Immunogenicity of a prototype enterotoxigenic *Escherichia coli* adhesin vaccine in mice and nonhuman primates

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) are the most common cause of bacterial diarrhea in young children in developing countries and in travelers. Efforts to develop an ETEC vaccine have intensified in the past decade, and intestinal colonization factors (CFs) are somatic components of most investigational vaccines. CFA/I and related Class 5 fimbrial CFs feature a major stalk-forming subunit and a minor, antigenically conserved tip adhesin. We hypothesized that the tip adhesin is critical for stimulating antibodies that specifically inhibit ETEC attachment to the small intestine. To address this, we compared the capacity of donor strand complemented CfaE (dscCfaE), a stabilized form of the CFA/I fimbrial tip adhesin, and CFA/I fimbriae to elicit anti-adhesive antibodies in mice, using hemagglutination inhibition (HAI) as proxy for neutralization of intestinal adhesion. When given with genetically attenuated heat-labile enterotoxin LTR192G as adjuvant by intranasal (IN) or orogastric (OG) vaccination, dscCfaE exceeded CFA/I fimbriae in eliciting serum HAI titers and anti-CfaE antibody titers. Based on these findings, we vaccinated *Aotus nancymae* nonhuman primates (NHP) with dscCfaE alone or admixed with one of two adjuvants, LTR192G and cholera toxin B-subunit, by IN and OG administration. Only IN vaccination with dscCfaE with either adjuvant elicited substantial serum HAI titers and IgA and IgG anti-adhesin responses, with the latter detectable a year after vaccination. In conclusion, we have shown that dscCfaE elicits robust HAI and anti-adhesin antibody responses in both mice and NHPs when given with adjuvant by IN vaccination, encouraging further evaluation of an ETEC adhesin-based vaccine approach. (249 words)

Keywords: Enterotoxigenic *Escherichia coli*; CFA/I fimbriae; CfaE; Vaccine; Mice; Nonhuman primates

INTRODUCTION

Globally, enterotoxigenic *Escherichia coli* (ETEC) is a leading cause of secretory bacterial diarrhea. Children in developing countries experience a majority of the attributable morbidity and mortality (1-3), while ETEC is also the principal cause of travelers' diarrhea (4, 5). Expression of intestinal colonization factors in concert with heat-labile (LT) and/or heat-stable (ST) enterotoxin constitutes the hallmarks of ETEC pathogenesis that distinguish it from other pathotypes of diarrheagenic *E. coli* (6). The substantial public health impact of ETEC disease has fostered escalating efforts to develop an effective vaccine.

Many candidate vaccines against human ETEC have undergone clinical evaluation, but none have reached broad licensure (7-14). Except for LT toxin-based-only vaccines, other approaches have targeted the prevalent ETEC colonization factors, wherein the intact fimbrial organelle has served as the immunogen in either purified form or as expressed on the surface of inactivated or live, attenuated bacteria. As molecular and atomic details have emerged on the structure and function of CFA/I and related class 5 fimbriae and other prevalent ETEC CFs (15-19), it has provided a framework for development of an adhesin-based protein subunit vaccine. CFA/I, the archetypal class 5 fimbrial CF, comprises a single copy of the minor subunit CfaE affixed to the tip of a polymerized helical stalk of the major subunit CfaB (16). This two-domain Ig-like subunit has been implicated in intestinal adherence, predicting a pivotal role early in pathogenesis (18, 20).

We engineered a stabilized derivative of CfaE by *in cis* donor strand complementation (17, 18). Donor strand complemented CfaE (dscCfaE) is functionally competent as an adhesin and upon rabbit immunization elicited antibodies that inhibit erythrocyte agglutination, a simplified model for intestinal adhesion (18, 21). Due to evidence that CFA/I and other genetically related

class 5 fimbriae are critical virulence determinants of ETEC diarrhea and prevalent in many endemic regions, we undertook the present studies to determine if dscCfaE is as effective as whole CFA/I fimbriae in eliciting anti-adhesive immune responses upon mouse mucosal vaccination. With affirmative results in mice, we conducted pilot experiments to assess immune responses after dscCfaE mucosal vaccination in nonhuman primates (NHP).

MATERIALS AND METHODS

Vaccine and adjuvant preparations. Native CFA/I fimbriae were purified from wild-type ETEC strain WS1933D (O71;H-; CFA/I; STh) (22). The strain was grown in liquid, minimal media at 37°C until the late logarithmic phase of growth. A CFA/I fimbrial preparation (>98% purity) was derived by serial heat treatment (62°C for 20 minutes) and ammonium sulfate precipitation (25% saturation). Treatment with Triton X-114 yielded a final product low in endotoxin (730 endotoxin units (EU) per mg). The recombinant CFA/I fimbrial tip adhesin dsc₁₉CfaE[His]₆ (referred throughout as dscCfaE) was produced and purified using a previously described expression clone and production methods (18). The dscCfaE lots ($\geq$ 95% purity) used here were low in endotoxin content (16 EU per mg). LTR192G (hereafter referred to as mutant LT or mLT) was produced from *E. coli* strain JM83(pLC326) (23). Endotoxin content of this material was 24 EU per mg. Cholera toxin B-subunit (CTB) (99% purity; $\leq$ 6 EU/mg) was purchased from Sigma-Aldrich (St. Louis, MO).

Mouse vaccination and sample collection. Mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Naval Medical Research Center. Female BALB/c mice, aged 6-8 weeks (Charles River Laboratories, Wilmington MA) were quarantined for 7 days before use. Groups of ten mice each were immunized on days 0, 14 and

28 by the intranasal (IN) route with 25 µg CFA/I or dscCfaE ± 1.5 µg mLT, or by the orogastric (OG) route with 250 µg CFA/I or dscCfaE ± 10 µg mLT. For each route, control groups were vaccinated with mLT only (at the same dose for each route) or PBS. For IN vaccination, mice were anesthetized with Isoflurane (Halocarbon Products Corporation, River Edge NJ), and the vaccine preparation (in 20 µl) was delivered by pipette into the external nares (10 µl per nare). For OG vaccination, gastric acidity was neutralized by gavage with 0.5 ml of 0.6 M sodium bicarbonate. Immediately thereafter, the vaccine preparation (in 0.5 ml) was delivered OG through a sterile plastic feeding tube. Blood was sampled by tail bleeding before the first vaccination (day 0) and at days 14 and 28. Two weeks after the third dose, (day 42), animals were anesthetized with ketamine-xylazine (Phoenix Scientific, Inc., St. Josephs MO), bled by cardiac puncture and euthanized by cervical dislocation. Clotted blood samples were centrifuged, and the separated serum was stored at -20°C until use.

***Aotus nancymae* source, selection, vaccination and sample collection.** Experiments involving *A. nancymae* nonhuman primates (NHP) were approved by the IACUC at the U.S. Naval Medical Research Unit No. 6 (NAMRU-6), Lima, Peru. Captive born *A. nancymae* were purchased from the Instituto Veterinario de Investigaciones Tropicales y de Altura, University of San Marcos, Peru. At NAMRU-6, animals were housed in an AALAAC-accredited Laboratory Animal Facility and cared for by the veterinary staff as described by Jones *et al* (24). Animals were quarantined for 60 days before vaccination. Animals previously used in an ETEC vaccination or challenge study, or with a reciprocal serum anti-CFA/I IgG titer >200 as measured by ELISA four weeks pre-vaccination were excluded. For IN vaccination, nine animals (4 males and 5 females), with a median age of 39 months (IQR, 19.5-41) and mean weight of 1292 g (SD, 219.7) were selected from the NAMRU-6 primate pool. In the OG vaccination experiment, 23

animals (10 males and 13 females) with a median age of 20 months (IQR, 20-22) and mean weight of 975.7 g (SD 131.5) were similarly selected.

For IN vaccination, animals were lightly anesthetized with ketamine hydrochloride (10 mg/kg weight, Ketalar, Parke-Davis), and the vaccine preparation (in 100 μ l) was delivered with a pipette (50 μ l per nare). Nine animals were randomly assigned into three groups that received the following vaccine regimens on days 0, 14, and 28 by the IN route: (1) 0.2 mg dscCfaE, (2) 0.2 mg dscCfaE plus 0.29 mg CTB, and (3) 0.2 mg dscCfaE plus 10 μ g mL_T. Blood samples were collected by femoral venipuncture at baseline (day 0) and on days 7, 21, 35, 42, 132, 217, 312 and 356 after the start of vaccination. For OG delivery, animals were anaesthetized with ketamine hydrochloride (10mg/kg) and treated with ranitidine (1.5mg/kg) and CeraVacx buffer (Cera Products, Inc., Columbia, MD) to neutralize gastric acidity. Vaccine preparations were administered in 5 ml PBS. Twenty animals were randomly assigned into four groups that received the following vaccine regimens on days 0, 14, and 28 by the OG route: (1) 1 mg dscCfaE, (2) 1 mg dscCfaE plus 1.46 mg CTB, (3) 0.5 mg dscCfaE, and (4) 0.5 mg dscCfaE plus 0.73 mg CTB. A control group of three animals received PBS alone. Blood samples were collected under anesthesia by femoral venipuncture at baseline (day 0) and on days 7, 21, 35 and 42 after the start of vaccination. Serum was separated from clotted blood samples and stored at -20°C until use.

Analysis of antibodies in mouse and *A. nancymae* serum samples by ELISA. Nunc, flat-bottom 96-well plates were coated with 100 μ l of dscCfaE or CFA/I diluted in PBS or carbonate buffer, and incubated either for one hour at 37°C and overnight at 4°C (mouse) or overnight at 37°C (NHP). Plates were washed with PBS, blocked with 5% fetal calf serum/PBS/0.05% Tween 20 for 60 min (mouse) or with 0.1% BSA/PBS/0.05% Tween 20 for 30 min (NHP) at

37°C, and washed with PBS 0.05% Tween 20. Starting at 1:50 (mouse) or 1:5 (NHP) dilutions, serum samples were serially diluted 3-fold, 100µl/well, and plates were incubated 90 min at room temperature (RT). After washing with PBS 0.05% Tween 20, plates were incubated with HRP-conjugated goat anti-mouse IgG or IgA (1:1000) for 120 min (CfaE IgA, mouse) or 90 min at 37°C (mouse) or HRP-conjugated rabbit anti-*A. nancymaae* IgG or IgA (1:4000) RT (NHP), washed, and developed with orthophenylenediamine, as per the manufactures instructions. *A. nancymaae* serum IgG antibody levels against CTB were analyzed by GM1 ELISA as previously described (25).

For all assays, A_{450nm} were read with a Multiskan Ascent microplate reader (Thermo Fisher Scientific, Waltham MA). The absorbance values for each sample were plotted, and a curve was fitted to the data points using Ascent Software (Thermo Scientific). The background for each plate was defined as the mean A_{450nm} of 8 wells incubated with all components of the ELISA except serum samples, and the cutoff for each plate was defined as the A_{450nm} background + 0.4. The dilution of each sample corresponding to the cutoff was interpolated from the graph, and the titer defined as the reciprocal dilution. Reciprocal titers <50 (mouse) and <5 (NHP), the lowest dilutions tested, were assigned values of 25 and 2.5, respectively, for computational purposes. Final titers represented the mean of duplicate analyses performed on separate days.

Hemagglutination inhibition assay. A hemagglutination inhibition (HAI) assay was used as the proxy for neutralization of fimbriae-mediated adhesion. Mouse and NHP serum samples were tested as previously described (15). CFA/I-ETEC strain H10407 and bovine erythrocytes were used in these assays. The HAI titer was defined as the reciprocal of the lowest dilution of serum that inhibited agglutination. Values below 1:32, the lowest dilution tested, were arbitrarily assigned a reciprocal titer of 16 for computational purposes.

Statistical analysis. HAI and ELISA titers were log₁₀-transformed for statistical analysis. Group titers were compared using a one-way analysis of variance (ANOVA), and over time using a repeated measures ANOVA or paired T test. A Tukey's post-hoc test was used for pairwise comparisons. All statistical analyses were performed using GraphPad Prism Version 6.0e for Mac OS X (Graphpad Software, San Diego, CA), and a *P* value <0.05 was considered significant.

RESULTS

Mouse mucosal vaccination with CFA/I fimbriae vs. dscCfaE adhesin. By both IN and OG vaccination, maximal anti-CfaE adhesin and anti-CFA/I fimbrial antibody titers were observed two weeks after the third dose (day 42) (Fig. 1). On day 42, higher serum anti-adhesin and anti-fimbrial antibody titers were observed in groups receiving the corresponding adhesin and fimbrial antigen, respectively. The highest serum anti-CfaE titers followed vaccination with dscCfaE + mLT by IN (all *P* < 0.001) and OG (all *P* < 0.01) administration (Fig. 2A, 2C). Likewise, the highest serum anti-CFA/I titers occurred in the groups vaccinated with CFA/I + mLT by both the IN (all *P* < 0.001) and OG (all *P* < 0.05, except comparison of CFA/I ± mLT, *p*=0.056) routes of administration (Fig. 2B, 2D).

By IN vaccination, adjuvanted groups receiving dscCfaE and CFA/I mounted the highest HAI responses, as measured two weeks after the third dose (Fig. 2E). In fact, co-administration of dscCfaE or CFA/I fimbriae with mLT resulted in significantly higher HAI titers compared to vaccination with the corresponding antigen without adjuvant by both IN (all *p*<0.01) and OG (all *p*<0.001) vaccination (Fig. 2E). With OG vaccination, the HAI titers were over 3-fold higher in the adjuvanted group that received dscCfaE as compared to CFA/I (*p*<0.001) (Fig. 2E).

A. nancymae mucosal vaccination with dscCfaE adhesin. In the IN vaccination experiment in *A. nancymae*, anti-adhesin antibody responses in all groups and anti-CTB antibody responses in the two adjuvanted groups generally peaked 7-14 days after the third dose (i.e., days 35-42) (Fig. 3). Comparing antibody titers at day 42, the two groups given dscCfaE with an adjuvant had higher mean anti-adhesin titers than the group receiving dscCfaE alone, though the only significant difference was in IgA anti-CfaE titers in the dscCfaE + CTB group vs. dscCfaE alone ($P < 0.05$) (Fig. 4A, 4B). Serum IgG anti-CTB titers at day 42 were significantly higher in the adjuvanted vs. non-adjuvanted groups (all $P < 0.01$) (Fig. 4C). Long-term follow-up of vaccine-specific serum IgG titers after IN vaccination showed that anti-CfaE and anti-CTB titers in the adjuvanted groups dropped by about a log after three months but persisted for at least a year after vaccination (Fig. 3A, 3C, 3E).

In the NHP OG vaccination experiment, none of the four groups mounted an appreciable anti-adhesin antibody response (Fig. 3B, 3D), while the groups to which CTB was co-administered showed similar increases in IgG anti-toxin titers, which peaked after the third dose (Fig. 3F). Examining antibody titers at day 42, elevations in IgG and IgA anti-CfaE titers were at best nominal in the OG-vaccinated groups (Fig. 4A, 4B). Serum IgG anti-CTB titers at day 42 were significantly higher in the adjuvanted vs. non-adjuvanted groups (all $P < 0.001$) (Fig. 4C).

Serum HAI responses were assessed two weeks after the third dose in both of the *A. nancymae* mucosal vaccination experiments. With IN vaccination, a significant increase over pre-immunization HAI titers was observed in the two adjuvanted groups (paired t test, $P < 0.01$, both groups), with no differences in post-vaccination titers among the three active vaccine groups (one-way ANOVA, $P > 0.05$) (Fig. 4D). In the OG vaccination experiment, HAI titers

were detected only in NHPs receiving dscCfaE with CTB, but were not significantly greater than baseline (paired *t* test, $P > 0.05$).

DISCUSSION

CFA/I fimbriae and other ETEC CFs have heretofore served as the standard vaccine components for elicitation of anti-bacterial immune responses against this important bacterial enteropathogen. Evidence indicating that the minor fimbrial tip subunit CfaE serves a key early role in mediating intestinal adhesion has provided the impetus for assessing its utility as an alternative vaccine antigen. Here, we have shown that mucosal vaccination of mice with dscCfaE elicited humoral immune responses that were significantly augmented by co-administration with mLT. When administered to mice with adjuvant by IN delivery, dscCfaE and CFA/I elicited similarly high serum HAI titers, which serve as a convenient measure of functional anti-adhesive antibodies. OG vaccination with dscCfaE in mice elicited significantly higher HAI titers than did CFA/I fimbriae when administered with adjuvant. By both routes, dscCfaE resulted in higher anti-adhesin antibody titers than did CFA/I fimbriae, which would be predicted based on the sparsity of CfaE as the tip-localized CFA/I fimbrial constituent. Conversely, CFA/I was more effective than dscCfaE in eliciting anti-fimbrial antibody responses. Comparing routes of delivery in the mice experiments, IN vaccination with CFA/I fimbriae or dscCfaE plus adjuvant resulted in higher HAI titers and antigen-specific antibody titers compared to OG vaccination, even with the use of 10-fold higher doses by the latter route. Based on the robustness of responses to dscCfaE in mice, we assessed its immunogenicity in NHPs, which may be more predictive of human responses. By IN vaccination, dscCfaE elicited optimal functional HAI titers and anti-adhesin IgG and IgA antibody titers when co-administered with either CTB or mLT as adjuvant, whereas OG administration either alone or with CTB

generated nominal HAI and anti-adhesin antibody titers.

Precedents for the suitability of minor adhesins of chaperone-usher pathway fimbriae as vaccines derive from studies of FimH and PapG, the tip-associated adhesins of type 1 fimbriae and P-fimbriae, respectively, both of which serve as virulence factors for urinary tract infections caused by uropathogenic *Escherichia coli* (UPEC). Vaccination with FimH prevented UPEC bladder infection in mouse and NHP disease models (26-28). Similarly, a PapG-based vaccine prevented pyelonephritis in a NHP disease model (29). Notably, the actual proteins used in these studies consisted of either a natural adhesion-competent truncate of FimH or a bimolecular complex of the adhesin and its cognate chaperone for both FimH and PapG, unlike our use here of a donor strand complemented derivative of CfaE. Interestingly, recent studies of FimH have demonstrated the existence of low- and high-affinity binding conformations and suggest that locking FimH into its low-affinity state results in enhancement of adhesion-neutralizing antibodies (30, 31). To date, structural evidence indicates that dscCfaE exists in a low-affinity conformational state, while a high-affinity state has been postulated but not proven (16, 17, 32).

There are advantages and disadvantages to the use of whole fimbriae and the derivative adhesin as components for an ETEC vaccine. Using CFA/I fimbriae as the pertinent example, the advantages of a fimbriae-based vaccine include its adaptability for incorporation into whole-cell killed and live, attenuated vaccines, of which there are candidates in clinical development (12, 13). There is also compelling evidence that CFA/I fimbriae serves as a protective antigen (33). On the other hand, CFA/I and related fimbriae are complex biopolymers that present challenges for standardization and are overwhelmingly composed of the stalk-forming major subunit, for which the isolated impact in disease prevention has not been specifically defined. These attributes are to be contrasted with those of the minor fimbrial tip adhesin CfaE, which as

recombinant dscCfaE provides a more homogeneous, definable vaccine component than CFA/I fimbriae and efficiently generates anti-adhesive immune responses as shown here. Moreover, greater conservation among CfaE and related Class 5 tip adhesins predicts that CfaE antibodies will cross-inhibit adherence of the closely related CS14 and CS4 fimbriae, all of which fall into the same subclass 5a (15). Analysis of pooled mouse serum from mice vaccinated IN with dscCfaE or CFA/I plus adjuvant showed that only the adhesin elicited heterologous HAI titers against CS14-ETEC (data not shown). Ultimately, the antigenic conservation of Class 5 fimbrial adhesins may be exploited to define a multivalent adhesin vaccine formulation containing a limited number of subclass-specific, representative Class 5 adhesins that confer broad protection against the eight known ETEC Class 5 fimbriae.

There are limitations to the studies presented herein. CTB but not mLT was co-administered with dscCfaE in the NHP OG vaccination experiments to provide a baseline for forthcoming NHP studies with holotoxin-like chimeras containing noncovalently linked CTB and dscCfaE (unpublished results). The shortcoming of this selection is that CTB failed to potentiate anti-adhesin responses, consistent with prior work indicating that it functions poorly as an adjuvant by the oral route (34). In a larger context, the use of IN and OG vaccination to evaluate the immunogenicity of dscCfaE with and without adjuvants was informative, but these mucosal routes may be unsuitable for clinical development of an ETEC adhesin vaccine. Since it would be desirable to incorporate an LT-like component into such a vaccine, IN vaccination would likely be proscribed given the recognition of Bell's palsy as a sequel to IN vaccination with LT and LT derivatives (35, 36). Oral vaccination with proteins often results in acid- and protease-mediated degradation, and in fact here we found OG vaccination with dscCfaE to be generally inferior to IN vaccination. Ultimately, utilization of oral or IN vaccination for an ETEC adhesin-

based subunit vaccine would have to address these challenges, or alternatively other clinically suitable routes of delivery would have to be defined. A limitation to interpretation of our results is the fact that we confined immunological analyses to measurement of serum antibodies. Because ETEC cause disease while presumably being confined to the intestinal lumen, greater importance is placed on intestinal antibodies where abrogation of ETEC adhesion and colonization is tantamount to disease prevention. Elsewhere, however, we have shown that mouse vaccination with dscCfaE or CFA/I co-administered with an LT-based adjuvant elicited vaccine-specific coproantibody responses (37).

Taken as a whole, our findings indicate that dscCfaE conveys key improvements over CFA/I fimbriae as an immunogen designed to elicit anti-adhesive immune responses and thus merits further investigation as the prototype for a refined adhesin-based vaccine approach against ETEC diarrhea. The immunogenicity findings in *A. nancymae* are of particular interest in light of related work wherein we have established a diarrhea model in this species with a well-characterized CFA/I-ETEC disease isolate (Hall ER et al, manuscript in preparation). This has enabled studies to evaluate the protective efficacy of dscCfaE in this model using IN vaccination for proof-of-principle. We are also exploring routes for effective delivery of dscCfaE and related ETEC adhesins that might circumvent some of the challenges imposed by mucosal immunization with protein subunit vaccines.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no financial, institutional or other relationships that might lead to bias or a conflict of interest.

FIGURE LEGENDS

Figure 1. Serum IgG antibody responses over time in mice vaccinated with dscCfaE and CFA/I. Ten mice per group were vaccinated with three doses (days 0, 14, 28) by the IN route with dscCfaE (25 μ g) $\pm$ mLT (1.5 μ g) or CFA/I (25 μ g) $\pm$ mLT (1.5 μ g) or PBS (not shown), or by the OG route with dscCfaE (250 μ g) $\pm$ mLT (10 μ g) or CFA/I (250 μ g) $\pm$ mLT (10 μ g) or PBS (not shown). For the IN vaccinated groups, **(A)** anti-dscCfaE and **(C)** anti-CFA/I end point ELISA titers are shown at two-week intervals from day 0 through 42. For the OG vaccinated groups, **(B)** anti-dscCfaE, and **(D)** anti-CFA/I titers are shown at two-week intervals over the same period. All values are the $\log_{10}$ titers $\pm$ SD, and the horizontal dotted lines denote the lowest dilution tested (1.7).

Figure 2. Comparison of serum antibody responses in mice post-vaccination with dscCfaE and CFA/I. Ten mice per group were vaccinated with three doses (days 0, 14, 28) by the IN route with dscCfaE (25 μ g) $\pm$ mLT (1.5 μ g) or CFA/I (25 μ g) $\pm$ mLT (1.5 μ g) or PBS, or by the OG route with dscCfaE (250 μ g) $\pm$ mLT (10 μ g) or CFA/I (250 μ g) $\pm$ mLT (10 μ g) or PBS. All titers shown are from serum taken two weeks after the third dose (day 42), with white and black bars representing dscCfaE given alone or with mLT, respectively. **(A)** Anti-dscCfaE IgG, **(B)** anti-CFA/I IgG, **(C)** anti-dscCfaE IgA, and **(D)** anti-CFA/I IgA end point ELISA titers are shown. For A-D, values are the $\log_{10}$ titers $\pm$ SD, and the horizontal dotted lines denote the lowest dilution tested (1.7). **(E)** Functional antibody titers are shown, as measured by the HAI assay using CFA/I-ETEC (H10407) and bovine red blood cells. Values are the median $\pm$ IQR. The dotted line denotes the lowest dilution of serum tested (1:32). Statistical significance was determined by using one-way ANOVA and Tukey's post test, for comparisons between antigens

given with and without adjuvants and between dscCfaE and CFA/I, for the same adjuvant condition. Asterisks denote a significant difference between responses in the indicated groups (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$). IN and OG vaccination experiments were analyzed separately.

Figure 3. Serum antibody responses over time in *A. nancymae* vaccinated with dscCfaE. In the IN vaccination study, three animals per group received three doses (days 0, 14, 28) of dscCfaE (200 μ g) alone, with CTB 292 μ g or with mLT (10 μ g). In the OG vaccination study, five animals per group received three doses (days 0, 14, 28) of a lower amount of dscCfaE (500 μ g,) $\pm$ CTB (730 μ g), an upper amount of dscCfaE (1000 μ g) $\pm$ CTB (1460 μ g), or PBS (not shown). For the IN vaccinated groups, (A) anti-dscCfaE IgG, (C) anti-dscCfaE IgA and (E) anti-CTB IgG end point ELISA titers are shown at two-week intervals from day 0 through 42 and then at longer intervals through day 356. For the OG vaccinated groups, (B) anti-dscCfaE IgG, (D) anti-dscCfaE IgA and (F) anti-CTB IgG titers are shown at two-week intervals from day 0 through 42 only. All values are the $\log_{10}$ titers $\pm$ SD, and the horizontal dotted lines denote the lowest dilution tested (0.7).

Figure 4. Comparison of serum antibody responses in *A. nancymae* post-vaccination with dscCfaE. In the IN vaccination study, three animals per group received three doses (days 0, 14, 28) of dscCfaE (200 μ g) alone, with CTB 292 μ g or with mLT (10 μ g). In the OG vaccination study, five animals per group received three doses (days 0, 14, 28) of a lower amount of dscCfaE (500 μ g,) $\pm$ CTB (730 μ g), an upper amount of dscCfaE (1000 μ g,) $\pm$ CTB (1460 μ g), or PBS. All titers shown are from serum taken two weeks after the third dose (day 42), with white, black

and gray bars representing dscCfaE given alone, with CTB or with mLT, respectively. **(A)** Anti-dscCfaE IgG, **(B)** anti-dscCfaE IgA, and **(C)** anti-CTB IgG end point ELISA titers are shown. For B-D, values are the $\log_{10}$ titers + SD, and the horizontal dotted lines denote the lowest dilution tested (0.7). **(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 median, with individual data points shown as closed circles (dscCfaE alone at the indicated amount), open circles (dscCfaE+CTB), and open triangles (dscCfaE+mLT). The dotted line denotes the lowest dilution of serum tested (1:32). Statistical significance was determined by using one-way ANOVA and Tukey's post test, for comparisons between dscCfaE given alone and with adjuvant and between different co-administered adjuvants (IN vaccination study). The asterisks denote a significant difference between the responses in the indicated groups (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). IN and OG vaccination experiments were analyzed separately.

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