Immunogenicity and Protective Efficacy against Enterotoxigenic *Escherichia coli* Colonization following Intradermal, Sublingual, or Oral Vaccination with EtpA Adhesin

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Enterotoxigenic *Escherichia coli* (ETEC) strains are a common cause of diarrhea. Extraordinary antigenic diversity has prompted a search for conserved antigens to complement canonical approaches to ETEC vaccine development. EtpA, an immunogenic extracellular ETEC adhesin relatively conserved in the ETEC pathovar, has previously been shown to be a protective antigen following intranasal immunization. These studies were undertaken to explore alternative routes of EtpA vaccination that would permit use of a double mutant (R192G L211A) heat-labile toxin (dmLT) adjuvant. Here, oral vaccination with EtpA adjuvanted with dmLT afforded significant protection against small intestinal colonization, and the degree of protection correlated with fecal IgG, IgA, or total fecal antibody responses to EtpA. Sublingual vaccination yielded compartmentalized mucosal immune responses with significant increases in anti-EtpA fecal IgG and IgA, and mice vaccinated via this route were also protected against colonization. In contrast, while intradermal (i.d.) vaccination achieved high levels of both serum and fecal antibodies against both EtpA and dmLT, mice vaccinated via the i.d. route were not protected against subsequent colonization and the avidity of serum IgG and IgA EtpA-specific antibodies was significantly lower after i.d. immunization compared to other routes. Finally, we demonstrate that antiserum from vaccinated mice significantly impairs binding of LT to cognate GM1 receptors and shows near complete neutralization of toxin delivery by ETEC *in vitro*. Collectively, these data provide further evidence that EtpA could complement future vaccine strategies but also suggest that additional effort will be required to optimize its use as a protective immunogen.

Enterotoxigenic *Escherichia coli* (ETEC) strains are among the most common causes of diarrheal illness in developing countries, where young children are most susceptible (1, 2). In addition, these pathogens are also a common cause of diarrhea in immunologically naive travelers (3) who venture to areas of endemicity where sanitation and clean water remain limited.

Given the significant impact of ETEC on global health, a vaccine to prevent these infections is a significant priority (4). However, despite decades of investigative efforts following the discovery of toxin-producing *E. coli* in patients with clinical illnesses indistinguishable from cholera (5), a vaccine that affords broad-based protection has yet to be developed.

All of the ETEC-specific virulence genes described to date are encoded on plasmids. These include the heat-labile and/or heat-stable enterotoxins that define this pathovar and the colonization factors (CFs). Most vaccines to date have primarily targeted these colonization factors, which include a broad array of fimbrial as well as afimbrial surface antigens thought to be essential for intestinal colonization and/or heat-labile toxin.

Unfortunately, the heterogeneity of CF antigenic structures presents a challenge to development of a broadly protective vaccine. While some antigens are more conserved and widely distributed geographically and across different phylogenic backgrounds (6), the inherent plasticity of *E. coli* genomes (7, 8) and the fact that many ETEC strains do not make one of the 26 different CFs described to date (9, 10) have prompted a search for additional antigens that might also be considered to complement existing vaccine development paradigms (11).

Among antigens under investigation as a putative vaccine candidate is EtpA. This plasmid-encoded secreted glycoprotein belongs to the two-partner secretion family of molecules that includes filamentous hemagglutinin (FHA), a component of acellular pertussis vaccines (12). Studies of EtpA to date have demonstrated that it plays a unique role in facilitating ETEC adhesion and toxin delivery to target intestinal epithelial cells (13). Moreover, experiments with mice have shown that EtpA promotes colonization of the small intestine and can serve as a protective antigen (13, 14).

Recent molecular characterization of strains from a variety of sources has also suggested that EtpA is sufficiently conserved to warrant further consideration as a potential vaccine antigen (7, 15, 16). Early proof-of-principal studies with EtpA were conducted using an intranasal route of vaccination with Protollin (14, 17–19) or heat-labile toxin (LT) (20). However, because LT is not safe for intranasal vaccination in humans (21, 22), and because an LT toxoid will likely be a key component of next-generation vaccines, here we investigated the immunogenicity and protective efficacy

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference(s)
Strains		
H10407	Wild-type ETEC strain O78:H11; CFA/1 LT$^+$ ST$^+$ EtpA$^+$	57, 58
jf570	*eltAB* LT deletion mutant of H10407	59
jf876	*lacZYA*::Km^r mutant of H10407	59
jf1696	TOP10(pJL017, pJL030) Amp^r Cm^r	24
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)7697 *galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pJL017	*etpBA* cloned into pBAD/myc-His A, with *etpA* in frame with myc and 6His coding regions; Amp^r	14
pJL030	*etpC* gene cloned into pACYC184; Cm^r	13

[a] Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant; Str^r, streptomycin resistant.

of EtpA when delivered by other routes using a double mutant (R192G L211A) heat-labile toxin (dmLT) as the adjuvant.

MATERIALS AND METHODS

Adjuvant and immunogen preparation. The double mutant (R192G L211A) heat-labile toxin (dmLT) (23) used in these studies was manufactured by the Bioproduction Facility at Walter Reed Army Institute for Research, Silver Spring, MD (BPR-1037-00, lot no. 1735) and was stored lyophilized at −20°C prior to use. dmLT was reconstituted to 1 mg/ml in sterile phosphate-buffered saline (PBS) immediately before use and then diluted as needed with PBS. Recombinant polyhistidine-tagged EtpA glycoprotein (rEtpA) was purified as previously described using metal affinity chromatography from culture supernatants of an *E. coli* TOP10 strain transformed with pJL017 and pJL030 (jf1696) (Table 1) (24). Briefly, overnight cultures grown from frozen glycerol stocks maintained at −80°C were diluted 1:100 into fresh Luria broth (LB) containing final concentrations of 100 μg/ml ampicillin and 15 μg/ml chloramphenicol and grown at 37°C to an optical density at 600 nm (OD$_{600}$) of ~0.6. Arabinose (0.0002%) was then added to induce EtpA expression. After 3 h of induction, cultures were harvested at 6,000 rpm at 4°C for 10 min, and supernatant was filtered and saved for subsequent purification. Supernatant was concentrated ~10-fold using a Pellicon concentrator with 30,000-molecular-weight (MW) cutoff (Millipore). After being loaded onto metal affinity columns (5 ml; HiTrap, GE), unbound protein was removed by washing with buffer containing 25 mM sodium phosphate, 300 mM sodium chloride (pH 7.4), and 0 to 100 mM imidazole. rEtpA was then eluted from the column in the same buffer over a gradient ranging from 100 to 600 mM imidazole. Eluate fractions containing rEtpA were pooled and then concentrated with an Amicon Ultra-15 Ultracel-100k centrifuge (Millipore), and the buffer was exchanged with PBS (pH 7.4) before being stored at −80°C. rEtpA and dmLT were combined immediately prior to vaccination.

Immunization protocols. Female CD-1 mice (5 to 8 weeks old) were purchased from Charles River Laboratories. Groups of 10 to 12 mice were vaccinated on days 1, 29, and 43 via the sublingual, intradermal (i.d.), or orogastric routes, with additional vaccination given on day 57 by the sublingual route only. Before vaccination, mice were lightly anesthetized with isoflurane. For orogastric vaccination, mice were first fasted for 2 h and then gavaged with 100 μl of NaHCO$_3$ (7.5%) to neutralize stomach acid. After 5 min, mice were then gavaged with the vaccine or controls in a final volume of 300 μl of PBS. For sublingual vaccination, 10 μl of the preparation was pipetted under the tongue, and the head was maintained up-

right until the mouse regained consciousness. For intradermal vaccination, an alcohol swab was used to wet and separate abdominal fur, after which 50 μl of the vaccine preparation or control solution was delivered intradermally using a 1/2-ml insulin syringe fitted with a 29-gauge needle. To confirm intradermal placement of the antigen, we performed test i.d. injections of tattoo ink in parallel sets of live CD-1 mice. Sections of skin were subsequently processed for hematoxylin and eosin staining and examination by light microscopy.

Orogastric vaccination employed 25 μg of dmLT in adjuvant-only controls and as the adjuvant with doses of either 200 or 400 μg of rEtpA. Sublingual vaccination used 5 μg of dmLT/mouse ± 10 μg of EtpA. Intradermal vaccination was done in two experiments with doses of either 100 ng of dmLT ± 250 ng of rEtpA or 1 μg of dmLT ± 2.5 μg of rEtpA.

Sample collection. Mouse fecal samples were collected 2 weeks after each boost. Six fecal pellets from each mouse were resuspended in 1.5 ml of fecal resuspension buffer (containing 10 mM Tris base, 100 mM NaCl, 0.05% Tween 20, and 5 mM sodium azide, pH 7.4) and stored at 4°C overnight. Mouse serum samples were collected either from abdominal aorta or from terminal cardiac bleeds using 25-gauge tuberculin syringes.

Bacterial strains and growth conditions. Strain jf876, a derivative of ETEC strain H10407 bearing a kanamycin resistance marker in the *lacZYA* locus (*lacZYA*::kan) (Table 1) was used in all colonization experiments as previously described (25). Briefly, jf876 maintained as a frozen glycerol stock at −80°C was used to inoculate sterile Luria broth (LB). After overnight growth at 37°C at 225 rpm, the culture was diluted 1:100 into fresh LB medium, grown to an OD$_{600}$ of ~0.3, and serially diluted to an inoculum of ~10^5 to 10^6 for challenge.

Intestinal colonization studies in mice. Intestinal colonization experiments were performed as previously described (25). Briefly, prior to challenge, mice were pretreated with streptomycin (5 g/liter) in drinking water for 24 h, followed by regular water for 12 h, and famotidine (50 mg/kg body weight) was given 1 to 3 h prior to challenge to neutralize stomach acid. Mice were then challenged with ~10^5 CFU of ETEC bacteria by oral gavage (25). Twenty-four hours after challenge, mice were sacrificed and two 3-cm sections of small intestine (ileum) were collected as previously described. Following incubation in saponin (5%) for 10 min, dilutions of intestinal lysates in PBS were plated onto Luria agar plates containing kanamycin (50 μg/ml). Following overnight incubation, bacteria were enumerated by counting kanamycin-resistant colonies. All experiments with mice were performed under protocols approved by the Animal Studies Committee at Washington University School of Medicine.

Immunologic assessment. A kinetic enzyme-linked immunosorbent assay (ELISA) was used to detect immune responses in both fecal and serum samples as previously described (15). Briefly, 96-well plates were coated with 0.1 μg/well of EtpA or GM1 gangliosides (Sigma; catalog no. G2375) in carbonate buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$, 0.2 g/liter NaN$_3$, pH 8.6) overnight at 4°C. After washing, dmLT (1 μg/ml in PBS) was added to wells containing GM1 gangliosides and incubated for 1 h at 37°C. Plates were washed and blocked with 1% bovine serum albumin (BSA) for 1 h at 37°C. Plates were again washed and then incubated with fecal extracts (undiluted) or with sera (diluted 1:100 in PBS containing 1% BSA). After incubation at 37°C for 1 h, plates were washed and incubated with horseradish peroxidase (HRP)-secondary antibody conjugates for 30 min at 37°C. After washing, TMB (3,3′,5,5′-tetramethylbenzidine)-peroxidase substrate (KPL) was added, and plates were immediately read at 650 nm to collect kinetic ELISA data (26). Data were analyzed using Gen5 software (BioTek) and are reported as V_{max} (milliunits per minute).

Serum EtpA antibody avidity determinations. Avidity indexes for EtpA-specific serum IgG and IgA antibodies were determined as previously described (27). Briefly, microtiter wells were coated with EtpA as described above. After being washed and blocked, plates were incubated with sera diluted 1:10,000 at 37°C for 1 h. Plates were washed and then incubated with either 6 M urea solution or PBS for 10 min at 37°C. After being washed, plates were incubated with either anti-mouse IgA or anti-
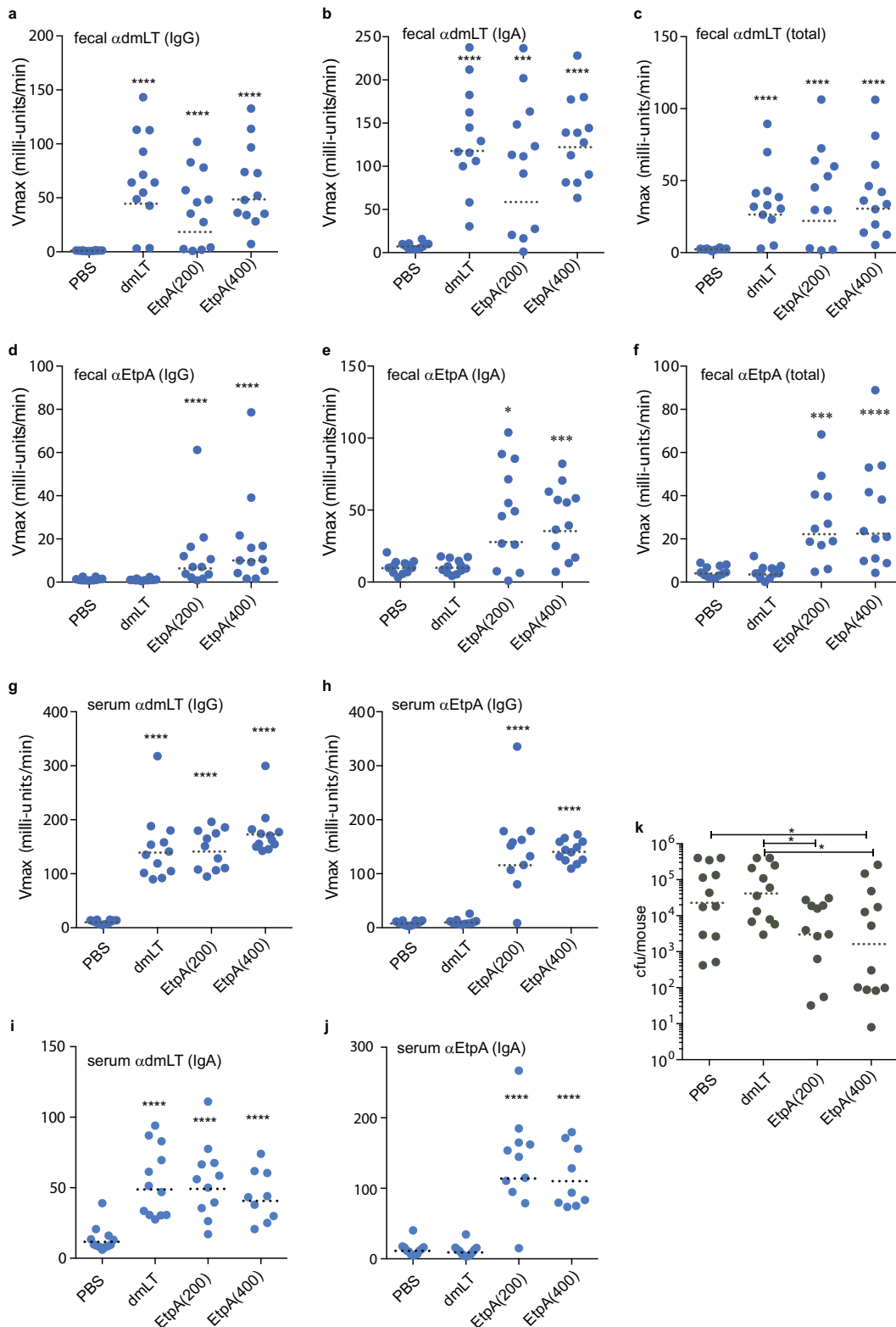
FIG 1 Immunogenicity and inhibition of intestinal colonization following oral immunization with EtpA adjuvanted with dmLT. (a to f) Kinetic ELISA data demonstrating fecal antibody responses to dmLT (a to c) and EtpA (d to f) following oral immunization with dmLT adjuvant alone (25 μg), 25 μg dmLT plus 200 μg rEtpA, or 25 μg dmLT plus 400 μg rEtpA. The antigen and antibody isotype tested are shown in the upper left-hand corner of each graph. Serum responses (IgG) at a dilution of 1:100 are shown for (g) dmLT and (h) EtpA, and panels i and j correspond to serum IgA responses. (k) Intestinal colonization in mice (grey symbols represent individual mice) following challenge with enterotoxigenic *E. coli*. Dashed horizontal lines in each figure panel represent geometric means. Comparisons to the PBS control group were made by Mann-Whitney nonparametric testing (*, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$).
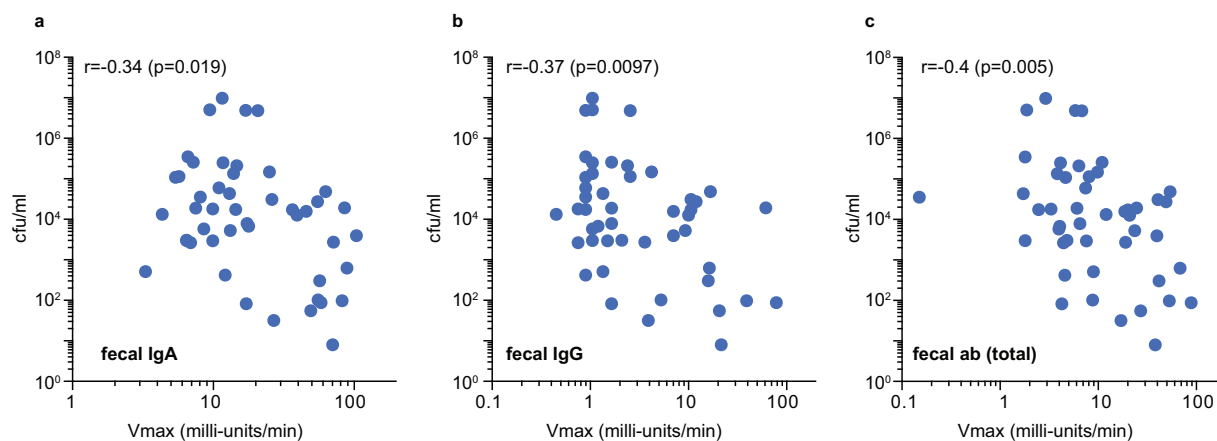
FIG 2 Correlation of anti-EtpA fecal antibody levels and intestinal colonization in mice following oral vaccination. (a) Comparison of IgA anti-EtpA fecal antibody levels and colonization. (b) Fecal IgG versus colonization. (c) Total fecal antibody (ab) (IgG, IgM, and IgA) versus colonization. The results of Spearman (nonparametric) correlation coefficient (r) and corresponding P value are shown in the upper left-hand corner of each graph. Each point represents data from a total of 47 mice, including PBS controls ($n = 12$), dmLT adjuvant-only controls ($n = 12$), mice administered dmLT plus 200 µg EtpA ($n = 11$), and mice administered dmLT plus 400 µg EtpA ($n = 12$).

mouse IgG HRP-conjugated secondary antibody, and responses were determined by kinetic ELISA as described above. Avidity indexes were then calculated as the ratio of the V_{max} (milliunits per minute) in the urea-treated wells to that in the untreated wells.

Toxin neutralization assays. To examine the ability of antisera to prevent binding of heat-labile toxin to its receptor, plates were coated with GM1 gangliosides at a 1-µg/ml final concentration in PBS overnight at 4°C. Serum from vaccinated or control mice was diluted 1:128 in PBS and then incubated 1:1 with LT at a final concentration of 1 µg/ml at 4°C overnight. After washing of plates with PBS, LT preincubated with serum was then allowed to bind to target gangliosides for 1 h at 37°C. Plates were then washed and blocked with 1% BSA for 1 h at 37°C. Bound LT was determined by anti-LT-B kinetic ELISA. Briefly, plates were incubated using primary anti-LT-B rabbit polyclonal antiserum diluted 1:1,000 in PBS containing 1% BSA at 37°C for 1 h and washed, followed by addition of goat anti-rabbit IgG-HRP conjugate diluted 1:5,000 in 1% BSA at 37°C for 30 min. After the final washing, plates were developed with TMB substrate and data collected kinetically as described above.

To examine the ability of antisera from immunized animals to prevent effective toxin delivery by ETEC, a 96-well tissue culture plate was first seeded with Caco-2 cells and the culture was grown at 37°C in 5% CO_2 until the cells formed a confluent monolayer. One microliter of a mid-logarithmic-phase Luria broth culture of H10407 was then added with 10 µl of sera from individual mice and incubated with target Caco-2 monolayers at 37°C in 5% CO_2. After 2.5 h, plates were washed with preequilibrated tissue culture medium, then incubated for an additional 2 h at 37°C in 5% CO_2, and finally washed 4 times with PBS. Cyclic AMP (cAMP) activation in target epithelial cells was then determined by ELISA (DetectX direct cyclic AMP kit; Arbor Assays, Ann Arbor, MI).

RESULTS

Immunogenicity and protective efficacy following oral vaccination with EtpA. Because dmLT has been given safely to human volunteers via the oral route (28), we first examined whether EtpA could afford protection when coadministered with dmLT as the adjuvant. Following oral immunization, mice mounted robust fecal IgA, fecal IgG, and total fecal antibody responses to the dmLT adjuvant, whether administered alone or in combination with EtpA (Fig. 1a to c). Likewise, we observed significant amounts of anti-EtpA fecal antibodies compared to either dmLT-only or PBS controls (Fig. 1d to f). Oral vaccination also resulted in demon-

strable increases in serum IgG and IgA antibodies against dmLT (Fig. 1g and i) and against EtpA (Fig. 1h and j).

Following oral vaccination, mice in the EtpA vaccine administration group had significantly lower levels of intestinal colonization than either PBS control mice or dmLT adjuvant-only controls (Fig. 1k). While mice immunized with the highest dose of EtpA exhibited the lowest geometric mean number of CFU of ETEC in the small intestine, the difference between EtpA low- and high-dose groups was not statistically significant.

Correlation between protection and fecal antibody production. Theoretically, vaccines against enteric pathogens should engender mucosal antibodies that neutralize the ability of the pathogen to colonize the host and/or to deliver its effector molecules. Because EtpA functions as an adhesin, we examined the correlation between the levels of fecal antibody levels achieved in individual mice following vaccination and intestinal colonization. Levels of intestinal IgG (Fig. 2a) and IgA (Fig. 2b) were both related to reductions in intestinal colonization, while the strongest correlation ($P = 0.005$) was observed with total fecal antibody levels (Fig. 2c).

Sublingual administration of EtpA with dmLT. Previous studies have shown that sublingual administration of antigens can induce both systemic and mucosal antibody responses with doses that are appreciably smaller than those required for oral administration (29). Similar to oral administration, sublingual vaccination with the combination of dmLT and EtpA also stimulated production of fecal IgG (Fig. 3a) and fecal IgA (Fig. 3b) as well as serum IgG (Fig. 3c) and serum IgA (Fig. 3d) antibodies to the dmLT adjuvant. Although sublingual vaccination resulted in modest increases in responses to EtpA in feces (Fig. 3e and f), there was no significant increase in serum antibodies (Fig. 3g and h), consistent with compartmentalization of the mucosal immune response. While vaccination of mice sublingually with EtpA adjuvanted with dmLT did afford some protection against colonization relative to unvaccinated mice (Fig. 3i), we did not observe a significant correlation between fecal antibody levels and the level of intestinal colonization (Fig. 3j).

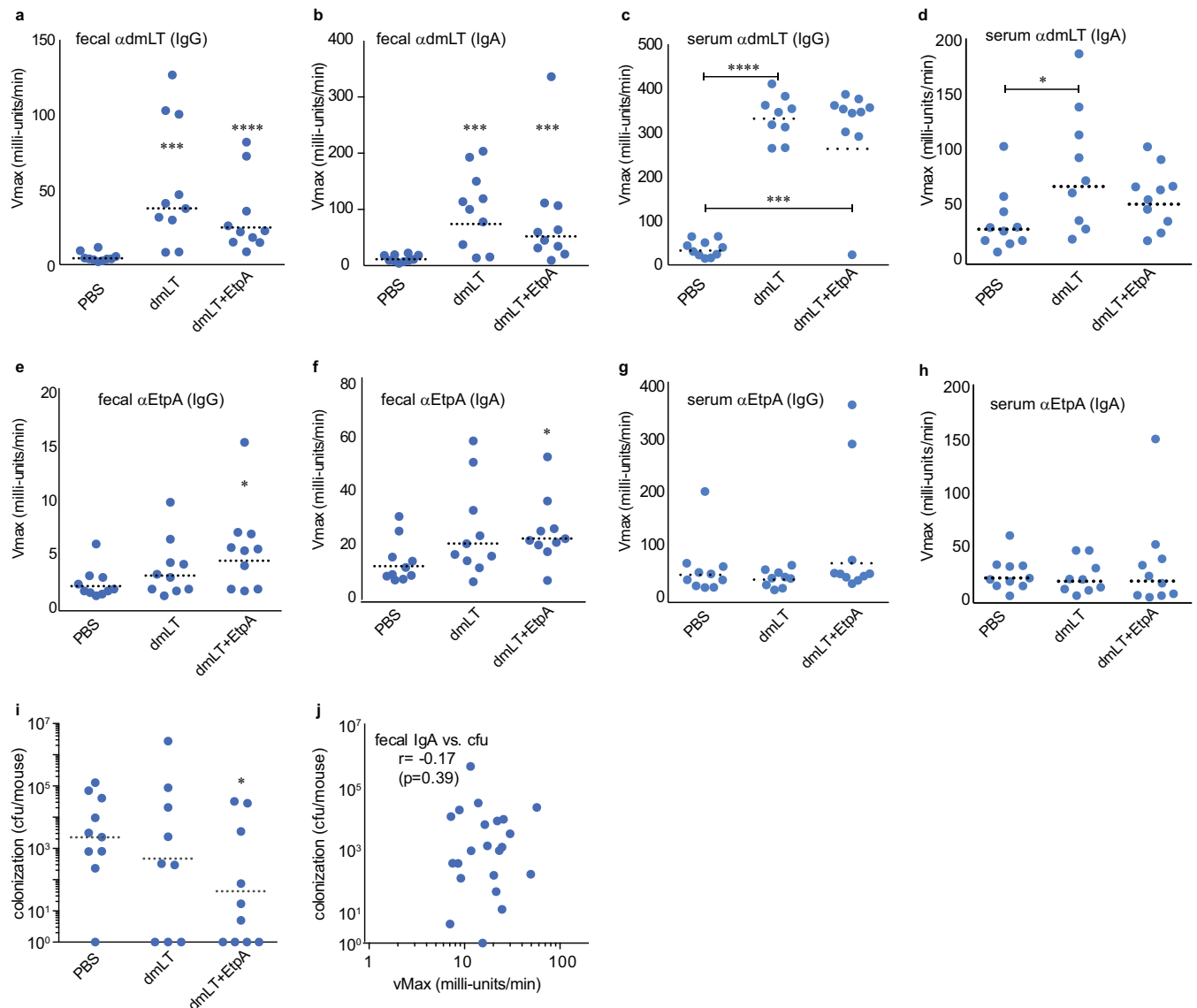**Intradermal administration of EtpA with dmLT.** The dose of

FIG 3 Immunogenicity and inhibition of intestinal colonization following sublingual immunization with EtpA adjuvanted with dmLT. (a and b) Fecal IgG (a) and fecal IgA (b) responses to the dmLT adjuvant. (c and d) Serum IgG (c) and serum IgA (d) responses to dmLT. (e and f) Fecal IgG (e) and fecal IgA (f) responses to rEtpA. (g and h) Serum IgG (g) and serum IgA (h) responses to rEtpA. (i) Colonization of mice following vaccination with rEtpA adjuvanted with dmLT versus dmLT alone or PBS controls. (j) Correlation between fecal IgA and intestinal colonization following sublingual vaccination. Comparisons between groups were made by Mann-Whitney nonparametric testing (*, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$).

vaccine required to achieve an immunologic response is typically several orders of magnitude smaller with i.d. vaccination than that required for other routes, offering significant dose sparing. Moreover, the emergence of needle-free technologies (30, 31) could make this route feasible for deployment to developing countries, where cost and ease of administration are important considerations. Therefore, we also examined intradermal vaccination with EtpA in mice. After verification of intradermal placement of potential immunogens (see Fig. S1 in the supplemental material), we were able to achieve high titers of both serum and fecal antibodies to both the adjuvant (Fig. 4a to d) and the EtpA immunogen (Fig. 4e to h) following intradermal administration. Curiously, however, we saw no significant protection against colonization when mice were vaccinated via the i.d. route (Fig. 4g), suggesting that

the route of administration could have a substantial impact on vaccine efficacy.

Because intradermal vaccination resulted in high titers of antibody that did not protect against intestinal colonization, we questioned whether the quality of antibody from mice vaccinated intradermally differed from that of mice vaccinated orally. Antibody avidity, which examines the functional affinity of antibody-antigen interactions, has been used as marker of B cell maturation and a surrogate of protective immunity to a number of important pathogens. Importantly, antibody avidity has been shown to correlate with the presence of antigen-specific memory B cells following *Vibrio cholerae* infection (27), potentially permitting efficient measurement of responses that might predict protection against a number of important diarrheal pathogens. Interestingly, the se-
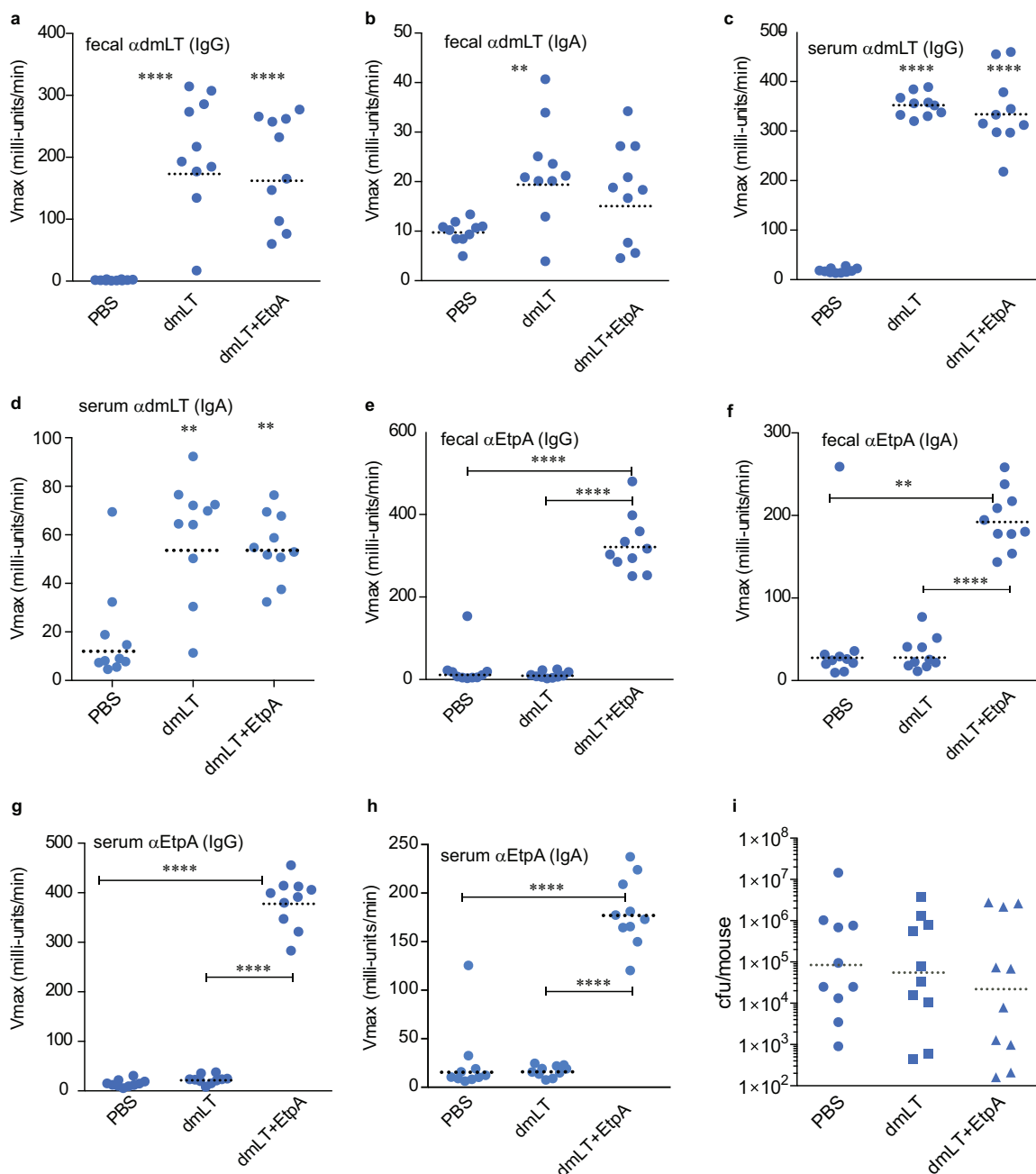
FIG 4 Intradermal vaccination with EtpA results in significant increases in fecal and serum antibody without substantial protection. Shown in panels a to h are kinetic ELISA data expressed as V_{max} (milliunits per minute) obtained with clarified fecal suspensions (at a 1:1 dilution) or serum (diluted 1:100). (a to d) Fecal IgG (a), IgA (b), serum IgG (c), and serum IgA (d) antibody responses to the dmLT adjuvant. (e to h) Fecal IgG (e), fecal IgA (f), serum IgG (g), and serum IgA (h) antibody responses to EtpA. (i) Mouse colonization following vaccination with EtpA and dmLT adjuvant compared to adjuvant-alone or PBS-only controls. Comparisons between groups were made by Mann Whitney (two-tailed) nonparametric testing (**, $P < 0.01$; ****, $P < 0.0001$).

rum IgG antibody avidity in the intradermally vaccinated mice was considerably lower than that in the mice vaccinated orally with EtpA ($P = 0.002$) (Fig. 5a), as was the EtpA-specific IgA avidity index (AI) (Fig. 5b). Collectively, these data suggest that further assessment of immunization routes, timing of administration, and doses of antigen and adjuvant will likely be important considerations in optimizing the protective efficacy of more recently described antigens, including EtpA.

Toxin neutralization by sera from vaccinated mice. Because

immunization using dmLT as the adjuvant resulted in significant production of anti-LT antibody, we also examined whether the antibodies were functionally relevant. As demonstrated in Fig. 6a, sera from mice immunized using dmLT prevented effective binding of wild-type heat-labile toxin to target GM1 gangliosides *in vitro* relative to sera from PBS control mice. Importantly, we found that antisera from either mice immunized with dmLT adjuvant only or from mice immunized with recombinant EtpA and dmLT yielded marked reduction in effective toxin delivery by the
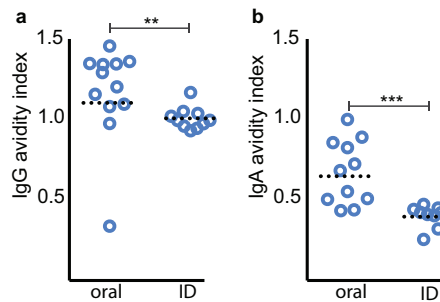
FIG 5 Avidity of EtpA-specific serum antibody following EtpA vaccination with dmLT varies with both dose and route of immunization. (a) EtpA-specific serum IgG avidity following oragastric immunization with rEtpA or intradermal (ID) vaccination. (b) EtpA-specific IgA avidity index after orogastric and intradermal vaccination. Statistical analysis results (**, $P = 0.002$; ***, $P = 0.0002$) reflect Mann-Whitney, two-tailed nonparametric comparisons.
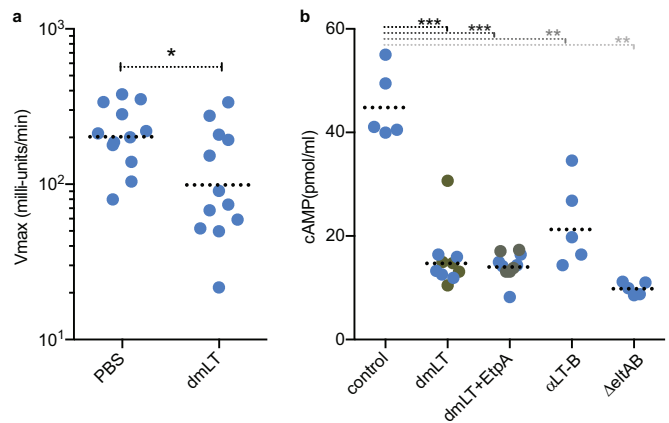

FIG 6 Toxin neutralization by sera from vaccinated mice. (a) Antisera from mice orally immunized with dmLT adjuvant prevent binding to GM1 gangliosides *in vitro*. Sera from unvaccinated (PBS-only) mice are shown as controls. All statistical comparisons were performed by Mann-Whitney 2-tailed nonparametric testing (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). (b) Sera from mice immunized with dmLT ± recombinant EtpA neutralize effective delivery of heat-labile toxin to target epithelial cells by ETEC H10407. Sera from PBS-only vaccinated control group mice are shown at left for comparison. Additional controls at right include anti-LT-B rabbit polyclonal antibody and the LT-negative ($\Delta eltAB$) strain.

ETEC strain H10407, as determined by activation of cAMP in target Caco-2 epithelial cell monolayers (Fig. 6b). These data provide further evidence vaccines combining novel adhesin and toxoid approaches could offer a viable strategy to protect against ETEC.

DISCUSSION

Construction of ETEC vaccines has been hampered in part by the extraordinary genetic plasticity of *E. coli* confounding efforts to define the ideal group of antigens that will achieve broad-based protection. Vaccines currently under study attempt to confront this challenge by using multiple live attenuated strains (32) or whole-cell killed strains that each present different CF antigens and are combined with mutant forms or subunits of heat-labile toxin (28). Interestingly, recent studies of more than 800 isolates from ETEC diarrhea cases in Bangladesh demonstrated that only approximately half of the strains expressed colonization factors (CFs) that could be identified by immunoassays (33), and new CF antigens continue to be identified by genomic sequencing (34).

The potential complexity of vaccines based exclusively on classical ETEC targets has driven investigation of other antigens common to the ETEC pathovar. Theoretically, these antigens, not currently part of ETEC vaccine platforms, could be incorporated into future iterations of vaccines to expand antigenic valency and perhaps act in concert with canonical ETEC vaccine targets to enhance efficacy. One potential alternative target is the two-partner secretion system that encodes EtpA. This system, originally discovered by transposon mutagenesis of the prototype H10407 strain (12), appears to be relatively conserved within the ETEC pathovar (7, 15, 16). EtpA-specific antibodies are present in human convalescent-phase sera, suggesting that this protein is expressed during the course of infection and is immunogenic (15, 35). These features and the molecular and functional similarity of EtpA to filamentous hemagglutinin (36), a component of acellular pertussis vaccines (37), have prompted preclinical investigation of the utility of EtpA as a protective antigen. Multiple studies with mice have now demonstrated that EtpA vaccination protects against colonization of the small intestine (14, 19, 20), thought to be a critical determinant of ETEC diarrheal illness. Another consideration driving the present studies is that ETEC and *Shigella* are among the most common bacterial pathogens causing serious di-

arrheal illness among young children in developing countries; therefore, we questioned whether subunit approaches presently being developed for *Shigella* effectors (38, 39) could be also applied to novel secreted ETEC antigens, including EtpA.

Because all prior studies of this target antigen to date have involved intranasal immunization, we examined whether we might be able to deliver EtpA by other means and elicit protective immune responses. These most recent studies provide further evidence that EtpA affords protection against intestinal colonization and that this antigen retains substantial immunogenicity when administered by a variety of different routes.

Perhaps not surprisingly, however, protective efficacy varied with the route of administration (40, 41), and despite substantial antibody responses following i.d. immunization, we did not achieve protection. One possible explanation for these seemingly discordant results is that the levels of quality of antibodies generated in different vaccination protocols could differ substantially. Antibody avidity is thought to represent the overall strength of binding by a polyvalent collection of antibodies to a variety of antigenic determinants and to reflect development of germinal center B cell antibody affinity maturation (42). A number of clinical studies have correlated antibody avidity with vaccine protective efficacy (43–45), and conversely low antibody avidity and poor affinity maturation have previously been associated with vaccine failures (46–50). The current studies seem to support the idea that antibody avidity could be an important parameter in evaluating antigen-specific memory responses and vaccine performance following immunization with novel and classical ETEC antigens (51).

The present data suggest that additional studies will need to take place to optimize both the route of administration, the doses of EtpA required to achieve protection, and conditions for coformulation with other antigens. Nevertheless, our studies suggest that it is possible to generate protective immune responses with the recombinant adhesin. The current studies are an extension of

earlier work with *Shigella* subunit proteins, and no attempt was made here to optimize immune responses to EtpA. Clearly, the doses of antigen delivered orally in these experiments would be impractical for effective immunization on a large scale. However, these studies suggest that enhanced delivery through an oral vaccine strain could be of benefit (52) and that EtpA could be used to complement existing canonical approaches to development of live attenuated ETEC vaccines (32). Not unexpectedly (53, 54), some routes of immunization resulted in highly compartmentalized responses. While serum antibody responses to EtpA were low following sublingual administration of antigen, we did observe measurable increases in fecal antibody (IgG and IgA) and sublingual immunization was protective. Application of emerging methods that enhance sublingual or buccal antigen delivery (55) could accelerate development of subunit vaccines that incorporate ETEC novel antigens.

Interestingly, emerging data do suggest that intradermal vaccination with mutant LT and ETEC fimbrial tip adhesins can protect against ETEC diarrhea in a human experimental challenge model (56), and i.d. immunization with secreted *Shigella* effectors adjuvanted with dmLT was protective against experimental challenge in mice (39). Therefore, the i.d. route could conceivably be used as a platform for development of hybrid subunit vaccines against important enteric pathogens. Nevertheless, further effort will be required to optimize this approach and refine antigen formulations with sufficient valency to achieve broad protection against ETEC and *Shigella*.

Collectively, these early data with EtpA support the concept that this antigen could complement ongoing approaches to vaccine development and expand the valency of ETEC vaccines. Additional efforts will need to focus on first defining and subsequently optimizing parameters that define protection mediated by this and other novel immunogens.

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