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Safety and immunogenicity of an improved oral inactivated multivalent enterotoxigenic *Escherichia coli* (ETEC) vaccine administered alone and together with dmLT adjuvant in a double-blind, randomized, placebo-controlled Phase I study☆

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ABSTRACT

Background: We have developed a new oral vaccine against enterotoxigenic *Escherichia coli* (ETEC), which is the most common cause of bacterial diarrhea in children in developing countries and in travelers.
Methods: The vaccine was tested for safety and immunogenicity alone and together with double-mutant heat-labile toxin (dmLT) adjuvant in a double-blind, placebo-controlled Phase I study in 129 Swedish adults. The vaccine consists of four inactivated recombinant *E. coli* strains overexpressing the major ETEC colonization factors (CFs) CFA/I, CS3, CS5, and CS6 mixed with an LT B-subunit related toxoid, LCTBA. Volunteers received two oral doses of vaccine alone, vaccine plus 10 μg or 25 μg dmLT or placebo. Secretory IgA antibody responses in fecal samples and IgA responses in secretions from circulating intestine-derived antibody secreting cells were assessed as primary measures of vaccine immunogenicity.
Results: The vaccine was safe and well tolerated; adverse events were few and generally mild with no significant differences between subjects receiving placebo or vaccine with or without adjuvant. As many as 74% of subjects receiving vaccine alone and 83% receiving vaccine plus 10 μg dmLT showed significant mucosal IgA responses to all five primary vaccine antigens and about 90% of all vaccinees responded to at least four of the antigens. Subjects receiving vaccine plus 10 μg dmLT responded with significantly increased intestine-derived anti-CS6 responses compared to subjects receiving vaccine alone.
Conclusions: The vaccine was safe and broadly immunogenic. dmLT further enhanced mucosal immune responses to CF antigens present in low amounts in the vaccine. Based on these encouraging results, the vaccine will be tested for safety and immunogenicity in different age groups including infants in Bangladesh and for protective efficacy in travelers.

Abbreviations: AE, adverse event; ALS, antibodies in lymphocyte supernatants assay; ASC, antibody secreting cell; CF, colonization factor; CT, cholera toxin; CTB, cholera toxin binding subunit; dmLT, double mutant LT; ETEC, enterotoxigenic *Escherichia coli*; GM, geometric mean; LT, heat labile toxin; LTB, heat labile toxin binding subunit; LCTBA, CTB/LTB hybrid protein; MEV, multivalent ETEC vaccine; mLT, single-mutant LT; PBMCs, peripheral blood mononuclear cells; PPS, per protocol analysis set; SAS, safety analysis set; ST, heat-stable toxin; SIgA, secretory IgA.

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1. Introduction

Although enterotoxigenic *Escherichia coli* (ETEC) is the most frequent bacterial cause of diarrhea in children in developing countries and the major cause of travelers' diarrhea, no vaccine is yet available against ETEC disease [1–3]. ETEC disease occurs after ingestion of ETEC leading to bacterial colonization of the intestinal mucosa by means of surface-expressed colonization factors (CFs) on the bacteria and production of a heat-labile toxin (LT) and/or a heat-stable toxin (ST) that induce watery diarrhea [3,4]. Immune protection is mediated by anti-CF and/or anti-LT antibodies produced locally in the intestine [2,5].

We have previously developed an oral vaccine consisting of inactivated ETEC bacteria expressing prevalent CFs and recombinantly produced cholera toxin binding subunit (CTB) [5,6]. This vaccine was shown to be safe and immunogenic in children and adults in endemic areas and conferred protection against moderate/severe diarrhea in adult travelers [5,7]. However, the protective efficacy in developing-country children was not significant and a full dose of vaccine, but not a quarter dose, induced vomiting in children 6–17 months old [2,8]. Therefore, we have now developed a modified second-generation oral ETEC vaccine with the aim to improve its immunogenicity without increasing the dosage and to be able to give a reduced dose to infants [5,9]. Our approach has been to construct recombinant *E. coli* strains expressing increased amounts of the most prevalent CFs [10] and to include a CTB/LTB hybrid protein (LCTB*A*), which induces stronger anti-LT responses than CTB in both mice and humans [11,12]. We have also broadened the coverage of the vaccine by including a strain expressing the prevalent colonization factor CS6 in immunogenic form [13]. This new multivalent ETEC vaccine (MEV) contains four different inactivated *E. coli* strains expressing substantially higher levels of CFA/I, CS3, CS5 and CS6 than in the first-generation vaccine, plus LCTB*A* [9]. In addition, we have evaluated the possibility to further enhance the immunogenicity of the vaccine by coadministration with the double-mutant LT (dmLT) adjuvant [14]. Our preclinical studies have demonstrated that addition of dmLT to MEV significantly improved both the anti-CF and anti-LT responses following oral immunization [9].

The primary objectives of this study were to evaluate the safety and mucosal immunogenicity of MEV and to explore if the immunogenicity of the vaccine might be further enhanced by addition of dmLT adjuvant. Serum anti-LT and toxin-neutralizing immune responses were determined as secondary and exploratory measures. These aspects were addressed in a Phase I clinical trial including 129 adult Swedish volunteers given either vaccine alone or together with two different dosages (10 μg and 25 μg) of dmLT; a matched control group received buffer only.

The results show that the vaccine was safe and well tolerated, both when given alone and in combination with dmLT adjuvant. The vaccine induced significant mucosal and intestine-derived immune responses to all major vaccine antigens and dmLT further enhanced mucosal immune responses to the CF antigens present in low amounts in the vaccine.

2. Materials and methods

For additional information, see Supplementary material.

2.1. Study design

This was a four-armed, randomized, double-blind, placebo-controlled, single-center Phase I trial. The study was approved by the Ethical Review Board in the Gothenburg Region, the Western Institutional Review Board, USA and the Swedish Medical Product Agency.

2.2. Randomization and masking

Healthy adult subjects, 18 to 43 years, were randomized into one of four groups (A–D); each group was given two oral doses two weeks apart of one of the following treatments: (A) vaccine buffer alone ($n = 34$), (B) MEV alone ($n = 35$), (C) MEV plus 10 μg dmLT ($n = 30$) or (D) MEV plus 25 μg dmLT ($n = 30$). A computer-generated randomization list was prepared by a statistician otherwise not involved in the study.

2.3. Vaccine

MEV (also called Etvax) consists of four inactivated recombinant *E. coli* strains (ETEX 21–24) which overexpress CFA/I, CS3, CS5 and CS6, respectively, mixed with LCTB*A* [9]. The CFA/I, CS3 and CS5 expressing strains, all based on a toxin-negative O78 ETEC strain, were inactivated with formalin and the CS6 expressing *E. coli* K12 strain with phenol to retain CF expression on the bacterial surface [10,13]. LCTB*A* is a recombinantly produced LTB/CTB hybrid protein in which seven amino acids in CTB have been replaced by corresponding amino acids of LTB [12]. dmLT (R192G/L211A) is an LT-derived protein which contains two genetic substitutions in the A subunit which eliminates the enterotoxic activity without removing the adjuvant activity [14].

2.4. Procedures

Volunteers received two oral doses of vaccine ± dmLT in bicarbonate buffer or placebo (buffer alone) two weeks apart (day 0 and day 14 ± 2). Fecal samples were collected on days 0, 7 ± 1, 14 ± 2, 19 ± 1, 21 ± 1 and 28 ± 2, blood samples for isolation of peripheral blood mononuclear cells (PBMCs) on days 0, 7 ± 1, 19 and 21 ± 1 and serum samples on days 0, 7 ± 1, 14 ± 2, 19 ± 1, 21 ± 1, 28 ± 2 and 40–56.

Safety was determined by evaluation of adverse event (AE) reports (diary cards and interviews) from day 0 until day 40–56, by clinical chemistry and hematology tests performed at screening and on days 7 ± 1 and 21 ± 1 and by physical examination at screening and on day 40–56. Solicited AEs listed in the study diaries were gastrointestinal symptoms (i.e. abdominal pain, nausea, vomiting, diarrhea, loose stools) plus fever.

Mucosal immune responses were evaluated by measuring intestine-derived antibody secreting cells (ASCs) and intestinal secretory IgA (SIgA) responses in fecal extracts. Systemic immune responses were analyzed by measuring serum antibody levels. PBMCs were isolated and used for ASC analyses by the antibodies in lymphocyte supernatants (ALS) and ELISPOT assays as described [11]. ASCs were detected by the ELISPOT technique using plates coated with in-house purified CFA/I, CS3, CS5 or GM1 ganglioside plus LTB or CS6 (Gift from F. Cassel) [6,11]. Fecal samples were immediately frozen at home by the subjects; fecal extracts were subsequently prepared and stored at −70 °C [11]. Antibody levels in ALS specimens, fecal extracts and sera were analyzed by ELISA using plates coated with CFA/I, CS3, CS5, CS6, GM1 plus LTB or O78 LPS [9,11]. Fecal antibody levels were determined as the antigen-specific SIgA titer divided by the total SIgA concentration of each sample [15]. LT toxin neutralization titers were determined using the Y1 adrenal cell assay [16].

2.5. Endpoints and statistical analyses

Safety endpoints were defined as absence of any vaccine-related serious AEs and not significantly higher frequencies of

Table 1
Subject demographics (safety analysis set).

	(A) Placebo ($n = 34$)	(B) Vaccine ($n = 35$)	(C) Vaccine + 10 μg dmLT ($n = 30$)	(D) Vaccine +25 μg dmLT ($n = 30$)	Total[a] ($n = 129$)
Age (years)					
Mean (SD)	24.5 (4.3)	26.1 (4.7)	25.8 (5.0)	24.5 (3.1)	25.2 (4.4)
Range	19–40	20–39	20–43	18–30	18–43
Gender (no and freq. of subjects)					
Female	15 (44%)	19 (54%)	17 (57%)	8 (27%)	59 (46%)
Male	19 (56%)	16 (46%)	13 (43%)	22 (73%)	70 (54%)

[a] 98% of the subjects were white Caucasians.

vaccine-related severe AEs in each of the vaccine groups than in the placebo group. Primary immunogenicity endpoints were defined as induction of immune responses in any of the vaccine groups in either of the primary assays proposed (fecal SIgA or ALS IgA) to at least four of the five primary vaccine components (CFA/I, CS3, CS5, CS6 and LTB).

The magnitudes of immune responses (fold rises) were calculated as the post-immunization divided by pre-immunization antibody levels. Statistical differences were evaluated using *t*-test (magnitudes, ELISA results), Mann–Whitney test (magnitudes, toxin neutralization results) and Fisher's exact test (frequencies) with Holm's correction for multiple testing [17]. Differences between vaccine groups and the placebo group were evaluated using one-tailed statistical tests; all other statistical tests were two-tailed. *P*-values <0.05 were considered significant.

3. Results

3.1. Study subjects

Of 161 subjects screened, 129 were enrolled with 30–35 subjects in each of the four study groups (Table 1 and Supplementary material; Fig. 1). The age and gender distributions were comparable in Groups A, B and C, but more males were randomized to Group D (Table 1).

3.2. Safety

Overall, MEV administered alone and in combination with dmLT was safe and well tolerated. No serious AEs were reported and the recorded AEs were mainly mild and not significantly different among any of the vaccine groups (B, C, D) and the placebo group (A). The addition of dmLT did not alter the safety profile. Altogether 89 solicited symptoms, deemed to be possibly or probably related to treatment, were recorded (Table 2); these AEs did not differ in either frequency or intensity between the different study groups. No significant changes of other clinical parameters, including serum chemistry and hematology, were observed in any of the volunteers.

3.3. Immunogenicity

3.3.1. Intestine-derived blood ASC responses

ASC responses against the primary vaccine antigens were studied by counting IgA ASCs by the ELISPOT method as well as by measuring antibody levels in lymphocyte secretions by the ALS method in the initial 43 randomized subjects. Since the frequencies of responses against all antigens were comparable using the two methods (data not shown), the ALS method was used in all subsequent study subjects as the sole measure of ASC responses.

The majority of the subjects in each of the vaccine groups (B, C, D) responded with increased IgA antibody levels to all five primary antigens in ALS specimens (Fig. 1 and Table 3); in contrast, only a few responders were recorded in the placebo group (A). Both the magnitudes of responses and frequencies of responders were significantly higher in all the vaccine groups than in the placebo group. Responses to all antigens peaked 5 days after the second dose in a majority of the vaccinees. Highest and most frequent responses were observed against LTB and CS3 in all vaccine groups. Evaluation of the effect of the dmLT adjuvant revealed significantly higher

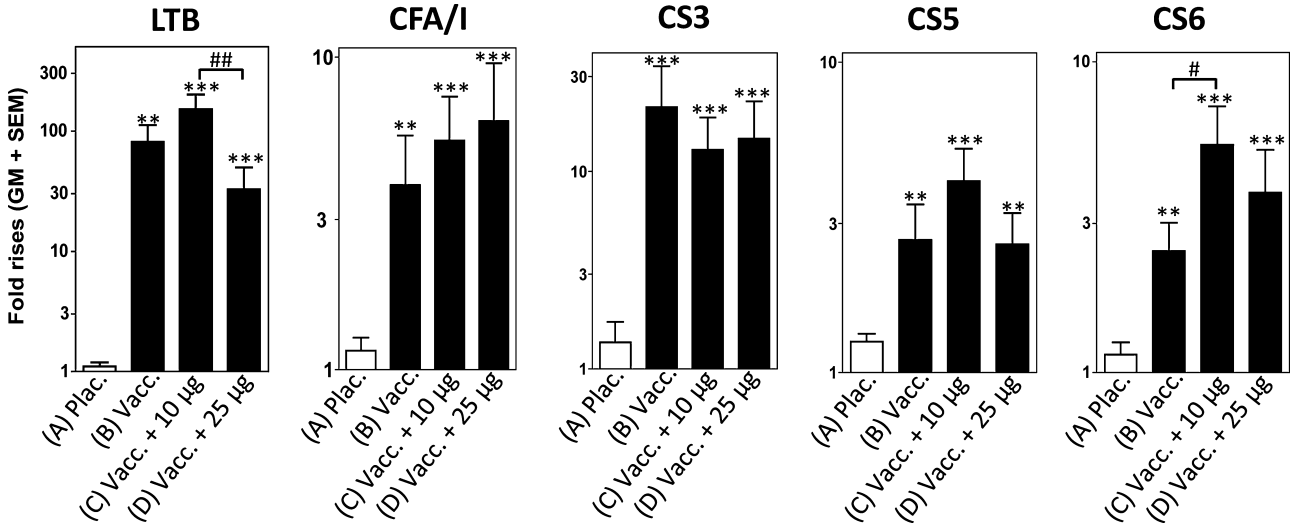

Fig. 1. Magnitudes of IgA responses against the five primary vaccine antigens (LTB, CFA/I, CS3, CS5 and CS6) in ALS specimens from volunteers receiving (A) placebo, (B) vaccine (MEV) alone, (C) vaccine plus 10 μg dmLT and (D) vaccine plus 25 μg dmLT. Maximum fold rises (geometric mean (GM) + SEM) in antibody levels after one or two doses of vaccine are indicated. *** $P < 0.001$ and ** $P < 0.01$ for comparisons of vaccine groups (B, C and D, respectively) with the placebo group (A). # $P < 0.05$ for comparison of Group C with group B. ## $P < 0.01$ for comparison of Group D with Group C.

Table 2
Numbers of solicited AEs with possible or probable relationship[a] with treatment (safety analysis set).

	(A) Placebo		(B) Vaccine		(C) Vaccine + 10 μg dmLT		(D) Vaccine + 25 μg dmLT	
	Dose 1 (n = 34)	Dose 2 (n = 34)	Dose 1 (n = 35)	Dose 2 (n = 34)	Dose 1 (n = 30)	Dose 2 (n = 30)	Dose 1 (n = 30)	Dose 2 (n = 28)
Nausea	3[b] (9%)[c]	6 [1][d] (18%)	6 (17%)	5 [2] (15%)	6 [1] (20%)	7 [4] (23%)	2 [1] (7%)	2 [1] (7%)
Vomiting	0	0	0	0	0	2 (7%)	0	1 (4%)
Diarrhea[e]	1 (3%)	0	0	0	1 (3%)	1 (3%)	0	0
Loose stools	2 (6%)	1 (3%)	3 (9%)	1 (3%)	2 (7%)	4 (13%)	5 (17%)	3 (11%)
Stomach ache	5 [1] (15%)	3 [1] (9%)	2 (6%)	4 (12%)	1 (3%)	1 [1] (3%)	7 (23%)	1 (4%)
Fever[f]	0	0	0	0	1 (3%)	0	0	0
Total	11 [1]	10 [2]	11	10 [2]	11 [1]	15 [5]	14 [1]	7 [1]

$P > 0.05$ for all comparisons of vaccine groups (B, C and D) with the placebo group (A) and comparisons between the vaccine groups.

[a] Study physicians judged the relation to immunization (unlikely, possible, probably, or unclassifiable) based on experiences from previous ETEC vaccine studies, including a temporal relationship with vaccination, i.e. within 72 h [7].

[b] Numbers of AEs of any intensity; mild, i.e. no interference with normal activity, or moderate, i.e. partial interference. No severe AEs, i.e. preventing normal activity, which were deemed possibly/probably related to vaccination, were recorded.

[c] Frequencies of AEs of any intensity.

[d] Numbers of AEs of moderate intensity are indicated in square brackets.

[e] Diarrhea was defined as three or more loose stools within 24 h; the reported cases of diarrhea were all mild, consisting of 3–4 loose stools within 24 h.

[f] Fever was defined as >37.7 °C orally or 38.0 °C rectally.

(2.3-fold, $P = 0.04$) magnitudes of ALS responses to CS6 in the group receiving vaccine plus 10 μg dmLT (C) than in the group receiving vaccine alone (B) (Fig. 1). Magnitudes and frequencies of responses to LTB, CFA/I and CS5 also tended to be higher in Group C than in Group B.

3.3.2. Fecal antibody responses

A majority of volunteers in each of the vaccine groups (B, C, D) responded with increased specific SIgA/total SIgA to all the primary antigens in fecal specimens (Fig. 2 and Table 3). Both the magnitudes and frequencies of responders were significantly higher in all of the vaccine groups than in the placebo group. Comparable frequencies of responders were observed after the first and second dose. No significant differences in frequencies or magnitudes of responses were recorded between the different vaccine groups.

3.3.3. Combined intestine-derived ASC and fecal antibody responses

Analysis of any mucosal immune response, i.e. fecal SIgA and/or ALS IgA responses against the primary antigens, showed that a high proportion (74–83%) of the vaccinees responded to all the 5 primary antigens, with the highest frequency in Group C, and 85–91% responded to ≥4 of the antigens (Table 4).

3.3.4. Serum antibody responses

The magnitudes and frequencies of serum IgA and IgG antibody responses against LTB were high in all vaccine groups (Fig. 3). The responses were higher after the second dose, peaking on day 21 (IgA) or day 21–28 (IgG) in most subjects. The frequencies and magnitudes of IgA and IgG responses in Group C were slightly higher than in Group B and significantly higher than in Group D. The LT neutralizing responses closely resembled the titer increases determined by ELISA (Fig. 3). Anti-LT serum antibody responses were also compared with those induced in recent trial of a first-generation ETEC vaccine containing CTB (for results of this comparison, see Supplementary material) [11]. The frequencies of IgA responses against the different CFs in serum were low (3–19%) and no significant differences between the different vaccine groups were seen (data not shown).

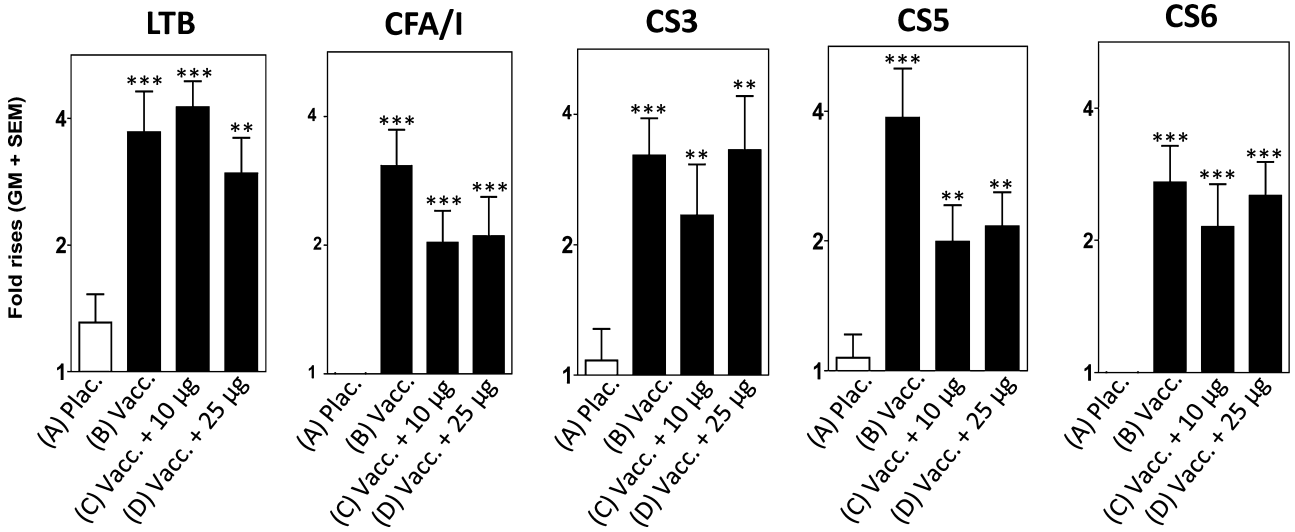

Fig. 2. Magnitudes of IgA responses (specific SIgA/total SIgA) against the five primary vaccine antigens in fecal specimens from volunteers receiving (A) placebo, (B) vaccine alone, (C) vaccine plus 10 μg dmLT and (D) vaccine plus 25 μg dmLT. Maximum fold rises (GM + SEM) in antibody levels after one or two doses of vaccine are indicated. *** $P < 0.001$ and ** $P < 0.01$ for comparisons of vaccine groups (B, C and D, respectively) with the placebo group (A).

Table 3
Frequencies of IgA responders[a] against the different primary vaccine antigens in ALS and fecal specimens (per protocol analysis sets).

	(A) Placebo	(B) Vaccine	(C) Vaccine + 10 μg dmLT	(D) Vaccine + 25 μg dmLT
ALS				
LTB	1/29 (3%, 0.1–18)[b]	26/29 (90%, 73–98)	28/29 (97%, 82–99)	22/26 (85%, 65–96)
CFA/I	1/24 (4%, 0.1–21)	15/27 (56%, 35–75)	20/28 (71%, 51–87)	17/24 (71%, 49–87)
CS3	2/24 (8%, 1–27)	24/27 (89%, 71–98)	23/28 (82%, 63–94)	21/24 (88%, 68–97)
CS5	1/24 (4%, 0.1–21)	15/27 (56%, 35–75)	19/28 (68%, 48–84)	14/24 (58%, 37–78)
CS6	3/24 (13%, 3–32)	15/27 (56%, 35–75)	20/28 (71%, 51–87)	15/24 (63%, 41–81)
Feces				
LTB	2/28 (7%, 0.9–24)	21/29 (72%, 53–87)	21/25 (84%, 64–95)	16/24 (67%, 45–84)
CFA/I	0/26 (0%, 0–13)	20/30 (67%, 47–83)	14/24 (58%, 37–78)	12/24 (50%, 29–71)
CS3	2/26 (8%, 1–25)	21/30 (70%, 51–85)	12/24[c] (50%, 29–71)	14/24 (58%, 37–78)
CS5	1/26 (4%, 0.1–20)	21/30 (70%, 51–85)	14/24 (58%, 37–78)	13/24 (54%, 33–74)
CS6	0/26 (0%, 0–13)	18/30 (60%, 41–77)	13/24 (54%, 33–74)	16/24 (67%, 45–84)

$P < 0.001$ for comparisons of all vaccine groups (B, C and D, respectively) with the placebo group (A) for all primary antigens in both ALS and fecal analyses.
[a] Cumulative response rates after one or two immunizations. Fold rises ≥2 were considered as responses in both ALS and fecal assays [6,11].
[b] Percentage of responders, 95% CI.
[c] If only subjects with a day 0 specimen were included in the analysis, 10/16 subjects (63%) responded to CS3 in fecal specimens. For all other antigens and groups, comparable frequencies were recorded if all subjects or only subjects with a day 0 specimen were included.

Table 4
Frequencies of IgA responders against different numbers of primary vaccine antigens in ALS and/or fecal specimens.

Frequency of subjects[a] responding to	(A) Placebo ($n = 20$) (%)	(B) Vaccine ($n = 23$) (%)	(C) Vaccine + 10 μg dmLT ($n = 23$) (%)	(D) Vaccine + 25 μg dmLT ($n = 20$) (%)
5 antigens[b]	0	74	83	75
4 antigens	0	17	4	10
3 antigens	5	0	4	0
2 antigens	10	4	4	10
1 antigen	5	0	4	0
0 antigen	80	4	0	5

[a] Only subjects from whom both fecal and ALS specimens were available.
[b] LTB, CFA/I, CS3, CS5, CS6.

3.3.5. *Immune responses against O78 LPS*

High rates of mucosal and serum antibody responses against O78 LPS were recorded in all vaccine groups. ALS responses were particularly frequent, with 96–100% of the vaccinated subjects responding (Table 5). Responses in Group D tended to be lower and less frequent than in Groups B or C. The antibody responses to O78 LPS were comparable after the first and the second dose in all sample types.

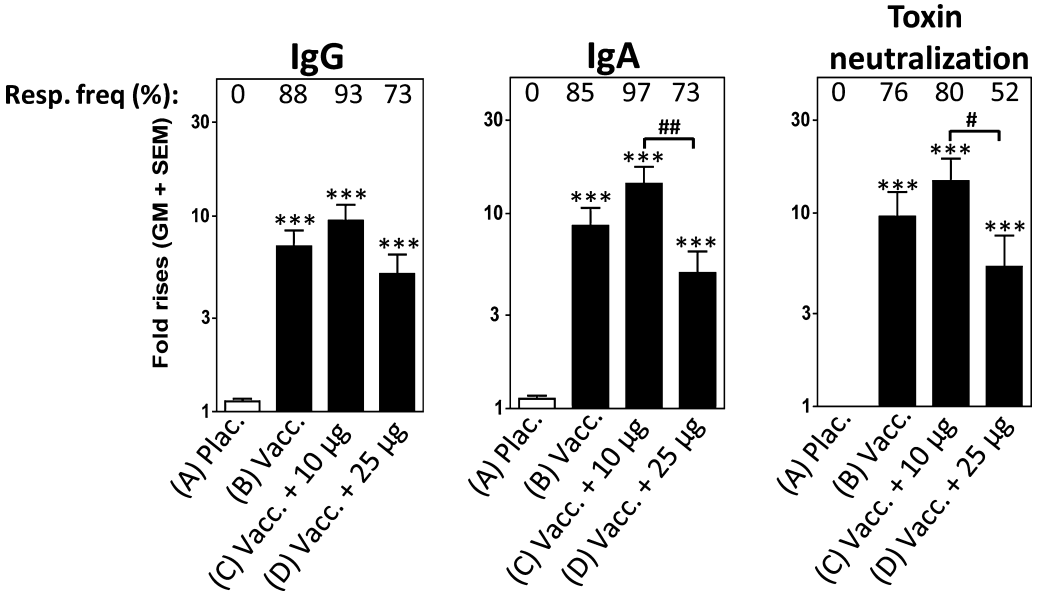

Fig. 3. Magnitudes of responses and frequencies of ELISA IgA and IgG responders against LTB and of LT neutralizing antibodies, determined by the Y1 adrenal cell assay, in serum from volunteers receiving (A) placebo, (B) vaccine alone, (C) vaccine plus 10 μg dmLT and (D) vaccine plus 25 μg dmLT. Maximum fold rises after one or two doses of vaccine are indicated (GM + SEM). IgA and IgG serum antibody responses were defined as twofold increases in post- compared to pre-vaccination samples [6,11]. Toxin neutralization responses were defined as fourfold increases in post- compared to pre-vaccination samples. *** $P < 0.001$, for comparisons of vaccine groups (B, C and D, respectively) with the placebo group (A). # $P < 0.05$ and ## $P < 0.01$ for comparison of Group D with Group C.

Table 5
Frequencies of IgA responders and magnitudes of responses against O78 LPS in ALS, fecal and serum specimens (per protocol analysis sets).

	(A) Placebo		(B) Vaccine		(C) Vaccine + 10 μg dmLT		(D) Vaccine + 25 μg dmLT	
	Resp. freq.[a]	Magn.[b]	Resp. freq.	Magn.	Resp. freq.	Magn.	Resp. freq.	Magn.
ALS	0/28 (0%, 0–0	1 (1–1)	29/29 (100%, 88–100)	44 (28–71)	28/28 (100%, 88–100)	55 (35–87)	24/25 (96%, 80–100)	34 (20–59)
Feces	4/26 (15%, 4–35)	1 (1–2)	21/30 (70%, 51–85)	4 (2–5)	17/24 (71%, 49–87)	3 (2–6)	13/24 (54%, 33–74)	2[d] (1–4)
Serum	2/33 (6%, 0.7–20)	1 (1–1)	26/34 (76%, 59–89)	4 (3–5)	23/30 (77%, 58–90)	5 (3–7)	20/26 (77%, 56–91)	3 (2–4)

$P < 0.001$ for comparisons of vaccine groups (B, C and D, respectively) with the placebo group (A) for both frequencies and magnitudes in all assays, with one exception, as indicated.

[a] Cumulative response rates after one or two immunizations. Fold rises ≥2 were considered as responses in all assays [6,11].
[b] Geometric means (95% CI).
[c] Percentage of responders, 95% CI.
[d] $P < 0.01$.

4. Discussion

The MEV (Etvax vaccine) was found to be safe and well tolerated. Only mild, and in a few instances moderate, AEs were recorded and neither the frequency nor intensity of AEs differed among subjects immunized with MEV or placebo. These results are consistent with data from several studies of the first generation ETEC vaccine as well as a prototype second generation ETEC vaccine, which were found to be safe and well tolerated in adults [6,7,11]. The MEV was also well tolerated when administered together with dmLT adjuvant, with no differences in frequency or intensity of AEs observed between subjects receiving MEV plus either dose of dmLT or MEV alone. These results support that the dmLT protein is more attenuated compared to single-mutant LT (mLT; LT(R192G)), an LT-derived adjuvant containing only one of the two mutations present in dmLT [18]. Thus, previous studies have shown that combinations of mLT, at comparable doses as used of dmLT in this study, and oral whole cell *Helicobacter* and *Campylobacter* vaccines, induced unacceptable gastrointestinal reactions ([19] and Bourgeois et al., unpublished data). The safety and tolerability of the MEV-dmLT combinations demonstrated in this trial support the rationale of further testing of such combinations in children and infants.

Evaluation of intestine-derived immune responses by the ALS method revealed strong responses against LTB in about 90% of the vaccinated subjects; these responses were about twofold higher in subjects given vaccine plus 10 μg of dmLT than vaccine alone. The vaccine also induced highly significant ALS responses against all of the CFs in 60–90% of the vaccinees as well as significant fecal SIgA responses to all five primary antigens in 60–80% of the immunized volunteers. These results confirm the encouraging results obtained when testing a prototype vaccine consisting of a CFA/I overexpressing strain and LCTB*A* in a previous Phase I trial [11] and support that the new vaccine, even in the absence of adjuvant, is highly immunogenic. The magnitudes of ALS responses against CS6, which is the CF antigen present in the lowest amount in MEV, were further increased in subjects receiving vaccine plus 10 μg of dmLT compared to those receiving vaccine alone. There was also a trend for higher ALS responses against CFA/I and CS5 in subjects receiving vaccine plus 10 μg of dmLT, whereas ALS responses against CS3, which is present in considerably higher amounts in MEV than the other CFs, were not enhanced by addition of adjuvant. These results are consistent with the dose-sparing effect of dmLT shown in mice immunized with decreasing doses of vaccine [9]. Thus, it is possible that the administration of a high dose of LCTB*A* and highly immunogenic CF-expressing bacteria may have masked some of the potential adjuvant activity of dmLT in this study.

Interestingly, when combining immune responses assessed by either or both of the two mucosal immunogenicity assays, about 80% of the subjects receiving vaccine alone or together with dmLT had responded to all five primary antigens and about 90% to at least four of the antigens. Thus, the primary hypothesis of the study, i.e., that at least 50% of the subjects in any of the vaccine groups should mount a mucosal immune response to at least four of the five primary vaccine antigens, was strongly supported and the results clearly exceeded the expectations. The comparatively high and frequent mucosal immune responses recorded against CS6 are particularly important since the first-generation formalin-inactivated ETEC vaccine did not induce any immune responses to this prevalent CF in humans [5]. Hence, our approach to use CS6 expressing bacteria inactivated with phenol, which preserves CS6 immunogenicity [13], rather than formalin has been successful.

Increased preimmunization antibody levels, i.e. titers above background levels, were detected in some of the subjects, particularly against the CS3 antigen (data not shown), suggesting previous exposure to ETEC or other microorganisms expressing immunologically related proteins. Previous exposure to such antigens, as well as different host genetic factors, may partially explain the variation in magnitude and breadth of immune responses observed in different vaccinees. Thus, it was recently shown that ETEC infection may induce memory B cells to ETEC CFs and LT that may mediate an anamnestic response to reexposure to ETEC [20] and probably also to corresponding antigens in MEV. Furthermore, we have previously shown that individuals with certain blood groups are more susceptible to infection with ETEC expressing certain CFs, and then most likely respond more strongly to corresponding vaccine antigens [21]. The influence of immunological memory and host genetics on immune responses to MEV will be addressed in follow-up studies.

Our finding of a positive effect of the lower dose of dmLT adjuvant on immune responses to antigens expressed in lower amounts supports the rationale to evaluate this adjuvant further. Of particular interest would be to assess the adjuvant effect in malnourished children in developing countries who are known to respond less well to oral vaccines [22]. Furthermore, previous studies with the first-generation ETEC vaccine have suggested that lower doses of vaccine might be needed to improve tolerability in younger age groups [8].

The observed lack of an effect of the higher dose of dmLT on the anti-LTB and anti-CF responses indicates the need to determine the optimal dosage of dmLT when given together with different vaccines in future clinical trials. The reason for the lack of an immune-enhancing effect of the higher dose of dmLT in this study is unclear. However, a related phenomenon was observed when a single, oral dose of dmLT was given to human volunteers where 100 μg was found to be less immunogenic than 50 μg doses [23]. Since studies to date have only investigated the influence of dmLT on immune responses shortly after immunization, it will also be important to evaluate the effect of different dosages of dmLT on long-term immunity, including immunological memory and protection against infection in future studies. The excellent safety of the vaccine-adjuvant combinations demonstrated in this trial will facilitate follow-on studies to optimize dmLT-vaccine formulations.

MEV also induced systemic IgA and IgG responses to LTB in serum in almost all vaccinated volunteers, with the highest

response rate (97%) in the group receiving vaccine plus 10 μg dmLT. Indeed, the combination of MEV with 10 μg dmLT gave rise to comparable anti-LTB responses, both in IgA and IgG, as induced by a fourfold higher dose of LCTBA in a previous study [11]. Interestingly, the anti-LTB responses determined by ELISA were closely mirrored by increases in LT neutralizing titers, supporting that anti-LTB responses reflect functional LT immunity. dmLT may also be capable of enhancing systemic anti-toxin immune responses, as suggested by the finding (see Supplementary material) that MEV plus 10 μg dmLT induced significantly higher LT neutralizing as well as anti-LT IgA and IgG antibody responses in serum than the first-generation ETEC vaccine containing a comparable dose of CTB.

As in previous studies of oral, inactivated as well as live ETEC vaccines in Swedish and American volunteers [5,24], IgA antibody responses against all of the different CFs in serum were infrequent and low. Serum IgA antibody responses induced by MEV against O78 LPS were, however, frequent. Fecal and ALS IgA responses against O78 LPS were also observed in a majority of vaccinees. Although O78 LPS is only expressed by about 10% of clinical ETEC isolates [25], these responses may add to the protective coverage of the vaccine since we have previously shown that anti-O antibodies may provide protection against ETEC expressing the homologous serogroup [5].

A combination of LT and CF antigens seems to be required for broad protective coverage. It has been estimated that a vaccine containing LT antigen and the most prevalent CF antigens, as those in MEV and in an oral, live ETEC candidate vaccine, ACE527, recently evaluated in humans [26], may have the potential to protect against at least 80% of all ETEC strains causing disease in humans [1,5]. In contrast, a vaccine based on LT antigen alone will not offer protection against ST-only ETEC strains and is likely to provide shorter duration of protective immunity [27].

Based on the excellent safety profile and capacity of MEV to induce highly significant mucosal immune responses against the most prevalent ETEC virulence factors, studies are planned to evaluate the safety and immunogenicity of the vaccine alone and in combination with different dosages of dmLT in descending-age groups in Phase I/II trials in Bangladesh and for protective efficacy in visitors to ETEC-endemic areas.

Contributors

AMS and AL were the principal investigators. AMS, AL, JH, LB, RW, JC, NC and BG participated in the design of the studies and interpretation of results. NC was responsible for production of the vaccine and JC had developed the adjuvant. AMS and AL were responsible for the immunological analyses, MH for the clinical assessments and analyses of AEs and MP for the statistical analyses. AMS and AL wrote the manuscript. All coauthors contributed to the critical review and revision of the manuscript and have seen and approved the final version.

Roles of the funding sources

The nonprofit organization PATH participated in the design of the studies, interpretation of results and reviewed the manuscript. The other funding sources only contributed financially to the study.

Conflict of interest statement

NC and BG are employees and minority shareholders of Scandinavian Biopharma Holding AB, which holds certain commercial rights to the vaccine tested in this study. AMS and JH are shareholders of the biotech company Gotovax AB that may receive a small royalty on sales of the ETEC vaccine if it becomes a commercial product. NC and AMS have patent PCT/EP2012/067598-PCT pending. NC, AMS and JH have patent PCT/EP2011/065784-PCT pending. JC has a U.S. Patent No. 6033673 licensed to Bill & Melinda Gates Foundation, PATH EVI and ETVAX. All other authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.10.069.

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