in mice immunized with dscCfaE and LT. Ten mice per group were immunized with two doses (days 1, 15) by the TCI route with dscCfaE (25, 5 or 1 μg) ± LT (5 μg), or PBS on wet patches, or by the IN route with dscCfaE (25 μg) + LT(R192G) (1.5 μg). (A) Anti-LT IgG, and (B) anti-LT IgA end point ELISA titers are shown. All titers shown are from serum taken two weeks after the second dose (day 30), with white or black bars/lines represent dscCfaE given alone or with LT, respectively. Intranasal and PBS controls are represented by gray bars/lines. Values are the mean $\log_{10}$ ELISA Units + SD. Statistical significance was determined by using one-way ANOVA and Tukey's multiple comparisons test, for comparisons between antigens given with and without adjuvants and the PBS group. Asterisks denote a significant difference between responses in the indicated groups ($^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$).

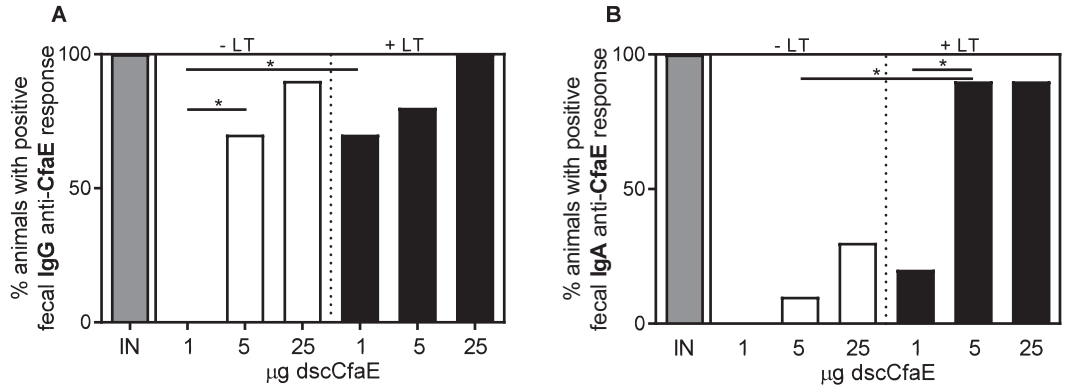

Fig. 3. Fecal anti-CfaE antibody responses in mice immunized with dscCfaE ± LT. Ten mice per group were immunized with two doses (days 1, 15) by the TCI route with dscCfaE (25, 5 or 1 μg) ± LT (5 μg), or PBS on wet patches, or by the IN route with dscCfaE (25 μg) + LT(R192G) (1.5 μg). Fecal samples were collected on day 30, two weeks following the second immunization. White and black bars represent dscCfaE given alone or with LT, respectively. Intranasal control is represented by gray bars. Samples were serially diluted and evaluated by ELISA for presence of anti-CfaE IgG (A) and IgA (B) antibody activity. Optical density measurements (O.D.) of the samples were recorded. Values graphed represent the percent of animals that had a positive fecal antibody response, where a positive response is that value greater than or equal to the mean plus two standard deviations of the responses in PBS control animals. Statistical significance was determined by using Fisher's exact test with a Bonferroni alpha correction (alpha = 0.007), for comparisons between antigens given with and without adjuvants and between dose groups. Asterisks denote a significant difference between responses in the indicated groups ($^{*}p < 0.007$).

opathogen may require a vaccine that incorporates key CF antigens [4] and we hypothesize that the genetically conserved tip adhesins would provide greater heterologous protection, reducing the required valency of a subunit vaccine [3]. Incorporation of key ETEC adhesins into an LT-based vaccine may strengthen coverage against LT+ only strains while extending coverage to key ST-expressing ETEC strains. Further, while we utilized wild-type LT in the present study for continuity with previous work, evaluation of protective efficacy of the more clinically safer LT mutants LTR192G and LTR192G/L211A applied transcutaneously is warranted. Our group has established a favorable stability profile of dscCfaE, and here we demonstrate that in the murine model, this antigen is highly immunogenic when given via the transcutaneous route. These findings support the further clinical evaluation of a combined dscCfaE-LT-based TCI ETEC vaccine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.08.057.

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