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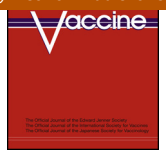

Development and preclinical evaluation of safety and immunogenicity of an oral ETEC vaccine containing inactivated *E. coli* bacteria overexpressing colonization factors CFA/I, CS3, CS5 and CS6 combined with a hybrid LT/CT B subunit antigen, administered alone and together with dmLT adjuvant

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ABSTRACT

A first-generation oral inactivated whole-cell enterotoxigenic *Escherichia coli* (ETEC) vaccine, comprising formalin-killed ETEC bacteria expressing different colonization factor (CF) antigens combined with cholera toxin B subunit (CTB), when tested in phase III studies did not significantly reduce overall (generally mild) ETEC diarrhea in travelers or children although it reduced more severe ETEC diarrhea in travelers by almost 80%. We have now developed a novel more immunogenic ETEC vaccine based on recombinant non-toxigenic *E. coli* strains engineered to express increased amounts of CF antigens, including CS6 as well as an ETEC-based B subunit protein (LCTB*A*), and the optional combination with a nontoxic double-mutant heat-labile toxin (LT) molecule (dmLT) as an adjuvant.

Two test vaccines were prepared under GMP: (1) A prototype *E. coli* CFA/I-only formalin-killed whole-cell + LCTB*A* vaccine, and (2) A "complete" inactivated multivalent ETEC-CF (CFA/I, CS3, CS5 and CS6 antigens) whole-cell + LCTB*A* vaccine. These vaccines, when given intragastrically alone or together with dmLT in mice, were well tolerated and induced strong intestinal-mucosal IgA antibody responses as well as serum IgG and IgA responses to each of the vaccine CF antigens as well as to LT B subunit (LTB). Both mucosal and serum responses were further enhanced (adjuvanted) when the vaccines were co-administered with dmLT. We conclude that the new multivalent oral ETEC vaccine, both alone and especially in combination with the dmLT adjuvant, shows great promise for further testing in humans.

1. Introduction

ETEC is the most common cause of bacterial diarrhea both among children in developing countries and in travelers to these regions [1]. ETEC causes disease by colonizing the small intestine by means of "colonization factors" (CFs), most of which are fimbriae [2], and by producing a cholera toxin (CT)-like heat-labile toxin (LT) and/or a non-immunogenic polypeptide heat-stable enterotoxin (ST). Both naturally acquired infection and oral-mucosal vaccination against LT or various CFs can induce protective immunity, and in animal models anti-LT and anti-CF antibodies cooperate to exhibit a synergistic protective effect [3–5].

There is a great need for an effective vaccine against ETEC for use both in children living in ETEC-endemic countries and in travelers. The serological diversity of ETEC with more than 100 O groups [6] and many different CFs [2] represents a great problem for vaccine development. However, several vaccine candidates mainly for oral administration and consisting of toxin-derived antigens alone or together with CF antigens, either purified or expressed on the bacterial surface, have been developed. These vaccines are in different stages of testing, and one vaccine, the oral cholera vaccine Dukoral

Abbreviations: CF, colonization factor; CT, cholera toxin B subunit; CTB, cholera toxin binding subunit; dmLT, double-mutant LT; ELISA, enzyme linked immunosorbent assay; ETEC, enterotoxigenic *Eschericia coli*; EV, ETEC vaccine; GMP, good manufacturing practice; i.g. immunization, intragastric immunization; LT, heat labile toxin; LTB, heat labile toxin binding subunit; PV, prototype vaccine.

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is licensed in some countries for use also against ETEC in travelers based on its short-term cross-protective effect against LT-mediated ETEC diarrhea [3,7,8].

We have earlier developed an oral ETEC vaccine consisting of formalin-inactivated ETEC bacteria expressing various prevalent CFs and recombinantly produced CTB (rCTB) [9,10]. This vaccine, given in a two- or three-dose regimen, was safe and induced intestinal-mucosal immune responses in 70–90% of Swedish, Bangladeshi and Egyptian vaccinees of different ages. However, when tested in phase III studies it did not significantly reduce overall (usually mild) ETEC diarrhea in travelers or children although it reduced more severe ETEC diarrhea in travelers by almost 80% [11]. It was also found that in infants 6–17 months of age a full dose of vaccine (but not a quarter-dose) induced with some frequency vomiting that would affect acceptability of the vaccine for use in infants [12].

We now describe the development and preclinical testing of a 2nd generation oral ETEC vaccine. The aim has been to improve the immunogenicity of the vaccine without increasing the dosage (and for the youngest age group hopefully being able to reduce it) by introducing several improvements compared to the 1st generation vaccine: (1) Use *E. coli* strains engineered to express increased amounts of CF antigens [13–15]; (2) Replace rCTB with a CTB/LTB hybrid B subunit protein (LCTB*A*) inducing a stronger anti-LT immune response [16]; (3) Optionally add a promising combined mucosal adjuvant and LT antigen, such as dmLT [17]. Increasing the CF antigen content per dose and evaluating the impact of adding a mucosal adjuvant to the vaccine formulation were also WHO's recommendations for improving this vaccine concept [18]. As described here, based on these principles we have prepared two ETEC vaccines under GMP and tested them preclinically: a prototype vaccine (PV) consisting of *E. coli* CFA/I-only formalin-killed bacteria + LCTB*A*, and a "complete" multivalent ETEC vaccine (EV) containing a cocktail of four inactivated recombinant *E. coli* strains expressing increased amounts respectively of CFA/I, CS3, CS5 and CS6 antigens in combination with the LCTB*A* protein. When tested in mice, oral immunizations with these strains individually have induced significantly higher intestinal as well as serum antibody responses against the various CFs than achieved with corresponding 1st generation vaccine strains [19]. Now, these vaccines given alone or together with dmLT, were tested in mice after oral immunizations for potential adverse reactions and stimulation of serum IgG and IgA and intestinal-mucosal IgA antibody responses to the different vaccine components. The results show that: (1) Both vaccines, also with dmLT, were well tolerated; (2) Both vaccines induced strong serum as well as intestinal antibody responses to each of the CF antigens included in the vaccines and to LT; and (3) The dmLT adjuvant significantly enhanced especially the intestinal immune responses to the various vaccine components.

2. Materials and methods

2.1. Vaccines and adjuvants

Two vaccines prepared under GMP were tested alone and together with GMP dmLT or with CT as adjuvants: (1) A monovalent prototype vaccine (PV) consisting of formalin-killed CFA/I-overexpressing *E. coli* + LCTB*A*, which has now also been evaluated in humans for its safety and immunogenicity [20] and (2) a multivalent CF-whole-cell + LCTB*A* ETEC vaccine (EV) containing four inactivated *E. coli* strains recombinantly expressing increased amounts respectively of CFA/I, CS3, CS5 and CS6 antigens mixed with the LCTB*A* protein. The preparation and characterization of vaccines and dmLT are described in the Appendix; CT was purchased from List Biological Laboratories (Campbell, CA, USA).

2.2. Immunizations and sample collections

Female BALB/c and for EV also C57/Bl6 (B6)mice (Charles River; 6–8 weeks of age; 5–10 mice/group) were used for "oral"/intragastric (i.g.) immunizations. When not otherwise specified, three rounds of immunization were given at 12–15 days intervals, each comprising two or three i.g. administrations on consecutive days of one-half or one-third of the total dose in 0.3 ml 3% (w/v) sodium bicarbonate through a baby feeding catheter; food was removed for 2–3 h before each administration. Unless specified differently, total vaccine doses in each round were for PV 4×10^8 inactivated bacteria (containing 8 μg CFA/I) + 13 μg LCTB*A*, given alone or with 15 μg CT or 25 μg dmLT, and for EV 1×10^9 inactivated bacteria (containing 10 μg CFA/I, 38 μg CS3, 6.4 μg CS5 and 1.5 μg CS6) + 13 μg LCTB*A* alone or together with 25 μg dmLT.

Bleedings were performed before the first, and one day before and 10–12 days after the last round of immunization, when fecal pellets were also collected and extracts prepared as described [21]. At the last time point when animals were sacrificed, and in a subset of mice sacrificed 11–12 days after the 2nd immunization round, the mice were perfused with a heparin-PBS solution to remove blood from the tissues, and jejunal tissue collected and extracted with a 2% Saponin-PBS solution (the Perfext method) [22]. Fecal and jejunal tissue extracts from unimmunized mice ("Nil") served as controls.

2.3. Immunological assays

IgA and IgG antibodies against CFA/I, CS3, CS5, CS6 and LTB in serum, and IgA antibodies to the same antigens in fecal and jejunal extracts were determined by ELISA; in fecal and jejunal extract samples total IgA was also measured by ELISA [14,15,23]. Antibody titers were estimated as the reciprocals of the extrapolated sample dilutions giving absorbance (A_{450}) of 0.4 above background, and for fecal and jejunal samples these titers were adjusted to the total IgA contents [19] and expressed as ELISA units [EU] per μg of total IgA. All antibody levels were $\log_{10}$ transformed; the log-transformed values were used for graphs and statistical analyses.

2.4. Statistical analyses

Statistical analyses between groups were conducted by Student's two-tailed *t*-test, with *P* values of <0.05 regarded as significant.

3. Results

3.1. Vaccines are stable and safe also with adjuvants

As further described in the Appendix, repeated dose toxicity studies in mice of PV, EV and EV mixed with dmLT, in dosages corresponding on a weight basis to up to 100 times the intended human dose were undertaken. All preparations were judged to be very well tolerated and safe for clinical studies in humans. This was further confirmed in the immunization experiments described below in which PV and EV, alone or with dmLT, were tested at even higher dosages and continued to be well tolerated.

As also further described in the Appendix, stability testing of stored PV, EV and their monovalent whole-cell bulk and LCTB*A* preparations as well as of dmLT has not indicated any product changes after 18–46 months surveillance of the various components.

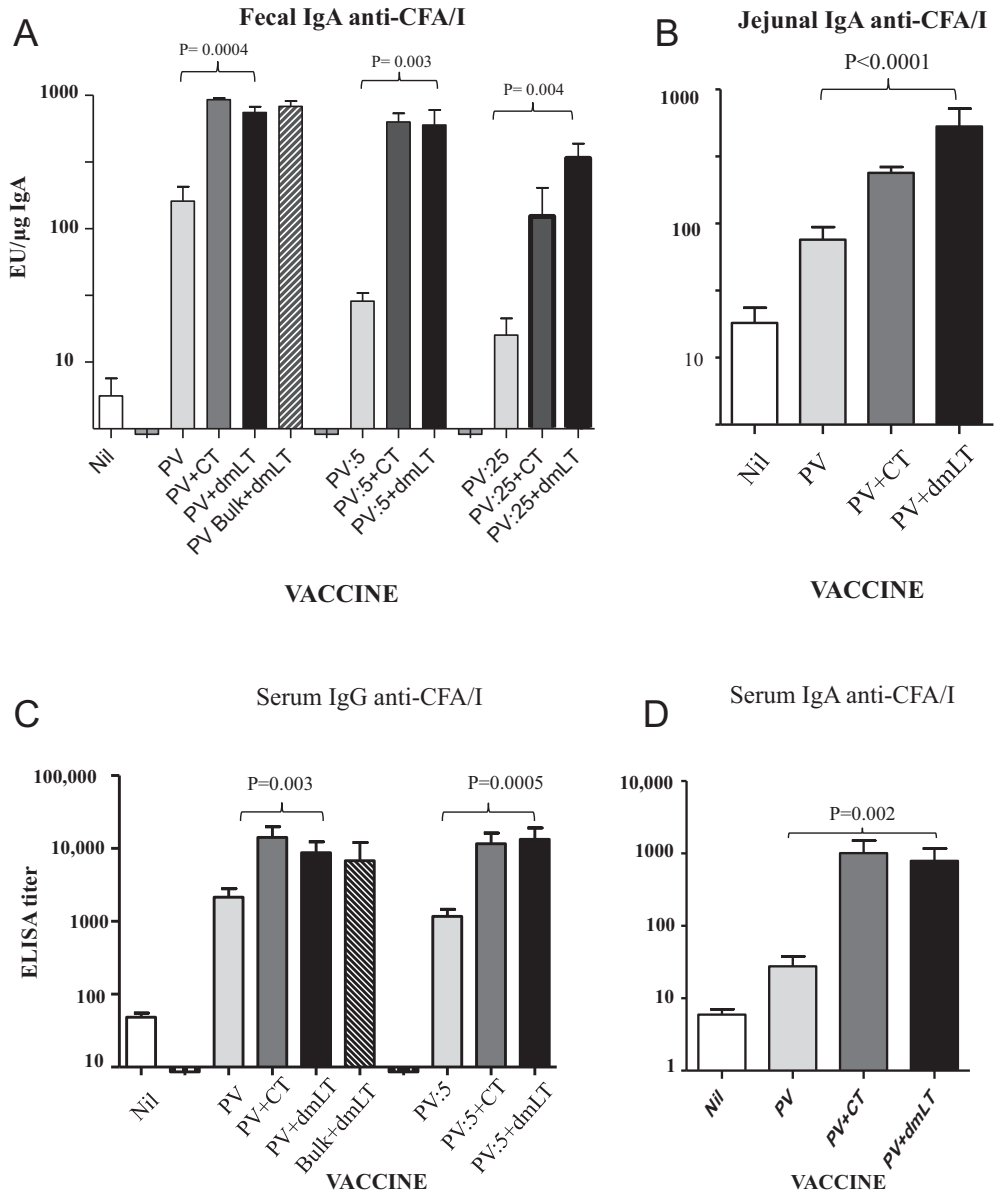

Fig. 1. Oral-i.g. immunization of mice with PV induces strong intestinal IgA and serum IgG and IgA antibody responses to CFA/I that are further increased when immunizations are performed with PV co-administered with dmLT or CT adjuvants: (A) Fecal IgA anti-CFA/I responses to three rounds of i.g. immunization with different dosages of PV (4×10^8 inactivated bacteria/13-μg LCTB*A* [PV] and 5-fold [PV:5] or 25-fold [PV:25] dilutions thereof) given alone or with 15 μg CT or 25 μg dmLT; PV bulk represents the PV bacterial component without any LCTB*A*, and Nil represents fecal extracts from unimmunized mice. (B) Anti-CFA/I IgA antibody responses in jejunal extracts. (C) Serum IgG, and (D) IgA anti-CFA/I antibody responses. Student's two-tailed *t*-tests were done when testing significances in antibody levels between indicated immunization groups (5–6 animals per group). The results are from one of three experiments giving similar results.

3.2. Oral immunization with PV elicits intestinal and serum antibody responses that are further adjuvanted by dmLT

Initial immunization studies were performed with the CFA/I-E. *coli* + LCTB*A* prototype vaccine (PV) tested in different dosages alone and with CT or dmLT as adjuvants.

Anti-CFA/I responses. As shown in Fig. 1A i.g. immunization with PV induced a dose-related fecal anti-CFA/I IgA antibody response, for the highest PV dose resulting in a 70-fold antibody rise compared to unimmunized controls. Co-administration with dmLT, to the same extent as with CT, further enhanced this response. Similar effects were seen in jejunal tissue extracts (Fig. 1B). Likewise, the immunizations with PV induced substantial IgG as well as IgA anti-CFA/I serum antibody responses which were further significantly and similarly enhanced by co-administration with dmLT or CT (Fig. 1C and D).

Anti-LTB immune responses. The intestinal-mucosal and serum antibody responses to LTB were also examined. Immunization with the highest PV dose induced significant intestinal as well as serum anti-LTB antibody levels compared to controls, which were much further enhanced by co-administration with either CT or dmLT (Fig. 2). Since the latter increases could have been due to the increased amounts of LTB antigen provided by the addition of dmLT, we tested in a separate experiment anti-LTB as well as anti-CFA/I responses after similar immunizations with PV + 25 μg rCTB and found that the responses then achieved were similar to those after immunization with PV alone and much lower than after immunization with PV + dmLT (not shown). We also tested the adjuvant effect of lower doses of dmLT and found that when added to PV 10- or 5-μg dmLT doses were only slightly inferior to the standard 25-μg dose, whereas a 2.5-μg dose had no significant adjuvant effect on either the anti-CFA/I or anti-LTB responses (not shown).

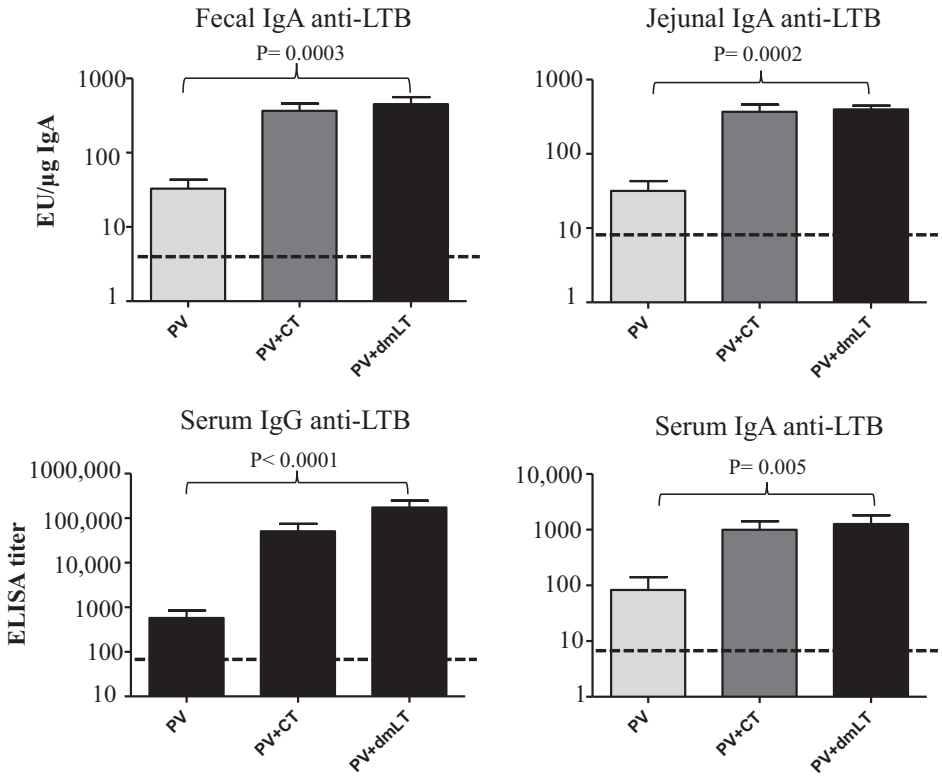

Fig. 2. Oral-i.g. immunization of mice with PV also induces strong intestinal IgA and serum IgG and IgA antibody responses to LTB that are further enhanced by co-administration with CT or dmLT. Immunizations and samples examined are the same as described in Fig. 1. Bars show the indicated anti-LTB antibody levels after immunization in comparison with the geometric mean + 2SD levels of for fecal and jejunal samples unimmunized controls or for serum preimmunization titers. Student's two-tailed *t* tests were done to test significances in antibody levels between indicated immunization groups. The results are from one of three experiments giving similar results.

3.3. Oral immunization with EV induces strong intestinal and serum antibody responses to all vaccine components that are further increased by dmLT

We undertook similar immunogenicity studies with the GMP-prepared multivalent CF-ETEC whole-cell + LCTB*A* vaccine (EV) alone and together with the GMP-prepared dmLT; we tested responses in both Balb/c and C57/Bl6 mice since we had previously found [15] stronger immune responses to the CS6 antigen in C57/Bl6 than in Balb/c mice.

Studies of fecal IgA antibody responses showed that the vaccine by itself induced significant 3-to 25-fold titer rises to each of the CFA/I, CS3, CS5, CS6 and LTB antigens in the vaccine, which were further significantly increased 3- to 100-fold by giving the vaccine together with dmLT (Fig. 3A). The IgA antibody responses in jejeunal extracts showed a similar picture (Fig. 3B). Confirming our previous report [15], we saw a stronger anti-CS6 response in C57/Bl6 compared to Balb/c mice in jejunal extracts and in serum (see below); this differed from the responses to the other vaccine antigens which were equal or slightly higher in the Balb/c mice (data not shown).

Analyses of serum antibodies also demonstrated strong IgG (Fig. 3C) as well as IgA (Fig. 3D) antibody responses to each of the vaccine antigens already after immunization with EV alone. Immunization together with dmLT tended to further enhance these responses, but to a lesser extent than for the fecal and jejunal responses and statistically significant only for serum IgA responses to CS3 and CS6 (in C57/Bl6 mice) and IgG and IgA anti-LTB responses.

We also compared the intestinal and serum antibody responses after two rounds of i.g. immunizations with those described after three immunization rounds. As seen in Table 1, substantial fecal, jejunal and serum antibody responses to all antigens were achieved already after two rounds of immunization, both with EV alone and even stronger with EV + dmLT. In general, the fold-increase rises in antibody titer were lower after two as compared to three rounds of immunization (Table 1).

4. Discussion

By constructing plasmids in which the genes for the different CF antigens were placed behind strong promotors, and incorporating these plasmids in *E. coli* K12 or a toxin-negative O78 ETEC strain, recombinant vaccine strains were engineered that expressed 4- to 10-fold higher levels of CFA/I and CS1-CS5 compared to the strains used in the 1st generation ETEC vaccine [19]. In addition, we engineered a novel vaccine strain producing a high level of CS6 [15], an antigen which is increasingly prevalent on clinical ETEC isolates [24,25] and was practically lacking in the previous vaccine. Likewise, immunization with the engineered LCTB*A* protein (a hybrid CTB/LTB molecule [16]) has induced a stronger anti-LTB response including stronger LT-neutralizing activity than achieved with the rCTB component of the 1st generation vaccine [16; N. Carlin et al. unpublished data]. Based on this, we describe here the development and preclinical evaluation of a new oral ETEC vaccine (EV) containing a cocktail of four inactivated recombinant *E. coli* strains expressing increased amounts of CFA/I, CS3, CS5 and CS6 antigens mixed with the LCTB*A* protein. This vaccine, whose composition makes it substantially more immunogenic than the previously tested vaccine, was prepared under GMP, as was also an earlier monovalent prototype vaccine (PV) containing only CFA/I-overexpressing inactivated bacteria together with LCTB*A* [20].

As described, first PV and then EV were tested in mice after oral immunizations for their safety and capacity to induce intestinal IgA and serum IgG and IgA antibody responses to the different vaccine

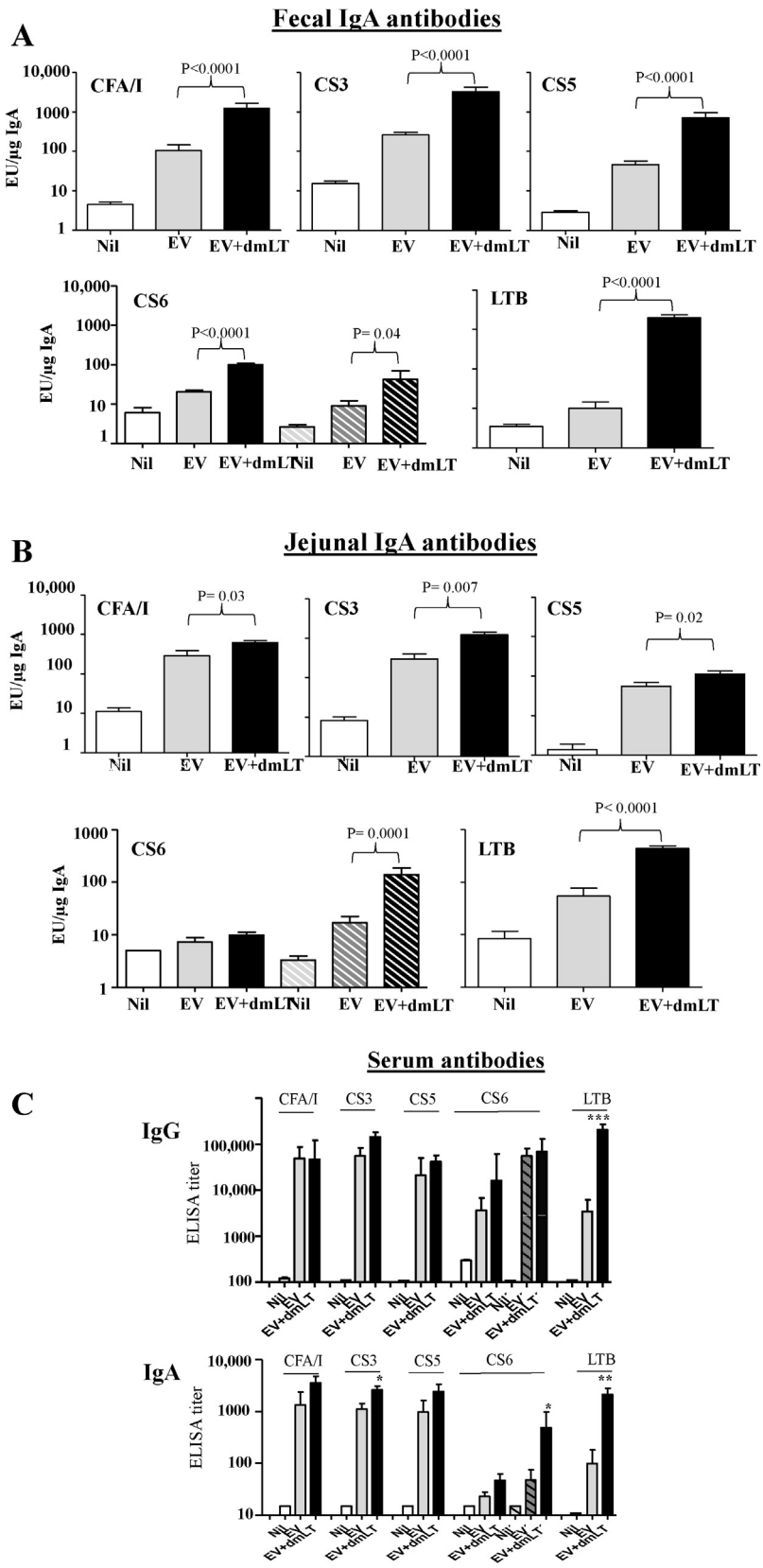

Fig. 3. Oral immunization with EV induces strong intestinal and serum antibody responses to all vaccine components that are further adjuvanted by dmLT. After three rounds of i.g. immunizations with EV alone, EV together with 25 μg dmLT or only bicarbonate buffer (Nil) IgA antibodies to CFA/I, CS3, CS5, CS6 and LTB were measured in (A) fecal extracts or (B) jejunal tissue extracts, and IgG and IgA antibodies to these antigens were also determined in serum (C). Results show antibody responses in Balb/c mice and for anti-CS6 also in C57/Bl6 mice (hatched bars). Student's two-tailed *t*-tests were done to test significances in antibody levels between indicated immunization groups; for serum samples *, ** and *** represent *p* values of <0.05, <0.01 and 0.001, respectively. Results shown are from one of two independent experiments giving closely similar results.

Table 1
Comparisons of fold-increase responses to different vaccine components after two versus three rounds of oral immunization with EV or EV + dmLT.[a]

	Immunization	a-CFAI		a-CS3		a-CS5		a-CS6 (B6)		a-LTB	
		2	3	2	3	2	3	2	3	2	3
Fecal IgA	EV	12	20	9	13	20	100	8 (25)	3 (5)	2	3
	EV + dmLT	63	200	50	200	25	300	30 (4)	25 (25)	250	630
Jejunal IgA	EV	3	30	11	63	16	50	1 (3)	1 (6)	1	7
	EV + dmLT	8	63	35	112	30	80	2 (16)	2 (50)	11	56
Serum IgA	EV	50	100	10	80	25	60	2 (2)	2 (3)	6	11
	EV + dmLT	100	250	60	250	80	150	2 (4)	4 (40)	200	8000
Serum IgG	EV	250	400	25	500	50	400	5 (40)	15 (500)	6	60
	EV + dmLT	400	400	500	1400	100	800	13 (16)	160 (600)	110	4000

[a] Values given are geometric mean fold-increase antibody levels over corresponding levels before immunization (serum) or in unimmunized controls (fecal and jejunal assays) in Balb/c mice; for antibodies to CS6 corresponding fold-increases in C57/Bl6 (B6) mice are also shown in parentheses.

components. An important study objective was also to determine whether co-administration of the vaccines with the mucosal adjuvant dmLT [17] could further enhance these immune responses without jeopardizing vaccine safety. On a per body-weight basis the dosages used were for PV 10–15 times the prospected human adult dose of the bacterial component and 30–50 times of LCTB*A*, and for EV 30–50 times the human dose of both inactivated bacteria and LCTB*A*. The dmLT adjuvant dose used in the mice would correspond to more than 1000 times a prospected human dose of 10–25 μg. The results show that both PV and EV, whether given alone or with dmLT, were extremely well tolerated, which was confirmed in extended toxicity studies according to European Pharmacopea recommendations for biological products in which PV and EV ± dmLT were tested in repeated doses up to 100 (PV) and 25 times (EV) the prospected human dose and found to be safe.

Oral immunizations with both PV and EV induced significant, usually strong serum IgG and IgA as well as intestinal IgA antibody responses to each of the CF antigens in the vaccines and also to LTB even when the vaccines were administered without any adjuvant. The strong IgA antibody responses in fecal and jejunal extracts are of special importance since they probably best reflect the protective efficacy of the vaccines [5,26]. Several previous studies have demonstrated a close correlation between IgA antibody levels measured in fecal and jejunal extracts [14,15,21], which was also seen in this study, and have also shown that the levels of IgA antibodies in jejunal extracts from perfused animals induced by the PV or EV vaccines, when compared to the IgA antibody levels in serum, are much too high to represent transudation from blood [26,27]. We are therefore confident that the IgA antibodies to the various vaccine components in fecal and jejunal extracts measured after i.g. immunizations with PV or EV largely, if not exclusively, reflect locally produced mucosal antibodies.

Of great potential importance for the further development and clinical testing of EV and other oral ETEC vaccines, co-administration of both PV and EV with dmLT adjuvant significantly enhanced especially the intestinal IgA responses to the various vaccine components. The dmLT is a genetically engineered LT protein containing two genetic substitutions (R192G/L211A) in the A subunit which appear to completely eliminate the enterotoxic activity of the parent LT without removing the adjuvant activity [17]. Several studies have shown that dmLT can function as a strong, and apparently non-toxic, adjuvant for stimulating both B and T cell immune responses in mice to various vaccine antigens [17,28,29]. A recent study has also demonstrated that at least 100 μg of dmLT could be given orally to humans without causing adverse reactions (L Bourgeois, personal communication). In the present study we show that when added to PV dmLT is similarly effective as the "gold standard" adjuvant CT in promoting both anti-CFA/I and anti-LTB antibodies. When different dosages of vaccine (PV) were tested, the adjuvant effect of dmLT appeared especially strong for the lower vaccine dosages. This suggests the potential for vaccine dose sparing when an effective adjuvant is added, which could be of special value for use of ETEC vaccines in younger age groups in which lower than adult vaccine doses may be needed. Since the increase in anti-LTB antibodies could have been explained by the increased amounts of LT-related antigen provided through the added dmLT rather than by a true adjuvant effect, we also tested the anti-LTB and anti-CFA/I responses after similar immunizations with PV together with 25 μg rCTB. These responses were much lower than after immunization with PV + dmLT and similar to those after immunization with PV alone, thus supporting the mucosal adjuvant activity of dmLT for both antibacterial and antitoxic immunity. The potency of dmLT as an adjuvant for both types of antibody responses was further underlined in the immunization studies with EV, where dmLT significantly enhanced the IgA mucosal immune responses to each of the four CF antigens as well as to LTB. Studies in progress will determine if the adjuvant also adds to the duration of these responses and/or to immunologic memory for later boosting. Also serum IgG and IgA antibody responses were enhanced by dmLT, but to a lesser extent than the intestinal-mucosal IgA responses. The latter may be explained by the already quite high serum antibody levels achieved with EV alone; it could also be that the adjuvant activity of dmLT in a more specific way promotes mucosal IgA responses.

We conclude that the described novel oral inactivated whole-cell/B subunit ETEC vaccines, containing inactivated *E. coli* strains overexpressing different CF antigens and the LCTB*A* B-subunit protein, have excellent safety and immunogenicity when tested in mice, and that especially the intestinal-mucosal IgA antibody responses are substantially increased even further by the dmLT adjuvant. Based on these results and those from a just completed study in humans with PV (without adjuvant) [20], phase I studies in adult Swedish volunteers are now under way to assess the reactogenicity and immunogenicity after two oral immunizations with EV alone and together with different doses of dmLT. Pending favorable results from that trial, we plan to proceed with extended immunization studies in adults, toddlers and infants in an ETEC-endemic setting.

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Conflict of interest: N. Carlin and B. Gustafsson are minority shareholders of Scandinavian Biopharma Holding AB, which holds certain commercial rights to the vaccines tested in this study.

Appendix A.

A.1. Preparation of vaccines

Monovalent CFA/I-ETEC/LCTBA prototype vaccine (PV). A CFA/I-overexpressing *E. coli* strain (V109) was generated with the aid of an expression plasmid containing the CFA/I operon under a strong tac promoter and a non-antibiotic selection marker, *thyA*, originating from *Vibrio cholerae* [13,19]. The plasmid was inserted into a host *E. coli* K12 strain (C600) whose chromosomal *thyA* gene had been deleted, thus allowing antibiotic free selection of plasmid-containing organisms by culturing the strain in thymine-deficient substrate. In non-GMP laboratory scale studies, the V109 strain expressed 5–15-fold more CFA/I than the reference 1st generation ETEC vaccine CFA/I strain as determined by an antigen-specific inhibition enzyme-linked immunosorbent assay (ELISA) [13]. When prepared under GMP, after growth of the strain V109 in CFA medium [30] in a 500-l fermentor at 37 °C for 6–8 h in presence of 1 mM IPTG, followed by extensive washing and inactivation with formalin [19] and renewed extensive washing, the final PV contained 600 μg CFA/I per human dose, 3-fold more than the reference vaccine.

LCTBA is a hybrid protein between LTB and CTB, in which seven amino acids in CTB have been replaced by corresponding amino acids of LTB [16]. This makes the LCTB*A* molecule closely similar to LTB with regard to immunogenicity including the generation of LT-neutralizing antibodies [16]. Purified LCTB*A* was prepared as described [20] under GMP from the culture medium of a nontoxigenic *V. cholerae* production strain harboring a multicopy expression plasmid and grown in a 500-l fermentor.

The final GMP-prepared PV contained 3×10^{10} formalin-inactivated CFA/I- *E. coli* bacteria (strain V109) mixed with 1.0 mg LCTB*A* in 6 ml saline (dispensed in glass vials representing one human dose).

Multivalent ETEC-CF/LCTBA vaccine (EV). A "complete" multivalent ETEC-CF/LCTB*A* vaccine (EV) was developed consisting of four inactivated recombinant *E. coli* strains (ETEX21-24) engineered to overexpress CFA/I, CS3, CS5, and CS6, respectively, mixed with LCTB*A*. Similar to the PV strain, these strains have in common that the CF antigens are expressed under a strong promoter from plasmids, modified to also harbor the *thyA* gene, and placed in host *E. coli* strains whose own chromosomal *thyA* gene has been deleted.

The vaccine strains producing CFA/I, CS3 or CS5 were inactivated with formalin [19]. The CS6-producing strain was instead inactivated with phenol since we have found that formalin but not phenol destroys the CS6 protein on the bacterial surface [15,31]. Individual inactivated vaccine bulks were then aseptically mixed with each other and with the LCTB*A* protein (the same preparation as used in PV) to yield a final EV with the following composition per planned human dose: ETEX21, 830 μg CFA/I on 2×10^{10} bacteria; ETEX22, 2970 μg CS3 on 2×10^{10} bacteria; ETEX23, 510 μg CS5 on 2×10^{10} bacteria; ETEX24, 120 μg CS6 on 2×10^{10} bacteria; LCTB*A* 1.0 mg; Phosphate-buffered saline q.s. ad 8 ml. These CF antigen levels are much higher than in the 1st generation ETEC vaccine, which per $2{,}5 \times 10^{10}$ bacteria contained 150–200 μg of CFA/I, CS3 and CS5 and <5 μg CS6.

Strain ETEX21 overexpressing CFA/I was developed using a recombinant plasmid expressing the CFA/I operon under tac promoter and also equipped with a *thyA* gene as described above for PV. Compared to PV, the host strain was an *E. coli* CFA/I (O78:K-,ST) which was originally isolated from a patient with watery diarrhea in Dhaka, Bangladesh, from which the plasmid(s) encoding the ST and CFA/I native genes were removed by natural selection [32]. Further modification was done by inactivating the *thyA* gene on the chromosome through insertion of a kanamycin resistance gene in the *thyA* gene. In a second round of chromosomal deletion, the kanamycin gene was then deleted of its first 200 nucleotides together with 200 nucleotides from the *thyA* gene making the strain kanamycin sensitive.

Strain ETEX22 overexpressing CS3 was developed using a recombinant plasmid expressing the entire CS3 operon under the rns promoter which in turn is under the lac operator. The bacterial host for this construct was the same *E. coli* O78:K- strain that was used for ETEX21. By similar steps as described for ETEX21, the non-antibiotic selection marker *thyA* was used to provide antibiotic free selection of plasmid-expressing organisms.

Strain ETEX23 overexpressing CS5 was developed using a recombinant plasmid expressing the entire CS5 operon under tac promotor, also harboring *thyA* as a non-antibiotic selection marker. The bacterial host for this construct is the same *E. coli* O78:K- strain used for ETEX 21 and ETEX 22.

E. coli strain ETEX24 overexpressing CS6 was developed using a recombinant plasmid expressing the entire CS6 operon under tac promotor. The bacterial host for this construct was the same modified *E. coli* K12 strain C600 as used in the PV.

After growth of each strain in a 500-liter fermentor at 37 °C for 6–8 h following IPTG induction, in CFA medium [30] for ETEX21, 22 and 24 and in modified Syncase medium [33] for ETEX23, the bacteria were inactivated, for ETEX21, 22 and 23 using formalin as described [19], and for ETEX24 instead using phenol [15] since it was found that phenol in contrast to formalin preserves the CS6 antigen on the bacterial surface [15].

Both PV and EV were prepared under GMP at Unitech Biopharma AB, Matfors, Sweden.

A.2. Preparation of dmLT adjuvant

The dmLT (R192G/L211A) adjuvant is based on two genetic substitutions in the A subunit (R192G/L211A) which apparently completely eliminates the enterotoxic activity of the parent LT without removing the adjuvant activity [17]. Initially, a single-mutant LT ("mLT," LT[R192G]) was constructed by substitution of arginine with glycine at position 192 of the A subunit. This genetic modification eliminated the ability of subunit A to be activated by trypsin cleavage and greatly reduced the enzymatic and biological activity of the toxin [34]. Further attenuation was accomplished by adding a second substitution, leucine to alanine at position 211 [17]. The double mutant LT (dmLT; R192G/L211A) has less than 0.1% of the enzymatic activity (ADP-ribosylation) of LT and induced no detectible fluid accumulation when given to mice by the intragastric route, while retaining immunogenicity and adjuvanticity [17]. The preparations used were produced and purified as described [17]; the lot used with EV was prepared under GMP at the Walter Reed Army Institute of Research Pilot Bioproduction Facility, Silver

Spring, MD, USA. Lyophilized dmLT preparations were diluted and stored at 1 mg/ml at 4 °C until mixed with PV or EV for the studies described.

A.3. Antigens for immunological assays

Purified CFA/I, CS3, and CS5 antigens were prepared as described [21,32, Unpublished data]. Purified CS6 antigen [35] was a kind gift from Dr F Cassel. LTB was prepared by Etvax AB from a recombinant plasmid expressed and purified in a manner similar to that of LCTBA. All antigens gave a single band in SDS gel electrophoresis and when tested in ELISA reacted specifically with matching monoclonal antibodies [23,31] but not with anti-O78 monoclonal antibody or hyperimmune sera against any of the host strains used in the PV or EV vaccines. Immunological specificity was further documented by ELISA studies showing that mouse antisera raised against individual CF-overexpressing *E. coli* strains reacted with the appropriate but not with any other CF antigen.

A.4. Stability and toxicology studies of vaccines

Monovalent PV and EV bulk preparations and LCTBA, as well as the final PV and EV vaccines stored at 4 °C and 25 °C are subjected to an ongoing 48-months ICH compliant stability study. Similarly, GMP-made dmLT in lyophilized vials stored at −20 °C is being tested in an ongoing 36-months stability study. No signs of product changes have been found to date (15–43 months for various components).

Repeated dose toxicity studies of both PV, EV, and EV with dmLT, in doses corresponding to up to 100 times the intended human dose on a weight basis were conducted in mice by Visionar AB, Uppsala, Sweden in accordance with European Pharmacopea GLP study criteria for biological products. Both when tested alone and together with dmLT, the vaccines were judged to be very well tolerated and safe for clinical studies in humans. No deaths or histological changes were found, and only at the highest dosages tested a few animals showed mild transient weight loss and/or a few minor biochemistry changes were recorded.

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