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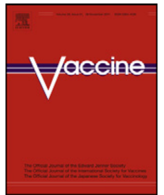

Clinical trial to evaluate safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* prototype vaccine containing CFA/I overexpressing bacteria and recombinantly produced LTB/CTB hybrid protein☆

A. Lundgren[a,*], S. Leach[a], J. Tobias[a], N. Carlin[b], B. Gustafsson[b], M. Jertborn[a], L. Bourgeois[c], R. Walker[c], J. Holmgren[a], A.-M. Svennerholm[a]

[a] *Gothenburg University Vaccine Research Institute (GUVAX), Department of Microbiology and Immunology, University of Gothenburg, Box 435, 405 30 Gothenburg, Sweden*
[b] *Etvax AB, Gunnar Asplunds allé, 171 63 Solna, Sweden*
[c] *PATH, 455 Massachusetts Avenue, NW, Washington, DC 20001, USA*

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A B S T R A C T

We have developed a new oral vaccine against enterotoxigenic *Escherichia coli* (ETEC) diarrhea containing killed recombinant *E. coli* bacteria expressing increased levels of ETEC colonization factors (CFs) and a recombinant protein (LCTB*A*), i.e. a hybrid between the binding subunits of *E. coli* heat labile toxin (LTB) and cholera toxin (CTB). We describe a randomized, comparator controlled, double-blind phase I trial in 60 adult Swedish volunteers of a prototype of this vaccine. The safety and immunogenicity of the prototype vaccine, containing LCTB*A* and an *E. coli* strain overexpressing the colonization factor CFA/I, was compared to a previously developed oral ETEC vaccine, consisting of CTB and inactivated wild type ETEC bacteria expressing CFA/I (reference vaccine). Groups of volunteers were given two oral doses of either the prototype or the reference vaccine; the prototype vaccine was administered at the same or a fourfold higher dosage than the reference vaccine.

The prototype vaccine was found to be safe and equally well-tolerated as the reference vaccine at either dosage tested. The prototype vaccine induced mucosal IgA (fecal secretory IgA and intestine-derived IgA antibody secreting cell) responses to both LTB and CFA/I, as well as serum IgA and IgG antibody responses to LTB. Immunization with LCTB*A* resulted in about twofold higher mucosal and systemic IgA responses against LTB than a comparable dose of CTB. The higher dose of the prototype vaccine induced significantly higher fecal and systemic IgA responses to LTB and fecal IgA responses to CFA/I than the reference vaccine.

These results demonstrate that CF over-expression and inclusion of the LCTB*A* hybrid protein in an oral inactivated ETEC vaccine does not change the safety profile when compared to a previous generation of such a vaccine and that the prototype vaccine induces significant dose dependent mucosal immune responses against CFA/I and LTB.

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1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) remains the most common cause of bacterial diarrhea in children in low-resource countries and in travelers to these countries [1,2]. Despite this, no effective vaccine for ETEC is available. ETEC causes disease by colonizing the small intestine through colonization factors (CFs) and producing heat-labile (LT) and/or heat-stable (STa) enterotoxins. Preclinical studies, as well as studies in humans, have indicated that locally produced intestinal IgA antibodies against LT and CFs are protective [3,4].

We have previously developed an oral ETEC vaccine consisting of a combination of recombinantly produced cholera toxin B subunit (rCTB) and formalin-inactivated ETEC bacteria expressing major CFs [5,6]. Extensive clinical evaluation of this first generation

Abbreviations: AE, adverse event; ALS, antibodies in lymphocyte supernatants; ASC, antibody secreting cell; CF, colonization factor; CT, cholera toxin; CTB, cholera toxin binding subunit; dmLT, double mutant LT; ELISA, enzyme linked immunosorbent assay; ELISPOT, enzyme linked immunospot assay; ETEC, enterotoxigenic *Eschericia coli*; GM, geometric mean; GMP, good manufacturing practice; HRP, horseradish peroxidase; LT, heat labile toxin; LTB, heat labile toxin binding subunit; PBMCs, peripheral blood mononuclear cells; PV, prototype vaccine; RV, reference vaccine; STa, heat stable toxin; sIgA, secretory IgA.

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* Corresponding author. Tel.: +46 31 7866213; fax: +46 31 7866205.
E-mail addresses: anna.lundgren@microbio.gu.se (A. Lundgren), susannah.leach@microbio.gu.se (S. Leach), joshua.tobias@microbio.gu.se (J. Tobias), nils.carlin@etvax.se (N. Carlin), bjorn.gustafsson@etvax.se (B. Gustafsson), marianne.jertborn@microbio.gu.se (M. Jertborn), lbourgeois@path.org (L. Bourgeois), rwalker@path.org (R. Walker), jan.holmgren@microbio.gu.se (J. Holmgren), ann-mari.svennerholm@microbio.gu.se (A.-M. Svennerholm).

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ETEC vaccine (rCTB-CF) showed that it was safe and induced significant mucosal immune responses except in children 6–17 months of age in endemic areas. In these children a full adult dose was associated with an increased frequency of vomiting [5,7–11]; however, a quarter of a full dose was safe and immunogenic [12]. The rCTB-CF vaccine provided significant protective efficacy (PE 77%, $P = 0.039$) against non-mild ETEC disease in American travelers to Mexico and Guatemala [13], but no significant protection (PE 20%) against ETEC diarrhea in Egypt in children 6–18 months of age with mostly mild disease [3].

We have now developed a second generation ETEC vaccine that contains inactivated *E. coli* bacteria expressing increased levels of the most prevalent CFs [14] plus the protein LCTB*A*, which is a hybrid between the binding units of LT and cholera toxin (CT) [15]. LCTB*A* has been shown to induce strong LT neutralizing immune responses in preclinical studies [15]. Mucosal IgA responses to LT and CFs have been shown to provide significant protection against infection with ETEC bacteria expressing homologous factors both in human and animal studies [16]. By using recombinant technology, several *E. coli* strains were produced that expressed 3- to 10-fold higher levels of major CFs than expressed on the wild type ETEC strains used in the original rCTB-CF ETEC vaccine [17–20]. The vaccine was made by constructing plasmids in which the genes for the different CF antigens were placed downstream strong promoters (e.g. *tac*) and incorporating these plasmids in non-virulent *E. coli* K12 bacteria or an LT negative O78 ETEC strain. These CF over-expressing strains have been shown to induce considerably higher mucosal and serum immune responses against corresponding CFs in pre-clinical animal studies than the previous vaccine strains [18–20].

We undertook the present double blinded phase I trial in adult Swedish volunteers to evaluate whether two different dosages of a prototype of this second generation ETEC vaccine, consisting of inactivated *E. coli* bacteria over-expressing one of the most prevalent CFs; i.e. CFA/I [14], administered together with LCTB*A*, is safe and induces stronger immune responses against CFA/I and LT than an inactivated reference vaccine containing the CFA/I positive strain and rCTB that were used in the first generation rCTB-CF ETEC vaccine.

2. Materials and methods

See Supplementary material for further information.

2.1. Study vaccines

Two different inactivated vaccine preparations were used: (1) the new prototype vaccine (PV) consisting of 3×10^{10} bacteria of the CFA/I overexpressing *E. coli* strain SBL109 [19] + 1 mg LCTB*A* [15] and (2) a reference vaccine (RV) consisting of 3×10^{10} bacteria of the CFA/I *E. coli* strain used in the 1st generation ETEC vaccine [5,21] + 1 mg rCTB [22] per dose. The strains and toxoids are described in further detail in Supplementary materials and methods section and in the cited references. The final good manufacturing practice (GMP) produced PV contained 600 μg CFA/I per dose and RV contained 200 μg CFA/I per dose.

2.2. Study objectives

The primary objective was to evaluate the safety of the PV given twice and in two different dosages (1× and 4×) and to compare the intestinal (fecal) and intestine derived (antibody secreting cell; ASC) immune responses induced by the PV against the RV. The secondary objective was to evaluate serum antibody responses.

2.3. Study design

This was a three-armed, randomized, double blind, comparator-controlled, single center phase I trial. Healthy adult subjects, 19–46 years old, were recruited from the Gothenburg area in Sweden. The study was performed in accordance with the Declaration of Helsinki and written informed consent was obtained before participation. The 60 enrolled subjects were randomized into one of three groups at a ratio of 1:1:1. Volunteers were immunized with two doses of RV (group A, 3×10^{10} CFA/I expressing wild type bacteria + 1 mg rCTB), PV at a lower dosage level (group B, 3×10^{10} CFA/I overexpressing bacteria + 1 mg LCTB*A*) or PV at a fourfold higher dosage level (group C; 4PV, 12×10^{10} CFA/I over-expressing bacteria + 4 mg LCTB*A*).

2.4. Immunizations and sample collection

Subjects were not allowed to eat or drink for 1 h before and after immunization. All volunteers received two oral doses of either vaccine 2 weeks apart (day 0 and day 14 ± 2). Serum samples were collected before the first vaccination and on days 7 ± 1, 14 ± 2, 21 (20–23) and 42–49. Heparinized blood for isolation of peripheral blood mononuclear cells (PBMCs) and fecal samples were collected on days 0, 7 ± 1, and (21) (20–23).

2.5. Safety evaluation

Safety was evaluated by physical examination at the screening visit and at the last follow up (days 42–49) and by clinical chemistry and hematology testing at the screening visit and on days 7 ± 1 and 21 (20–23). The study subjects recorded adverse events (AEs) in study diaries.

2.6. Evaluation of immune responses

PBMCs were isolated from heparinized blood by density gradient centrifugation of Ficoll-Paque (GE Healthcare Bio-Sciences, Sweden). For antibodies in lymphocyte supernatant (ALS) assays, 2×10^6 PBMCs per well were cultured in 96-well plates. Supernatants were collected after 72 h and enzyme inhibitors added before storage at −70 °C [23]. ASCs were detected by enzyme linked immunospot (ELISPOT) technique [8]. Fecal samples were collected and immediately frozen at −20 °C at home by the subjects. Fecal extracts were prepared as described and stored at −70 °C [7]. Antibody levels in serum, fecal extracts and ALS specimens were analyzed by enzyme linked immunosorbent assay (ELISA) using plates coated with CFA/I or GM1 ganglioside plus LTB [5].

2.7. Statistical analyses

Differences between pre- and post-immunization antibody levels within groups were evaluated using a paired *t*-test. Correlation analyses were performed using the Pearson test. Comparisons of responses in different groups were performed using an unpaired *t*-test. Analysis of response rates and frequencies of AEs was performed using the Fisher's exact test. To control for the two comparisons used to address the primary objectives (comparisons between responses in subjects receiving PV and RV and between subjects receiving 4PV and RV) Bonferroni correction was applied; results significant after correction ($P < 0.025$) are indicated ([B]).

3. Results

3.1. Study subjects

Seventy-two subjects were screened for possible enrollment into the study (Supplementary Fig. 1); among these, 60 were

Table 1
Subject demographics (safety analysis set).

Characteristics	Reference vaccine (RV) (n = 20)	Prototype vaccine (PV) (n = 20)[a]	4× prototype vaccine (4PV) (n = 19)	Total (n = 59)
No. (%) of subjects				
Sex				
Male	9 (45%)	10 (50%)	10 (54%)	29 (49%)
Female	11 (55%)	10 (50%)	9 (47%)	30 (51%)
Age (years)				
Mean (SD)	30 (9)	26 (7)	24 (5)	27 (7)
Range	19–46	19–44	20–40	19–46

[a] One female subject aged 23 years in the prototype vaccine group was excluded from the study before administration of the second dose due to suspected gastroenteritis and was thus not included in the per protocol analysis set.

enrolled with 20 in each vaccination arm. The age and gender distributions were comparable in the three vaccination arms (Table 1). Among the enrolled volunteers, 58 completed the study per protocol (Supplementary Fig. 1). The safety analysis set included 20 volunteers immunized with RV, 20 with PV and 19 with 4PV. The per protocol analysis set included 20 volunteers immunized with RV, 19 with PV and 19 with 4PV.

3.2. Safety

No serious adverse events occurred in any of the volunteers. No subjects experienced any immediate (within 60 min) post-immunization reactions and no significant adverse changes of blood pressure, pulse or other parameters were observed at the follow-up visit.

Among the total 119 AEs recorded, 33 were deemed to be possibly or probably related to immunization (Table 2); among these, most (28) were of gastrointestinal origin. AEs deemed to be possibly or probably related to immunization occurred at comparable low frequencies in the three study groups (Table 2). The vast majority of these AEs (82%) were of mild intensity, while the remaining AEs were of moderate intensity. None of the volunteers had diarrhea during the study. Mild or moderate nausea was experienced on the day of vaccination or on the following day by three volunteers immunized with RV (after dose 1 or 2), two with PV (after dose 1) and four with 4PV (one volunteer after dose 1, one volunteer after dose 2 and two volunteers after both doses). Slightly more AEs were reported after the first than the second dose. A few deviations in hematology and blood chemistry from base line values were noted, but all were regarded as clinically non-significant.

3.3. Immunogenicity

3.3.1. Mucosal immune responses in fecal samples

We have previously determined antigen specific intestinal (fecal or lavage) antibody titers in relation to total IgA levels in the samples [7,24]. In this study, the total sIgA levels were comparable before and after vaccination in volunteers receiving RV or PV whereas samples from volunteers given 4PV contained as a mean twofold higher levels of sIgA on day 21 compared to before immunization (P = 0.01). Since we found comparable levels of total protein in samples from all groups and little variation between the different samples collected from the same individual at different time points, antibody levels in feces were not compensated for levels of total sIgA.

Before immunization, the LTB-specific sIgA antibody titers in fecal extracts were low (Fig. 1A). Immunization resulted in significantly increased LTB-specific sIgA levels in all groups. Responses to LTB were more frequent after the second dose (Table 3). A majority of volunteers immunized with PV developed fecal sIgA responses against LTB; the responses were slightly higher than those induced by RV (Fig. 1A). The responses in volunteers receiving 4PV were significantly higher and more frequent than in volunteers receiving RV (Fig. 1A) and also more frequent after the first dose compared to subjects receiving RV (P = 0.049, Table 3).

Immunizations also resulted in increased fecal sIgA levels against CFA/I in all groups (Fig. 1B), both after the first and second dose (Table 3). The frequencies of responders were slightly higher among volunteers receiving PV compared to RV. The strongest responses were induced by 4PV, both with regard to frequency and magnitude (Fig. 1B). A higher proportion of volunteers receiving 4PV responded after the first dose compared to subjects receiving RV (Table 3).

All groups exhibited good agreement between sIgA responses to LTB and CFA/I in fecal extracts with 89% concordance among responses to the two antigens.

3.3.2. ALS responses

In recent studies, the ALS method has often replaced the ELISPOT assay for measurement of ASC responses [23,25,26]. Since in initial studies we found significant correlations between the levels of

Table 2
Adverse events with a possible or probable relation to vaccination (safety analysis set).

Adverse event	No. (%) of subjects					
	Reference vaccine (RV) n = 20		Prototype vaccine (PV) n = 20		4× prototype vaccine (4PV) n = 19	
	Dose 1	Dose 2	Dose 1	Dose 2	Dose 1	Dose 2
Nausea	1 + 1[a] (10%)	1 (5%)	2 (10%)	0	2 + 1[a] (16%)	2 + 1[a] (16%)
Vomiting	0	0	0	0	0	1 (5%)
Abdominal pain	1[a] (5%)	0	1 (5%)	0	0	1[a] (5%)
Diarrhea	0	0	0	0	0	0
Loose stool(s)	2 (10%)	1 (5%)	1 (5%)	1 (5%)	1 (5%)	0
Flatulence/bubbly stomach	1 (5%)	0	1 (5%)	0	3 (16%)	1 (5%)
Regurgitation	1 (5%)	0	0	0	0	0
Headache	1[a] (5%)	0	1 (5%)	0	0	0
Dry mouth	0	0	0	0	1 (5%)	1 (5%)
Tiredness	0	0	1 (5%)	0	0	0

[a] Six AEs were of moderate intesity, as indicated ([a]). All other AEs were of mild intensity. The six moderate AEs were experienced by five individuals; three receiving RV (one subject experienced nausea, one abdominal pain and one headache after the first dose) and two receiving 4PV (one subject experienced nausea after both the first and the second dose and one subject abdominal pain after the second dose).

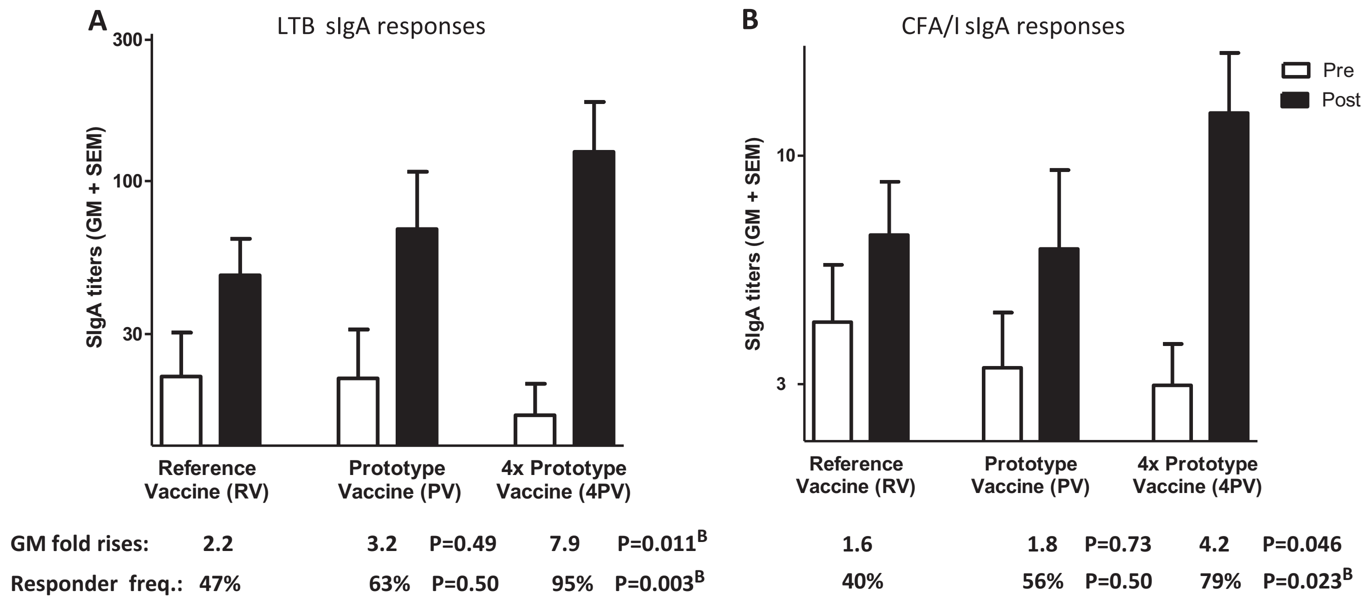

Fig. 1. Titers (geometric mean + SEM) of sIgA antibodies specific for LTB (A) and CFA/I (B) in fecal extracts prepared from specimens collected before (white bars) and after (black bars; maximum levels of antibodies detected after administration of one or two doses) immunization. Geometric mean (GM) fold rises (magnitudes of responses) and cumulative responder frequencies are indicated. A few fecal samples were excluded from the analysis due to low content of total sIgA or unspecific binding to ELISA plates (see Table 3). Responses in subjects receiving the prototype vaccine at 1× or 4× dosage (PV or 4PV) were compared to responses in subjects receiving the reference vaccine (RV) by an unpaired *t*-test. Statistically significant differences after Bonferroni correction are indicated ([B]).

LTB- and CFA/I specific antibodies in ALS samples and numbers of ELISPOT ASCs (Fig. 2A and B), we used the ALS method throughout the study.

Before immunization, LTB-specific IgA ALS levels were low (Fig. 2C). Immunizations resulted in significant increases in LTB-specific IgA ALS titers in all groups. Responses were more frequent after the second vaccine dose (Table 3) and almost all volunteers responded to LTB after two immunizations (Table 3 and Fig. 2C). The magnitudes of ALS responses were about twofold higher in subjects receiving PV or 4PV compared to RV (Fig. 2C).

Immunizations also gave rise to significant increases in IgA ALS responses against CFA/I in all groups (Fig. 2D); similar frequencies of CFA/I responses were seen after doses 1 and 2 (Table 3). Frequencies as well as magnitudes of responses were comparable in volunteers receiving RV and 4PV (Fig. 2D).

Comparable responder frequencies, but slightly higher (as a mean 1.5-fold) magnitudes of responses against CFA/I and LTB were detected by ELISPOT compared to ALS.

Among the subjects immunized with PV or RV, 70–80% responded to CFA/I in fecal or ALS or both samples and 95% of subjects receiving 4PV responded to CFA/I in at least one of these tests.

3.3.3. *Serum antibody responses*

Immunizations resulted in significant serum IgA antibody responses to LTB in almost all volunteers (Fig. 3A). Responses were more frequent after the second dose (Table 3); the responses reached maximum levels on day 21 and declined by day 42 (Fig. 3A). Post-vaccination IgA titers on day 21 were significantly higher in subjects receiving PV (twofold increase) and 4PV (threefold increase) compared to subjects immunized with RV. Both frequencies and magnitudes of responses were higher in subjects immunized with either dose of PV compared to RV.

The LTB-specific IgG responses in serum were slightly lower and less frequent than the IgA responses (Fig. 3B and Table 3) and remained at comparable high levels from day 21 through day 42 (Fig. 3B). The magnitudes of anti-LTB IgG responses were also higher in subjects receiving PV and 4PV compared to those receiving RV.

Table 3
Number of responders after administration of one and two doses of vaccine and the cumulative response rate in reslation to day 0 (per protocol analysis set[a]).

Assay	Reference vaccine (RV)			Prototype vaccine (PV)			4× prototype vaccine (4PV)		
	Dose 1	Dose 2	Cumulative	Dose 1	Dose 2	Cumulative	Dose 1	Dose 2	Cumulative
Faeces[b]									
LTB sIgA	5/19 (26%)	6/19 (32%)	9/19 (47%)	5/16 (31%)	10/16 (63%)	10/16 (63%)	12/19 (63%)	16/19 (84%)	18/19 (95%)
CFA/I sIgA	5/20 (25%)	5/20 (25%)	8/20 (40%)	5/16 (32%)	8/16 (50%)	9/16 (56%)	9/19 (47%)	8/19 (42%)	15/19 (79%)
ALS[b]									
LTB IgA	12/20 (60%)	19/20 (95%)	19/20 (95%)	12/19 (63%)	19/19 (100%)	19/19 (100%)	13/19 (68%)	19/19 (100%)	19/19 (100%)
CFA/I IgA	9/20 (45%)	12/20 (60%)	15/20 (75%)	7/19 (37%)	5/19 (26%)	10/19 (53%)	11/19 (58%)	9/19 (47%)	15/19 (79%)
Serum[c]									
LTB IgA	8/20 (40%)	16/20 (80%)	16/20 (80%)	7/19 (37%)	17/19 (89%)	18/19 (95%)	10/19 (53%)	19/19 (100%)	19/19 (100%)
LTB IgG	8/20 (40%)	14/20 (70%)	14/20 (70%)	9/19 (47%)	16/19 (84%)	16/19 (84%)	11/19 (58%)	16/19 (84%)	16/19 (84%)
CFA/I IgA	4/20 (20%)	5/20 (15%)	5/20 (25%)	0/19 (0%)	2/19 (11%)	2/19 (11%)	2/19 (11%)	3/19 (16%)	4/19 (21%)

[a] A few fecal samples were excluded from the analysis due to low content of total sIgA or unspecific binding to ELISA plates.
[b] Responses in fecal samples and in ALS samples to dose 1 were determined on day 7 and to dose 2 on day 21.
[c] Serum responses to dose 1 were determined on day 7 or day 14 and serum responses to dose 2 on day 21 or day 42.

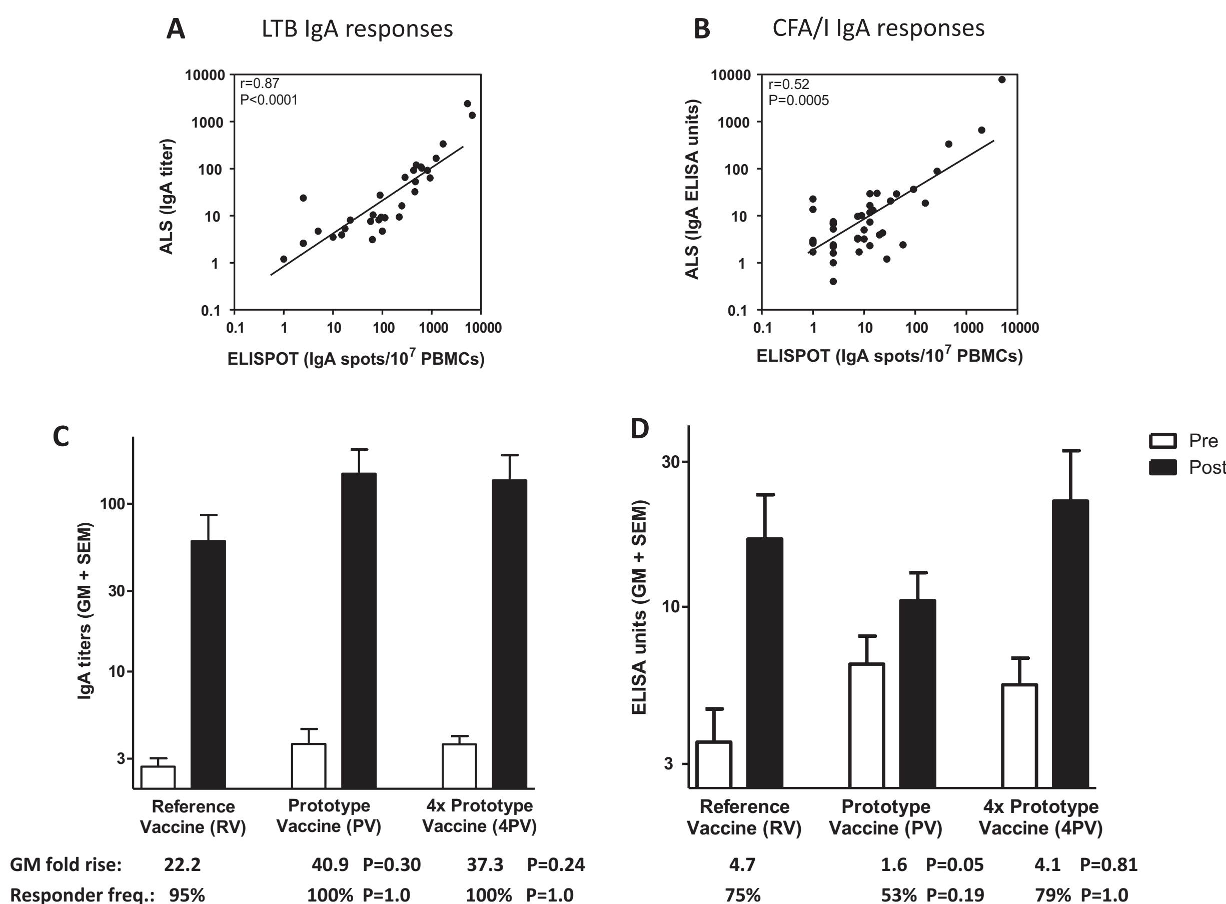

Fig. 2. Levels of IgA antibodies specific for LTB (A and C) and CFA/I (B and D) in ALS samples. (A and B) Comparisons of specific antibody levels in ALS specimens and numbers of ASCs detected by ELISPOT in a subset immunized volunteers. (C and D) Specific antibody levels (GM + SEM) were analyzed before (white bars) and after (black bars; maximum levels after one or two doses) immunization. GM fold rises (magnitudes of responses) and cumulative responder frequencies are indicated (per protocol analysis set, $n = 58$). Responses in subjects receiving the prototype vaccine at 1× (PV) or 4× dosage (4PV) were compared to responses in subjects receiving the reference vaccine (RV) by an unpaired *t*-test.

Immunizations only induced serum IgA responses to CFA/I in10–25% of volunteers (Table 3) and no differences were detected between the different immunization groups.

4. Discussion

In this study we show that the new inactivated whole cell prototype ETEC vaccine administered in two oral doses 2 weeks apart was safe and immunogenic at both dosage levels tested. The numbers of AEs deemed to be possibly or probably related to immunization were low in all three study groups and similar to results from previous trials with the first generation ETEC vaccine [5,13] or buffer alone [5]. Most AEs were mild and no severe reactions were reported. For these reasons, we conclude that administration of 3–12 × 10^{10} inactivated *E. coli* bacteria over-expressing CFA/I as well as 1–4 mg of LCTB*A* is safe and well tolerated.

Previous studies in animals and epidemiological data indicate that mucosal IgA responses to CFs and LT can protect against ETEC disease [27–29] and that these responses may cooperate synergistically for protection [6,16]. In this study, we used several different approaches to evaluate mucosal IgA responses. Using fecal extracts, frequencies of responses against LTB as well as CFA/I tended to be higher in subjects receiving PV than RV and both types of responses were significantly higher and more frequent among subjects receiving 4PV than RV. sIgA levels were examined to exclude the risk of measuring IgA antibodies transudated from serum. At variance with previous studies [7,24], we did not adjust vaccine specific antibody levels for total sIgA because sIgA concentrations were approximately twofold higher in post- than pre-immunization samples from volunteers receiving 4PV. This suggests that administration of a higher dose of bacteria, with a high content of *E. coli* lipopolysaccharide, may potentially boost the production of intestinal sIgA. Since the total protein concentrations in samples collected from the same individuals at different time points only differed marginally, vaccine specific titers in fecal extracts were reported without compensation.

We also analyzed intestinally derived ASC responses. We chose the ALS assay as our primary analysis method since initial results demonstrated good agreement between the ALS and ELISPOT responses, confirming previous reports of the suitability of using ALS for evaluating ASC responses to both LTB and CFA/I [26]. Since the ALS method allows analysis of a large number of pre and post-immunization samples collected over extended periods of time in the same test as well as reanalysis of samples, we used this method throughout the study.

We found significant ALS responses to both LTB and CFA/I in all vaccination groups. Almost 100% of subjects in all groups responded to LTB, consistent with the high response rates to CTB recorded by ELISPOT in previous trials [5,7]. The magnitudes of the anti-LTB ALS responses were, on average, twofold higher in subjects receiving PV as compared to RV, but the responses did not increase further in subjects receiving 4PV. About 80% of subjects receiving RV and 4PV responded to CFA/I as detected by the ALS method, which was comparable to the ASC responses against CFA/I recorded

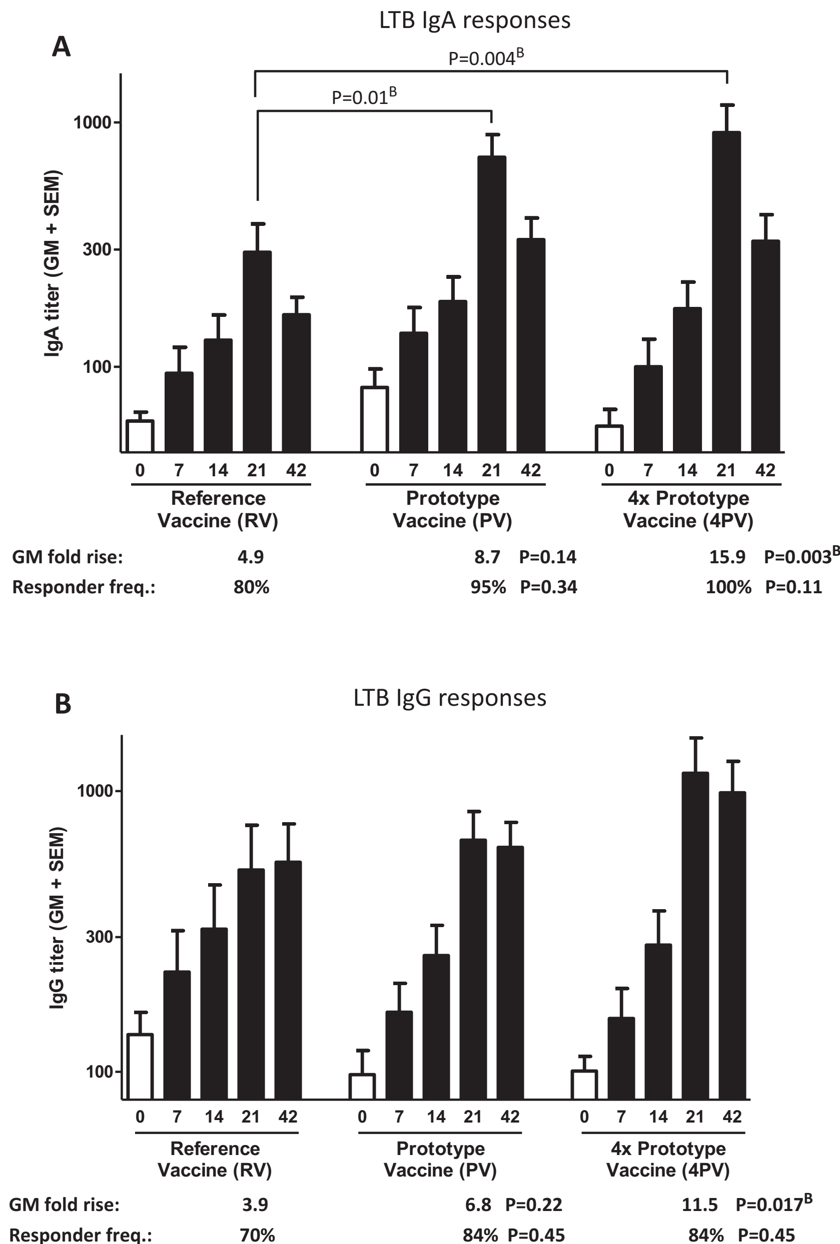

Fig. 3. IgA (A) and IgG (B) antibody responses against LTB in serum samples. Antibody titers (GM + SEM) were analyzed before (white bars) immunization and at 7, 14, 21 and 42 days after immunization (black bars). GM fold rises (magnitudes of responses) and cumulative responder frequencies 1 week after administration of the second vaccine dose (day 21) are shown below the graphs (per protocol analysis set, $n = 58$). Responses (magnitudes and responder frequencies) in subjects receiving the prototype vaccine at 1× (PV) or 4× (4PV) dosage were compared to responses in subjects receiving the reference vaccine (RV) by an unpaired *t*-test, as indicated below the graphs. Post-immunization titers on day 21 were also compared, as indicated in the graph (A), by an unpaired *t*-test. Statistically significant differences after Bonferroni correction are indicated ([B]).

by ELISPOT in previous ETEC vaccine studies [5,7]. In contrast to the fecal antibody responses, only small differences between ALS responses induced by RV, PV and 4PV were recorded. Recent results from our laboratory however indicate that whereas ALS responses peak 7 days after administration of the first dose of an oral vaccine, ALS responses reach maximal levels some days earlier after a second vaccine dose and rapidly decrease thereafter (to be published), suggesting that we may have underestimated maximal ALS responses at least threefold in the present study. In contrast to the relatively transient ASC responses in blood, previous studies of anti-CTB and anti-CFA/I antibody responses in fecal and lavage samples collected after oral ETEC and cholera vaccination have shown that intestinal antibody levels remain elevated for longer periods of time [7,30].

Immunization with PV also induced IgA and IgG responses to LTB in serum in almost all volunteers; the post-immunization anti-LTB IgA levels on day 21 being significantly higher after immunization with PV compared to RV and significantly increased in subjects receiving 4PV. Similar strong responses to LTB were also observed for serum IgG responses. In contrast, only a few subjects responded to CFA/I in serum in any of the study groups, consistent with results from our previous ETEC vaccine trials [5,7].

Taken together, our results show that the inactivated prototype ETEC vaccine is safe and induces mucosal antibody responses to both CFA/I and LTB. In addition, preliminary analyses show that immunization with LCTB*A* in the PV gives rise to comparable antibody titers to LTB and CTB (unpublished data). ALS response rates to LTB and CFA/I were comparable to those recently reported

after immunization with live attenuated ETEC bacteria [25]. Similar to our results, serum antibody responses to CFA/I were also low after immunization with the live attenuated vaccine [25]. However, while the PV induced systemic anti-LTB IgA responses in almost 100% of the volunteers, the live attenuated ETEC vaccine induced serum IgA antibody responses to LTB at a lower magnitude and frequency (58%) [25]. Our results also support a dose dependency for antibody responses against CFA/I and LTB. Hence, we have made efforts to further increase the over-expression of CFs in subsequent ETEC vaccine preparations [17]. Since administration of a full adult dose of *E. coli* bacteria has been associated with increased frequency of vomiting in previous studies in small children in developing countries [12], we will in subsequent studies also explore the possibility to further enhance immune responses to CFs and LTB using a lower dose of vaccine in combination with an adjuvant which may be better tolerated in the youngest age group. The recently described adjuvant double mutant LT (LT(R192G/L211A); dmLT) [31] is highly promising for such studies.

Based on the encouraging data from this study of the prototype vaccine, we have produced a "complete" multivalent ETEC vaccine consisting of LCTB*A* and four inactivated strains expressing 4–10 times higher levels of four of the most prevalent CFs [14], i.e. CFA/I, CS3, CS5 and CS6, than the ETEC strains in the first generation inactivated ETEC vaccine [19,20,32]. In preclinical studies this multivalent vaccine has induced strong mucosal and systemic immune responses to LTB and each of the vaccine CFs, which all were further significantly enhanced by coadministration with dmLT adjuvant [18]; these findings support the use of this adjuvant in subsequent trials. We are currently evaluating the multivalent vaccine in a clinical trial in Swedish adults, alone and in combination with dmLT. If successful, the optimal formulation identified for the multivalent vaccine will be tested for safety and immunogenicity in young children in ETEC endemic low-resource countries as well as in travelers to such countries.

Disclosure statement

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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.2012.12.063.

References

[1] World Health Organization. Diarrhoeal diseases. http://www.who.int/vaccine_research/diseases/diarrhoeal/en/index4.html [consulted October 2012].
[2] Qadri F, Svennerholm AM, Faruque AS, Sack RB. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin Microbiol Rev 2005;18(3):465–83.
[3] Walker RI, Steele D, Aguado T. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic *E. coli* (ETEC) disease. Vaccine 2007;25(14):2545–66.
[4] Svennerholm AM, Lundgren A. Recent progress toward an enterotoxigenic *Escherichia coli* vaccine. Expert Rev Vaccines 2012;11(4):495–507.
[5] Jertborn M, Ahren C, Holmgren J, Svennerholm AM. Safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* vaccine. Vaccine 1998;16(2–3):255–60.
[6] Svennerholm AM, Tobias J. Vaccines against enterotoxigenic *Escherichia coli*. Expert Rev Vaccines 2008;7(6):795–804.
[7] Ahren C, Jertborn M, Svennerholm AM. Intestinal immune responses to an inactivated oral enterotoxigenic *Escherichia coli* vaccine and associated immunoglobulin A responses in blood. Infect Immun 1998;66(7):3311–6.
[8] Jertborn M, Ahren C, Svennerholm AM. Dose-dependent circulating immunoglobulin A antibody-secreting cell and serum antibody responses in Swedish volunteers to an oral inactivated enterotoxigenic *Escherichia coli* vaccine. Clin Diagn Lab Immunol 2001;8(2):424–8.
[9] Savarino SJ, Hall ER, Bassily S, Brown FM, Youssef F, Wierzba TF, et al. Oral, inactivated, whole cell enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine: results of the initial evaluation in children. PRIDE Study Group. J Infect Dis 1999;179(1):107–14.
[10] Savarino SJ, Hall ER, Bassily S, Wierzba TF, Youssef FG, Peruski Jr LF, et al. Introductory evaluation of an oral, killed whole cell enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Egyptian infants. Pediatr Infect Dis J 2002;21(4):322–30.
[11] Qadri F, Ahmed T, Ahmed F, Bradley Sack R, Sack DA, Svennerholm AM. Safety and immunogenicity of an oral, inactivated enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Bangladeshi children 18–36 months of age. Vaccine 2003;21(19–20):2394–403.
[12] Qadri F, Ahmed T, Ahmed F, Begum YA, Sack DA, Svennerholm AM. Reduced doses of oral killed enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine is safe and immunogenic in Bangladeshi infants 6–17 months of age: dosing studies in different age groups. Vaccine 2006;24(10):1726–33.
[13] Sack DA, Shimko J, Torres O, Bourgeois AL, Francia DS, Gustafsson B, et al. Randomised, double-blind, safety and efficacy of a killed oral vaccine for enterotoxigenic *E. Coli* diarrhoea of travellers to Guatemala and Mexico. Vaccine 2007;25(22):4392–400.
[14] Isidean SD, Riddle MS, Savarino SJ, Porter CK. A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. Vaccine 2011;29(37):6167–78.
[15] Lebens M, Shahabi V, Backstrom M, Houze T, Lindblad N, Holmgren J. Synthesis of hybrid molecules between heat-labile enterotoxin and cholera toxin B subunits: potential for use in a broad-spectrum vaccine. Infect Immun 1996;64(6):2144–50.
[16] Svennerholm AM. From cholera to enterotoxigenic *Escherichia coli* (ETEC) vaccine development. Indian J Med Res 2011;133(2):188–96.
[17] Tobias J, Svennerholm AM. Strategies to overexpress enterotoxigenic *Escherichia coli* (ETEC) colonization factors for the construction of oral whole-cell inactivated ETEC vaccine candidates. Appl Microbiol Biotechnol 2012;93(6):2291–300.
[18] Holmgren J, Blomquist M, Bourgeois L, Carlin N, Clements J, Ekman A, et al. Preclinical evaluation of an oral inactivated ETEC vaccine, based on E. coli overexpressing CFA/I, CS3, CS5, and CS6 together with LTB/CTB subunit, and of the adjuvant effect of co-administration with dmLT. Vaccines for enteric diseases 2011; Cannes, France.
[19] Tobias J, Lebens M, Bolin I, Wiklund G, Svennerholm AM. Construction of non-toxic *Escherichia coli* and *Vibrio cholerae* strains expressing high and immunogenic levels of enterotoxigenic *E. coli* colonization factor I fimbriae. Vaccine 2008;26(6):743–52.
[20] Tobias J, Holmgren J, Hellman M, Nygren E, Lebens M, Svennerholm AM. Over-expression of major colonization factors of enterotoxigenic *Escherichia coli*, alone or together, on non-toxigenic *E. coli* bacteria. Vaccine 2010;28(43):6977–84.
[21] Gothefors L, Ahren C, Stoll B, Barua DK, Orskov F, Salek MA, et al. Presence of colonization factor antigens on fresh isolates of fecal *Escherichia coli*: a prospective study. J Infect Dis 1985;152(6):1128–33.
[22] Sanchez J, Holmgren J. Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. Proc Natl Acad Sci USA 1989;86(2):481–5.
[23] Lundgren A, Kaim J, Jertborn M. Parallel analysis of mucosally derived B- and T-cell responses to an oral typhoid vaccine using simplified methods. Vaccine 2009;27(33):4529–36.
[24] Wenneras C, Qadri F, Bardhan PK, Sack RB, Svennerholm AM. Intestinal immune responses in patients infected with enterotoxigenic *Escherichia coli* and in vaccinees. Infect Immun 1999;67(12):6234–41.
[25] Harro C, Sack D, Bourgeois AL, Walker R, Denearing B, Feller A, et al. A combination vaccine consisting of three live attenuated enterotoxigenic *Escherichia coli* strains expressing a range of colonization factors and LTB is well tolerated and immunogenic in a placebo-controlled double-blind phase I trial in healthy adults. Clin Vaccine Immunol 2011;18(12):2118–27.
[26] Carpenter CM, Hall ER, Randall R, McKenzie R, Cassels F, Diaz N, et al. Comparison of the antibody in lymphocyte supernatant (ALS) and ELISPOT assays for detection of mucosal immune responses to antigens of enterotoxigenic *Escherichia coli* in challenged and vaccinated volunteers. Vaccine 2006;24(18):3709–18.

[27] Peltola H, Siitonen A, Kyronseppa H, Simula I, Mattila L, Oksanen P, et al. Prevention of travellers' diarrhoea by oral B-subunit/whole-cell cholera vaccine. Lancet 1991;338(8778):1285–9.
[28] Clemens JD, Sack DA, Harris JR, Chakraborty J, Neogy PK, Stanton B, et al. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. J Infect Dis 1988;158(2):372–7.
[29] Qadri F, Saha A, Ahmed T, Al Tarique A, Begum YA, Svennerholm AM. Disease burden due to enterotoxigenic *Escherichia coli* in the first 2 years of life in an urban community in Bangladesh. Infect Immun 2007;75(8):3961–8.
[30] Svennerholm AM, Sack DA, Holmgren J, Bardhan PK. Intestinal antibody responses after immunisation with cholera B subunit. Lancet 1982;1(8267):305–8.
[31] Norton EB, Lawson LB, Freytag LC, Clements JD. Characterization of a mutant *Escherichia coli* heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. Clin Vaccine Immunol 2011;18(4):546–51.
[32] Tobias J, Svennerholm AM, Carlin NI, Lebens M, Holmgren J. Construction of a non-toxigenic *Escherichia coli* oral vaccine strain expressing large amounts of CS6 and inducing strong intestinal and serum anti-CS6 antibody responses in mice. Vaccine 2011;29(48):8863–9.