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Review

Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic *E. coli* (ETEC) disease

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is the most common bacterial cause of diarrhoea in the world, annually affecting up to 400,000,000 children under 5 years of age living in developing countries (DCs). Although ETEC possesses numerous antigens, the relatively conserved colonization factor (CF) antigens and the heat labile enterotoxin (LT) have been associated with protection and most vaccine candidates have exploited these antigens. A safe and effective vaccine against ETEC is a feasible goal as supported by the acquisition of protective immunity. The success of an ETEC vaccine targeting infants and children in DCs will depend on a combination of maximally antigenic vaccine preparations and regimens for their delivery which will produce optimal immune responses to these antigens. Vaccine candidates having a high priority for accelerated development and clinical testing for eventual use in infants would include inactivated ETEC or *Shigella* hybrids expressing ETEC antigens as well as attenuated ETEC strains which express the major CF antigens and LT toxin B-subunit, as well as attenuated *Shigella*, *Vibrio cholerae* and *Salmonella typhi* hybrids engineered to deliver antigens of ETEC. Candidates for an ETEC vaccine would have to meet the minimal requirement of providing at least 50% protection against severe disease in DCs during the first 2 years of life. The critical roadblock to achieving this goal has not been the science as much as the lack of a sufficiently funded and focused effort to bring it to realization. However, a Product Development Partnership to overcome this hurdle could accelerate the time lines towards when control of ETEC disease in DCs is substantially closer.

Keywords: Enterotoxigenic *Escherichia coli*; Vaccine; Diarrhoeal disease

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1. Introduction

On any given day an estimated 200,000,000 people worldwide will suffer from the effects of gastroenteritis, the amount of diarrhoeal water being passed as a result will equal the amount of water passing over Victoria Falls in 1 min. Over a year, approximately 2,000,000 of these people living in developing countries (DCs) will die as a result of these diseases, predominantly children in the first years of life [1–3]. Multiple pathogens are responsible for this suffering and death in DCs, but enterotoxigenic *Escherichia coli* (ETEC) is regarded as the most common bacterial cause of diarrhoea. A vaccine to control ETEC in children living in DCs could have a significant impact on morbidity and mortality due to diarrhoeal diseases among this population. For example, in a recent study of the importance of ETEC in DCs, Wenneras and Erling [4] found that carriage of ETEC was associated with diarrhoea in children less than 5 years old. In their analysis, 280 million episodes of diarrhoea due to ETEC were estimated annually in this age group, and 50 million children of this age were considered to be asymptomatic carriers. Although mortality from diarrhoeal diseases has decreased in recent years [5], the incidence of this disease remains the same and thus contributes significantly to the suffering and anticipated loss of human potential in children suffering from these diarrhoeal diseases [6–8].

The heavy burden of ETEC disease on young children and infants in many DCs is illustrated by recent studies in Bangladesh and Egypt. The recent study of an Egyptian birth cohort found an extremely high incidence of diarrhoeal disease among infants living in the Nile Delta area of Egypt (8.8–4.6 episodes per child per year over the first 2 years of life) [9]. Ninety percent of this birth cohort experienced their first episode of pathogen-associated diarrhoea by 14 months of age and ETEC accounted for 66% of these first episodes. The incidence of ETEC diarrhoea increased from

Table 1
Distribution of relatively conserved antigens found in isolates of ETEC[a]

Colonization factor antigens			
CFA/I	33% of isolates	66% ST, 33% ST/LT	
CFA/II	23% of isolates	85% ST/LT	CS1 and 2 never found together or in absence of CS3; CS3 also occurs alone
CFA/IV	21% of isolates	80% ST	CS4 and 5 never found together or in absence of CS6; CS6 frequently occurs alone
Enterotoxin antigens			
LT	25% of strains produce LT only, whereas 53% produce LT or LT/ST		
ST	75% of strains produce ST or ST/LT		

[a] Data in this chart are taken from the report by Wolf [20].

1.7 episodes per child per year in the first 6 months of life to 2.3 episodes per child per year in the second 6 months and declined thereafter.

The importance of ETEC among other common enteric pathogens is also illustrated by recent data from Bangladesh (D. Sack, personal communication). Between January 1996 and September 2001, an interval when ETEC testing was included in the routine surveillance of diarrhoeal diseases in children less than 5 years of age admitted to the hospital, an estimated 89,050 cases of ETEC diarrhoea, 138,300 cases of cholera and 146,450 cases of rotavirus diarrhoea were treated. If only those children with some or severe dehydration are included, the comparable numbers were 39,365, 113,985, and 27,623. Although rotavirus diarrhoea was more common in this age group, the more severe cases of diarrhoeal illness tended to be either cholera or ETEC. During most years, rotavirus and cholera were the most commonly isolated organisms (about 25–30% of cases) followed by ETEC (15%) and *Shigellae* (4%). Although there are more ETEC diarrhoea cases seen in older individuals, according to the population-based data from Matlab, the rates of ETEC infection is 10-fold higher in the age group 0–3 years than in any other age group.

Like many diarrhoeal diseases, ETEC is a consequence of inadequate sanitation which is not likely to be resolved in the near-term future in many DCs. Immunoprophylaxis offers a promising approach to help alleviate this problem, but the development of a successful vaccine against ETEC remains elusive. Two major intertwined technical problems contribute to this deficiency. One involves the identification of safe and immunogenic preparations of antigens with the ability to confer broad spectrum protection against disease. The second is a consequence of the challenge of achieving effective mucosal immunization, which has been observed to be a hurdle to immunization of adult volunteers in industrialized countries against a variety of pathogens. As will be discussed below, the infant population in DCs have unique complications of their own which must be overcome if mucosal immunization against ETEC or other pathogens in this population is to be achieved.

2. Feasibility of an ETEC vaccine

A vaccine against ETEC should certainly be feasible as prior natural infections confer demonstrable immunity [10–12]. As with other enteric infections, the incidence of ETEC diarrhoeal illness decreases with age, suggesting the acquisition of natural immunity [9,13]. This phenomenon is also seen in travelers who spend sufficient time in endemic areas [14] and volunteers re-challenged with homologous strains of ETEC [15].

Nearly two decades ago, investigators at the University of Maryland evaluated the first oral, live, attenuated ETEC vaccine candidate [16,17]. The candidate strain, E-1392-75-2A, was derived from a wild-type ETEC that had spontaneously lost the enterotoxin-bearing virulence plasmid but retained the CFA/II genetic determinants (CS1 and CS3). Vaccination with a single dose of 5×10^{10} organisms of this strain was associated with significant protection (75% vaccine efficacy) in a single, small volunteer challenge study using an enterotoxin positive strain [18,19]. Anti-colonization immunity was responsible for this protection. Further evaluation of this strain was, however, suspended due to a 15% rate of mild post-immunization diarrhoea.

3. Vaccine candidates against ETEC

A complex antigenic repertoire is expressed on the surface of ETEC, providing many antigens for consideration in vaccine design. The occurrence, distribution and associations of these antigens have been extensively reviewed [20]. These antigens include lipopolysaccharide (O serogroup), flagella (H serogroup) and colonization factor (CF) antigens. In addition, strains of the organism produce heat labile enterotoxin (LT) and heat stable enterotoxin (ST), alone or in combination. Based on Wolf's [20] analysis, toxin-based and colonization factor antigen-based vaccines would have the broadest coverage with the fewest components. LT, CFA/I, and CF components including CS3 and CS6, together in a sufficiently immunogenic formulation, would be expected to cover most strains worldwide. The distribution of the relatively conserved antigens found in ETEC isolates is presented in Table 1. Although the O and H antigens may contribute to protection, too, there are too many of these groups to be practical in a vaccine.

Most ETEC vaccine efforts to date have focused on the LT and CF, or pathotype, antigens. Based on knowledge of the natural acquisition and development of immunity against ETEC and some understanding of its antigenic components as discussed above, it has been possible to pursue a number of

Table 2
Approaches to vaccine development

Vaccine approach	Developer	Remarks	Status	References
Cholera WC/BS	SBL Vaccine	3–9 months protection in Bangladeshi women and children after 2 or 3 oral doses at 6-week intervals; contains 1 mg B-subunit and 10^{11} inactivated *V. cholerae* cells	Licensed as Dukoral®	Clemens et al. [23]
LT patch	IOMAI	Some evidence of disease amelioration in challenge study; may need safety adaptation for infants; potential for use in prime-boost regimen; 50 μg LT holotoxin given days 0, 21, 42	Current clinical study	McKenzie et al. [27]
LTB edible	Arizona State Univ. Prodigene	Oral doses generate immunity to LT-B subunit; significant development issues remain	Proof of principle study	Tacket [33], Haq et al. [34], Mason et al. [35], Tacket et al. [36]
Toxin conjugate vaccines	NICHD	Conjugate with LT or another antigen given parenterally	Preclinical	Szu et al. [43,44]
Inactivated ETEC				
Formalin-inactivated cells	SBL/U. of Goteborg	Safe and immunogenic; 2×10^{10} each of 5 different strains expressing major CFs and also 1 mg cholera toxin rBS; 2 doses 2 weeks apart; some protection in travelers, but not in Egyptian infants	Undergoing reformulation	Ahren et al. [46], Svennerholm and Steele [2]
Colicin treated cells	Baylor College of Medicine	Colicin inactivated cells provided some protection against challenge	Inactive	Evans et al. [52]
PhiX174 protein E-mediated lysis	Bird-C/Vital Probes Inc.	Uses protein E-mediated lysis of cell to inactivate; immunogenic in mice	Preclinical	Lubitz et al. [54], Jalava et al., [56]; Eko et al. [57]
Inactivated Shigella vector for ETEC antigen	Bird-C/Vital Probes Inc.	Induces immune response to ETEC antigens expressed by *S. flexneri* 2a; effective intranasally, orally or by TC immunization	Preclinical	Osorio et al. [58]
Attenuated ETEC				
CFA/II toxin mutant	CVD	Some reactogenicity, strong protection	Inactive	Levine [16,17]
HolaVax	CBL	Safe and immunogenic, no significant protection, model to be modified before repeat of challenge	Current clinical trials	Turner et al. [61], McKenzie et al. [59]
Shigella hybrid	CVD	*S. flexneri* CVD 1208 immunogenic in animals and possibly sufficiently attenuated	Current clinical trials	Barry et al. [66,67], Kotloff et al. [68]
	DoD	Not sufficiently attenuated	Seeking better attenuation	Ranallo et al. [69]
V. cholerae hybrid	Berna Biotech	Well tolerated, newer formulation developed including CFA/I, CS3, CS6 expressed in CVD103-HgR	Preclinical	Favre et al. [70]
	Avant Immunotherapeutics	ETEC antigens expressed in Peru-15	Preclinical	K. Killeen, AVANT Immunotherapeutics
S. typhi hybrid	Microscience	Safe and immunogenic for LTB expressed by attenuated vaccine construct ZH9	Current clinical trials	Khan et al. [71]; Sizemore et al. [12]
Hybrid constructs expressing ETEC ST	Tulane Univ; Beijing Institute of Biotechnology	ST-Neutralizing immune response to ST/LT-B fusions expressed in attenuated bacteria administered orally; safety remains to be determined; potential for broad coverage	Preclinical	Cardenas and Clements [73], Zheng [72]
Fimbrial antigen microspheres	DoD	Poor immune response in humans	Inactive	Tacket et al. [88], Katz et al. [90]
CS6/TC immunization	DoD	LT induced immune responses to itself And CS6	Inactive	Yu et al. [91], Guerena-Burgueno [92]
Fimbrial tip adhesin vaccines	DoD	Induced higher functional antibody titers in mice than native fimbriae	Preclinical	Anantha et al. [93]
DNA/vectored vaccine	Biomedical Sciences Institute	Prime with DNA and boost with live salmonella vaccine gave synergistic immune response to CFA/I; protected mice	Preclinical	Lasaro et al. [96,97], Alves et al. [95]

avenues towards development of an ETEC vaccine (Table 2). The approaches that have been taken to identify an ETEC vaccine may be grouped in a number of different ways, but for consideration here they fall into one of 6 groups: toxin-based vaccines; inactivated whole cell vaccines; attenuated ETEC; hybrid (vectored) vaccines; fimbrial antigen vaccines; prime/boost approaches.

3.1. Toxin-based vaccines

After small intestinal colonization, ETEC produce the heat stable and/or a heat labile enterotoxins that stimulate intestinal fluid secretion by differing pathways. ST is a low molecular weight, non-immunogenic peptide, and LT is a bipartite oligomeric protein that is structurally and antigenically related to cholera toxin (CT) [21]. Synergistic combinations of antigens may be desirable in a vaccine. Studies in a rabbit model have shown that a combination vaccine that stimulates both anti-bacterial and anti-toxic immunity confers greater protection against ETEC than vaccines that stimulate only one or the other type of immunity [22].

3.1.1. Cholera WC/B subunit

The antigenic similarity between the B subunit of cholera toxin and the LT of ETEC led to an assessment of whether the combined cholera toxin BS/whole-cell oral vaccine against cholera could also protect against ETEC. In a double-blind field trial among rural Bangladeshi women and children 67% fewer episodes of LT-ETEC diarrhoea were noted in the BS-WC group than the group given whole cell vaccine without the B subunit [23]. This protection waned between 3 and 9 months after oral vaccination with at least 2 doses of vaccine. Short-term protection in this study was most apparent against severe LT-ETEC diarrhoea. This vaccine was also shown to induce short-term protection against LT-ETEC in travelers to Morocco [24] and Mexico [25], and is now licensed as Dukoral® (SBL, Sweden) in 15 countries.

3.1.2. Labile toxin patch

Transcutaneous immunization (TCI) is an innovative approach involving topical application of an antigen and adjuvant to intact, hydrated skin using a simple occlusive patch [26]. In preclinical studies conducted by IOMAI Corporation (Gaithersburg, USA), LT and closely related cholera toxin were found to be potent immunogens and adjuvants when given transcutaneously to mice, with protection against CT oral challenge. IOMAI has recently completed a clinical trial of LT holotoxin vaccine given by TCI, with subsequent test of protective efficacy by challenge with a prototype pathogenic ETEC strain E24377A, performed in collaboration with the Center for Immunization Research, Johns Hopkins University (Baltimore, USA) [27].

The recently presented results of this trial indicated that TCI with 50 μg of LT alone given on the upper arm on days 0, 21 and 42 induced substantial systemic and intestinal anti-toxic antibody responses as measured by ELISA and by a toxin neutralization assay. Immunization did not confer protection against diarrhoea upon E24377A ETEC challenge, although some evidence of disease amelioration was apparent. The volunteers were challenged with 6×10^8 cfu of the LT^+/ST^+ ETEC strain, which may be several logs higher than expected numbers of organisms encountered during natural infection [28–30]. If so, protection may be more clear-cut in a field study in travelers. This possibility may be further supported by the effectiveness, although short-term, of Dukoral®. These results may indicate that anti-LT immunity should be considered as an adjunct in development of an effective vaccine, but that it must be combined with additional protective antigens (e.g., CFs or derivatives) to effectively prevent disease caused by ETEC encountered in settings of natural exposure. Animal data suggesting that LT toxin may also serve as an accessory colonization factor for LT and LT/ST ETEC strains further indicate that it may be important to include in future ETEC vaccine formulations [31,32].

3.1.3. Plant-derived LT-B

Transgenic plants offer a novel strategy for oral delivery of vaccine antigens [33]. This approach has many of the advantages of orally-administered vaccines and has been used to deliver the LT-B subunit. Mice fed transgenic potatoes encoding the gene for LT-B developed antigen-specific serum IgG and mucosal IgA [34]. In subsequent studies, mice immunized with LT-B via ingestion of the tuber material were challenged with LT [35]. None of these mice were completely protected against fluid accumulation, but the potato vaccine provided a significant reduction in fluid accumulation. No antibodies were seen in mice fed non-transformed potatoes and these mice did not have reduction in fluid accumulation in the patent mouse assay. Human volunteers fed this potato-derived vaccine also mounted vigorous immune responses to the LT-B [36].

Results similar to the potato-derived vaccine have been obtained in mice immunized by feeding with transgenic corn meal [37,38]. These data offer proof of concept that transgenic enteric vaccines could be safe and immunogenic in humans, but significant development will be needed before this approach is practical for infants in DCs.

3.1.4. Toxin conjugate vaccines

LT and ST could be developed for parenteral immunization as conjugate vaccines. ST occurs in 75% of ETEC strains, either alone or in combination with LT [20]. There is a significant correlation of ST-expressing ETEC and diarrhoea [39–42]. This approach is being investigated at the National Institute of Child Health and Disease (Bethesda, USA). Cholera toxin or its B-subunit conjugated with the capsular polysaccharide of *Salmonella typhi* elicited high titers of anti-toxin IgG in mice, with higher neutralization activity obtained with the holotoxin than with its B-subunit [43,44]. For this approach to be applied to parenteral immunization with LT it was essential that the toxicity of the enterotoxin be reduced [45]. This problem also applies to the ST of ETEC,

but previous experience with other toxins would suggest that its toxicity could be greatly reduced through chemical conjugation with LT or another antigen ([43], unpublished data).

3.2. Inactivated whole cell vaccines

3.2.1. Formalin-inactivated ETEC WC/B subunit

A killed whole-cell ETEC vaccine (2×10^{10} each of 5 strains expressing CFA/I, CS1, CS2, CS3, CS4, CS5) co-administered with 1 mg of recombinant cholera toxin B-subunit (rCTB) is the one vaccine that has been carried furthest along a clinical development pathway [2,46]. This vaccine was developed at the University of Göteborg, Sweden and refined and manufactured by SBL Vaccin (Stockholm, Sweden). The US Department of Defense (US-DoD) brought the ETEC/rCTB vaccine into advanced development where evaluation culminated in a Phase 3 efficacy trial in Egyptian infants in 2002 [47–49]. The results of this trial indicated that the vaccine was not efficacious in preventing diarrhoea due to ETEC that expressed a vaccine-shared antigen [47,50].

In keeping with findings from the trial in young Egyptian children, the primary analysis of two trials in adult volunteers in Latin America showed no significant protection against ETEC diarrhoea, although post hoc subgroup analyses did indicate that the vaccine protected against moderate to severe forms of ETEC traveler's diarrhoea and the subset of subjects with anti-CTB serum IgA titers above 1358 were protected against ETEC diarrhoea of any intensity [51]. If means can be found to enhance immunogenicity of critical antigens in the preparation or to use a regimen to adjuvant the preparation, it may be possible to enhance the efficacy of this vaccine.

3.2.2. Genetically inactivated ETEC

Immuno-protection against ETEC is afforded by natural clinical infection in which the host is exposed to the complex antigenic repertoire on the bacterial cell [52,53]. To safely mimic this phenomenon, an oral whole cell vaccine consisting of ETEC cells was rendered incapable of replication by treatment with colicin E2 [53]. The colicin is a potent DNA endonuclease which destroys both chromosomal and plasmid DNA without damage to bacterial cell integrity or antigenicity. In contrast to cells altered by heat or chemical treatment, these cells should be antigenically as close to live cells as possible. Using ETEC strain H-10407; ST^+LT^+, 078:H11:CFA/I for this approach, 9 out of 10 vaccines in a double-blind, placebo-controlled study responded with increased anti-CFA/I intestinal IgA, and 8 were protected against a challenge dose of the live homologous organism which produced diarrhoea in 89% of the placebo-treated volunteers [52,53].

This vaccine was used in a study to compare protection against both homologous and heterologous serotypes [53]. Approximately 75% efficacy was achieved in groups challenged with either a CFA/I-positive 063:H strain or a CFA/II-positive 06:H16 strain. None of 16 vaccines who had responded to both CFA/I and LT became ill upon challenge while both of the vaccines who had not responded to either antigen did. These data suggest that ETEC heterologous with respect to O, H, and CFA may share other antigens which contribute to a protective intestinal immune response.

PhiX174 protein E-mediated lysis of bacterial enteric pathogens may offer another means of genetic inactivation. Chemical and physical treatment of cells has also been avoided for inactivation through the use of PhiX174 protein E-mediated lysis of Gram-negative bacteria, thereby producing what are known as cell "ghosts" [54–56]. In this process, gene E of phage PhiX174 codes for a membrane protein which fuses inner and outer membranes of Gram-negative bacteria, forming a transmembrane lysis tunnel. As a result, the remaining bacterial internal space is devoid of nucleic acids, ribosomes or other constituents, while the inner and outer membrane structures of the "ghosts" are well preserved. Among the applications of this approach, rabbits have been vaccinated intra-gastrically with *Vibrio cholerae* "ghosts", which led to markedly increased levels of vibriocidal antibodies and protection against diarrhoea and death following challenge with fully virulent *V. cholerae* [57]. A CFA/I-expressing ETEC "ghost" as well as a "ghost" of *Shigella flexneri* 2a which expresses CFA/I and CS3 (CVD1203) have been prepared by Bird-C in Vienna, Austria, and are now undergoing preclinical evaluation by scientists at the US-FDA [58]. Both of these "ghost" preparations induce IgG antibodies against CFA/I when administered to mice and the "ghost" of *Shigella* was also immunogenic for CS3.

3.2.3. Inactivated shigella vectors for ETEC antigens

Formalin-inactivated *Shigellae* are immunogenic in animals and man [58,59] and offer safe platforms with which to deliver a variety of antigens to the host. Work with CVD1203 [60], a *S. flexneri* 2a construct expressing CFA/I and CS3, has demonstrated that this inactivated organism elicits immune responses to the ETEC antigens when given nasally or orally [58]. If the immune responses to the ETEC and the *Shigella* antigens are sufficient, this cellular component could protect against 3 enteric bacterial pathogens.

3.3. Attenuated ETEC

3.3.1. CFA/II positive mutant

Escherichia coli E 1392-75-2a, a CFA/II positive mutant wherein the genes encoding LT and ST are spontaneously deleted from the CFA/II plasmid [16,17], was described above. This organism, although too reactogenic for general use, demonstrated the protection potential for attenuated ETEC cells [18].

3.3.2. Attenuated ETEC

A program to develop a live, attenuated ETEC vaccine (HolaVax) was initiated by Acambis Corporation (Cambridge, UK). Starting with ETEC strain E1392-75-2A, investigators introduced 2 or 3 specific gene deletions for further attenuation without affecting CFA/II antigen expres-

sion [61]. Two clinical trials to evaluate the safety and immunogenicity of these two candidate vaccine strains were conducted at the Johns Hopkins University Vaccine Testing Unit (Baltimore, USA) in collaboration with investigators at the US Naval Medical Research Center (Bethesda, USA) [61–63]. Both vaccines were found to be safe, while the strain with 3 defined gene mutations (PTL-003) elicited better immune responses as measured by peripheral blood IgA antibody secreting cell responses to CFA/II and showed more prolonged intestinal colonization. A vaccination-challenge study with PTL-003 in adult volunteers was conducted as a proof of principle for this vaccine approach. Findings from this trial indicated a lack of significant protection against the primary disease outcome of diarrhoea, but there was some suggestion of disease attenuation in the vaccinated group [63].

Interpretation of the results centered on concerns that the vaccination regimen may have been suboptimal, coupled with speculation that the challenge dose of virulent CFA/II ETEC may have been excessive. In the original study done by the CVD with strain E1392-15-2A, prior to the further attenuations induced in the HolaVax vaccine, volunteers were vaccinated with one dose of 5×10^{10} cfu and challenged with 5×10^{8} cfu of strain E24377A/5. In the study with the further attenuated PTL-003, volunteers were vaccinated with 2 doses of 2×10^{9} cfu and challenged with 3×10^{9} cfu. Also a day 0 and 28 immunization regimen may have induced a better anti-CFA/II antibody response than the day 0 and 10 regimen used in the trial [63].

Using wild-type ETEC strains, Acambis constructed similarly attenuated ETEC strains that express other prevalent CFs including CFA/I, CS1 + CS2 + CS3 and CS4 + CS5 + CS6, each strain expressing LTB. Additional Phase 1 trials of a CFA/I-ETEC live, attenuated strain were conducted in the United Kingdom demonstrating a favorable safety and immunogenicity profile and that a cryopreserved formulation of the vaccine construct was as immunogenic as the fresh, plate grown vaccine preparation [62]. In early 2004, Acambis announced the discontinuation of their efforts to develop a live, attenuated ETEC vaccine. HolaVax was subsequently out-licensed to Cambridge Biostability Ltd. (Cambridge, UK) and this company has been pursuing further development of the vaccine. It is anticipated that a Phase 1 clinical trial of a final, multivalent (3-strain) vaccine (that collectively expresses seven CFs and LTB) will be performed. If favorable results are generated, a Phase 2b vaccination-challenge clinical trial should be considered to determine the level of protective efficacy in the inpatient volunteer challenge model.

3.4. Hybrid (vectored) vaccines

3.4.1. Attenuated Shigella-ETEC hybrids

a. *CVD hybrid*. A group at the University of Maryland Center for Vaccine Development (CVD, Baltimore, USA) has used the *Shigella flexneri* 2a attenuated strain CVD 1204 (auxotrophic for guanine, *guaBA*) as the live attenuated backbone for development of this hybrid vaccine concept [64,65]. One recombinant strain was engineered to co-express CFA/I along with non-toxic mutated derivatives of LT. In guinea pig studies, the majority of animals immunized with one of the constructs showed systemic and mucosal antibody responses to CFA/I while the anti-LT response was suboptimal. Using the Sereny test model of *Shigella*-induced keratoconjunctivitis in guinea pigs, the hybrid vaccines were shown to confer protection against wild-type *Shigella flexneri* 2a challenge. The goal of this candidate is a multivalent formulation of 5 shigella strains (*S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6, *S. sonnei*, and *S. dysenteriae* type 1) expressing major CFs and LT antigen.

In recent work the group has separately introduced recombinant plasmids into *S. flexneri* 2a CVD1208 (*guaBA*, *sen*, *set*) and comparable attenuated strains of *Shigella sonnei* and *Shigella dysenteriae* type 1 that collectively express CFA/I, CS1, CS2, CS4 and genetically detoxified LT (LTK63), and elicited immune responses to each of the components after guinea pig mucosal immunization with the cocktail of hybrid strains [66,67]. Clinical trials with such vaccines will be important to demonstrate the degree of attenuation obtained with this approach. Based on a Phase 1 trial comparing CVD 1204 and CVD 1208, the *sen* and *set* gene deletions lead to a well-tolerated and immunogenic *Shigella* vaccine candidate [68].

b. *US-DoD hybrid*. Investigators at the Walter Reed Army Institute for Research (WRAIR, Silver Springs, USA) have also pursued a related strategy to develop a live-attenuated hybrid *Shigella*/ETEC vaccine [69]. The *Shigella flexneri* 2a vaccine construct SC602 has been used as the backbone for SC608, a shigella strain expressing antigens from ETEC. Some effort has been devoted to the development of stabilized plasmids (based on *asd* balanced lethal mutations) for expression of ETEC CF antigens. This group has introduced recombinant plasmids into SC602 that express different antigens consisting of LT-CFA/I major subunit fusions, LT-CFA/I consensus peptide fusions, and CFA/I chaperone-major subunit protein combinations. Immunization studies in small animals have shown detectable responses to the heterologous ETEC antigens. Further evaluation of such vaccines has been postponed until the development and testing of a new generation of live, attenuated *Shigella* vaccines has been accomplished.

3.4.2. Attenuated Vibrio cholerae-ETEC hybrid

a. Berna Biotech (Switzerland), developed a prototype *V. cholerae*-vectored vaccine into which the CFA/I gene cluster was integrated into the chromosome. The base strain is CVD103-HgR (licensed as Orochol® by Berna Biotech), a live, attenuated vaccine derived from *V. cholerae* 569B with a deletion in the cholera toxin A

subunit, and mercury resistance gene insertion into the hemolysin locus. One cited advantage of this approach is the extensive and favorable safety track record of CVD103-HgR. Further development of this vaccine concept by Crucell Berna Biotech is in process and new constructs in which CVD 103-HgR expresses colonization factors CFA/1, CS3, and CS6 have been developed and offer promise for future trials [70].

b. AVANT Immunotherapeutics Inc. has clinically evaluated Peru-15 as a live attenuated, oral, single-dose cholera vaccine candidate in 7 clinical trials (6 in US and 1 in Bangladesh) [Kevin Killeen, AVANT Immunotherapeutics, personal communication]. Cumulatively, Peru-15 was proven well tolerated and highly immunogenic in >400 human subjects and has been funded by the International Vaccine Institute (IVI) to advance through a Phase III field study. AVANT's ETEC vaccine candidate, strain Peru-15 pCTB, is a derivative of Peru-15 carrying the GlnA balanced-lethal plasmid pMEG-2350 with CTB expressed from the strong constitutive Ptrc promoter and secreted in large quantities (>60-fold) compared to Peru-15 (single copy of chromosomal borne CTB). In preclinical murine and rabbit studies, Peru-15 pCTB induced a strong anti-CTB antibody response with titers significantly higher than Peru-15. This construct is actively being developed as a 'first generation' ETEC vaccine. AVANT is also generating additional Peru-15 vectored ETEC vaccine candidates that express CS6, CFA1 and CS3. Studies are underway to evaluate the immunogenicity of these 'second generation' ETEC vaccine candidates.

3.4.3. Attenuated Salmonella typhi-ETEC hybrid

Microscience, Ltd. (Wokingham, UK) developed a live, attenuated *S. typhi*-vectored ETEC vaccine. A live, attenuated vaccine construct (ZH9) was generated from wild-type *S. typhi* strain Ty2 by deletions in an aromatic biosynthesis pathway gene (*aroC*) and pathogenicity island-2 (*ssaV*) gene. In published work, ZH9 was used as the base strain for incorporation of the gene expressing LTB and this *S. typhi*-vectored ETEC vaccine candidate (ZH9/LT-B) was shown to elicit anti-LT serum and mucosal immune responses in orally immunized mice [71]. A Phase 1 clinical trial of the *S. typhi* attenuated ETEC vaccine was completed in the United Kingdom in 2003, and indications were that 70% of subjects showed a positive immune response to the heterologous ETEC antigen, presumably LTB [12].

3.4.4. Hybrid constructs expressing ETEC ST

The genetic determinants encoding CS3 and LT-B/ST fusion toxin were manipulated for expression in an attenuated *Shigella flexneri* [72]. The expressed ST antigen raised antibodies in mice which were able to neutralize the biological activity of native ST. Earlier work with this approach [73] described construction of a fusion peptide in which an eight amino acid, proline-containing linker was included between the LT-B and ST moieties of ETEC. An *aroA* mutant of *Salmonella dublin* was transformed with a plasmid containing this genetic construct and shown to express antigenic determinants of both LT-B and ST. Sera and mucosal secretions from mice immunized orally with this strain were able to neutralize the biological activity of native ST in the suckling mouse assay. The possibility of applying a similar immunogenic construct of ST to immunoprophylaxis against ETEC-induced diarrhoea remains to be explored. In addition to any latent toxicity in humans, it will also need to be determined that an immune response to ST does not interfere with intestinal cell functions in humans, since ST is an analog of the peptide guanylin whose receptor is widespread on epithelial cells [20].

3.5. Vaccines based on fimbrial antigens

To date, over 25 human ETEC CFAs have been reported [74,75]. Structurally these virulence determinants constitute complex biopolymeric filaments on the bacterial surface mediating adherence to the intestinal mucosa [72] and can be divided into a smaller number of biogenetically related families within which member structures show considerable antigenic variability. The CFs are mainly fimbrial or fibrillar proteins, although some, such as CS6, are not [74,76,77]. With the exception of CFA/I, all CFs are designated as coli surface antigen (CS). CFs have been subdivided into different families:

a. CF I-like group including CFA/I, CS1, CS2, CS4, CS14, and CS17.
b. CS 5-like group including CS5, CS7, CS18, and CS20.
c. Unique group including CS3, CS6, and CS10-12.

The CFs most commonly found on diarrheagenic strains include CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS14, CS17, and CS21 [74,76,77].

Some human vaccine and passive immunization studies have shown that ETEC CFs can induce protection [78–82]. Early trials in travelers to DCs with a formalin-inactivated vaccine made up of components expressing the major colonization factors and the recombinant B-subunit of cholera toxin were encouraging [83]. In more recent efficacy trials with this vaccine in travelers, protection was only seen against moderate to severe episodes of diarrhoea and protection against ETEC disease of any severity was only seen in those subjects with anti-CTB serum IgA titers >1385 [51]. This vaccine showed essentially no protective efficacy in 6–18 month old Egyptian children [47–49]. It is of note that the latter data could be a consequence of the difficulty of inducing immune responses in this group, but could also indicate that other antigens than CFAs and *V. cholerae* CTB are needed. In a cohort study of the natural history of childhood intestinal infections in Guinea-Bissau, where ETEC is endemic, Steinsland et al. [11] concluded that protection seen against ETEC was larger than that induced by CFAs, suggesting that for breastfed children living in endemic areas,

antigens other than the CFAs may be needed in the vaccine in order to induce protection.

In spite of the questions raised above, recent data do lend support to the concept of using CFs in a vaccine against ETEC. Among Egyptian children 9–17 months of age, serum levels of IgG antibody against CFA/I were good markers of protection against ETEC strains expressing this CF [84] which may indicate that the level of antibody titers obtained against CFAs could be critical, and that therefore the level of CF antigen expressed on the cell surface and the regimen for its delivery may be important to generate antibody responses correlating with protection. In these children protection was noted against CFA/I-ETEC diarrhoea if reciprocal antibody titers were >76. A similar correlation was not noted for antibodies against CS3 and CS6. This finding does not preclude the importance of these antigens; it only indicates that serum levels of IgG antibodies are not a good correlate of protection against disease by ETEC expressing these antigens. In support of this possibility, Dr. C. Carpenter [85] was able to associate anti-CS3 IgA titers with protection in volunteers participating in a challenge study. In her studies, anti-CS3 titers >155 were associated with protection against moderate to severe illness and a significant reduction in the median weight of diarrhoea stools passed after challenge. When pre-challenge CS3 IgA titers were evaluated with regard to protection induced with an attenuated ETEC vaccine strain, there was a correlation between these titers and milder symptoms post challenge.

3.5.1. Fimbrial antigen microspheres

Until recently, a primary approach to ETEC vaccines in the US-DoD was to develop and evaluate monovalent vaccines composed of the 3 most important CF targets (CS6, CS3 and CFA/I) formulated as purified, intact fimbriae incorporated into microspheres (microencapsulation in poly-D,L-lactide-co-glycolide [PLGA]) and given by the oral route [86]. Animal studies using this delivery system demonstrated that microspheres of defined sizes are taken up and processed by Peyer's patches and stimulate local and systemic immune responses [87]. In the first human study of microencapsulated ETEC subunit vaccine, purified CFA/II (CS1 + CS3) was given to study volunteers at the University of Maryland (Baltimore, USA) as a 1 mg dose four times via a duodenal tube [88]. The vaccine was well tolerated and induced a mucosal immune response in half of the vaccinated volunteers. When challenged with a virulent CFA/II ETEC strain 2 months later, all of 10 unvaccinated and 7 of 10 vaccinated volunteers developed diarrhoea, indicating a lack of significant protection. That two of the three volunteers in the vaccine group who did not develop diarrhoea upon challenge had the highest IgA anti-CFA/II antibody secreting cell responses, suggests that improvements in this immune response might result in a protective effect.

A vaccine consisting of purified microencapsulated CS6 (meCS6) was found in mouse studies to be highly immunogenic when given by the intranasal route [89]. In contrast, when given via intra-gastric tube, there was minimal uptake of antigen and non-detectable immune responses. In a subsequent clinical trial the safety and immunogenicity of two different oral formulations of CS6 were compared in a three-dose regimen. Microencapsulated CS6 as well as free CS6 were administered perorally, both with and without buffer [90]. Orally administered meCS6 was found to be safe but elicited suboptimal immune responses, which served as the basis for a follow-on Phase 1 trial in which the mucosal adjuvant, genetically detoxified mutant LT (LTR192G), was added to microencapsulated CS6 to potentiate anti-CS6 immune responses. A Phase 1 clinical trial was completed comparing peroral meCS6 versus LTR192G/meCS6 and appropriate controls on two different immunization schedules (every other day, 4 dose-regimen versus every 2 week 3-dose regimen). This study showed that the vaccine with or without adjuvant by either regimen was safe but did not induce the predetermined criteria for immune responses, which would have provided the basis for further evaluation in a vaccination-challenge study. Further clinical development of this vaccine candidate has been suspended.

3.5.2. CS6 delivered by transcutaneous immunization

In preclinical studies conducted by IOMAI (Gaithersburg, USA) in mice and guinea pigs, LT co-administered with CS6 stimulated favorable gut immune responses to both antigens [91]. In a phase I study jointly conducted by the Walter Reed Army Institute for Research) WRAIR and IOMAI, TCI with LT was free of local and systemic toxicity and induced systemic and mucosal IgG and IgA anti-LT antibodies. A phase I trial was conducted at WRAIR to assess the adjuvant effect of LT holotoxin when co-administered with CS6 by TCI. While CS6 alone induced undetectable immune responses, co-administration with LT elicited significant responses to both antigens [92]. Development of a local delayed type hypersensitivity reaction in many of the subjects receiving the antigen combination raised some concern about acceptability. A subsequent trial to assess the comparability of adjuvant effect with LT versus its mutated derivative LTR192G and optimal adjuvant dose for stimulating anti-CS6 responses demonstrated that the adjuvant activity of LTR192G was similar to native LT in terms of enhancement of CS6 immune responses. As with CS6/LT co-administration, CS6/LTR192G given by the transcutaneous route led to a mild DTH skin reaction in a majority of subjects, with rates apparently related to the adjuvant dose.

3.5.3. Fimbrial tip adhesin vaccines

A research program to develop a sub-fimbrial ETEC subunit vaccine that targets the adhesive protein component of the major CFs of importance in human disease has been initiated by US-DoD. Since the actual adhesin is typically a minor constituent of the fimbrial polymer, investigators postulated that the recombinant adhesin will more efficiently elicit protective anti-adhesive antibodies than will whole fimbriae. Within the Class 5 family of eight ETEC fimbriae (including

CFA/I), the minor adhesive proteins reveal greater structural conservation than the major subunit constituents that form the bulk of the fimbrial stalk [93]. Moreover, antibodies to the minor subunit inhibit in vitro binding of ETEC bacteria that express the homologous as well as heterologous CFs within the same subclass. These findings suggest the possibility of vaccine coverage against all eight Class 5 fimbriae with as few as three subfamily-specific adhesin antigens. Addition of the CS3 fibrillar adhesin to such a cocktail in a quadrivalent preparation along with an enterotoxoid component (based on LT) could conceivably offer sufficiently broad protection for an effective ETEC vaccine.

Immunogenicity of recombinant CFA/I adhesin (*dscCfaE*) was compared in a mouse mucosal immunization model to that of native CFA/I fimbriae [94]. When given by either the oro-gastric or intranasal route in conjunction with a mucosal adjuvant (genetically detoxified LTR192G), *dscCfaE* elicited robust serum IgG and IgA antibody responses. Moreover, this adhesin elicited significantly higher functional antibody titers in serum, as measured by the hemagglutination inhibition assay, when compared to immunization with native CFA/I fimbriae. These findings have provided the impetus for down-selection of the *dscCfaE* antigen as the lead vaccine component for dose-ranging mucosal immunogenicity studies in animals. Other fimbrial subunit vaccine components are under development.

3.6. Prime/boost approaches with DNA vaccine/vector

A strategy involving priming with DNA and boosting with a live recombinant *Salmonella* vaccine has been studied as a means to induce immune responses to fimbrial antigen of ETEC [95–97]. Mice primed with intramuscularly delivered CFA/I-encoding DNA vaccine followed by two oral immunizations with a live recombinant *Salmonella typhimurium* vaccine strain expressing the ETEC antigen had a synergistic response to the induced CFA/I-specific systemic and secreted antibody levels which could not be obtained with either immunization strategy alone [96,97]. In subsequent studies [97], this prime-boost regimen increased the ability of serum antibodies to inhibit the adhesive properties of the CFA/I fimbriae expressed by live bacteria. Addition of the mucosal adjuvant LTR192G to the *Salmonella* vaccine strain further enhanced the synergistic effects of the vaccine regimen. Further, dams given the prime-boost regimen transferred complete passive protection to suckling neonates challenged with a virulent ETEC strain. Of note is the fact that such an approach might be difficult to implement in a DC infant population.

4. Routes of administration of ETEC vaccines for children in DCs

Current evidence suggests that stimulation of immunity within the gut mucosal compartment is important for protection against ETEC diarrhoea [98]. Furthermore, most of the possible near-term candidates for an ETEC vaccine are not tailored for delivery by non-mucosal routes, nor has effectiveness of an ETEC vaccine been demonstrated to-date by a parenteral route only. Consequently, the greatest efforts have been devoted to making vaccines for oral delivery. While this appears to be the preferred route of administration, other routes for stimulating gut-associated immunity should be explored. One reason for this is that the immunogenicity of oral vaccines may be reduced in children living in DCs. For example, Indonesian children living in poor conditions required 5×10^9 cfu of the live oral cholera CVD103-HgR to obtain a level of sero-conversion obtained with a 10-fold lower dose in North Americans and Europeans [99,100]. This phenomenon has also been noted for oral polio vaccine and the RIT 4237 attenuated bovine rotavirus vaccine candidate [101–104]. Moreover, serum antibody responses to the B subunit of cholera toxin component of the ETEC and cholera killed whole cell vaccines were higher in children from industrialized countries than in those in children living in DCs [105].

The refractoriness to vaccination could to be associated with colonization differences between the intestines of children in wealthy countries and those living under poor conditions in DCs. The proximal small intestine of children in industrialized countries is virtually free of *Enterobacteriaceae*. Their bacterial counts are low (less than 10^4 organisms/ml of aspirated intestinal fluid), while those from children in DCs typically have much higher bacterial colonization of their proximal small intestine [106–109]. More recently Adelberth et al. [110] studied the intestinal flora of infants living in Pakistan and showed that poor hygienic conditions result in an unstable and diverse enterobacterial flora, which may facilitate enteric infections as well as lower responsiveness to oral vaccines. This overgrowth is accompanied by architectural changes in the intestinal mucosa (environmental enteropathy) of children, due to ingestion of large numbers of microorganisms in their environment. The consequence of this for orally-administered vaccines could be the production of factors which inhibit the vaccine organisms or factors which impair antigen uptake and subsequent immune responses. That intestinal parasites can contribute to impairment of immune responses to orally delivered vaccines was demonstrated following Albendozole treatment of children harboring *Ascaris lumbricoides*. This treatment to remove the worms, improved the immunogenicity of CVD 103-HgR [111]. Micronutrient deficiencies may, in addition to overgrowth of micro-organisms in the intestine, also contribute to the blunted immune response in children in DCs [112].

Research to find ways to overcome the immunization barrier in the infant intestine is needed. This may be partly obtained by increasing the dose or number of doses given. It was mentioned above that a 10-fold increase in dose of CVD103-HgR could improve titers. In some cases, however, higher numbers of live cells per dose may be problematic for children living in DCs. It should also be noted that the few

"unsuccessful" live vaccines that have been given orally to children in DCs are given in a single-dose regimen. It remains possible that had these vaccines been given as 2–3 doses they may have been more effective.

Multiple dose regimens for oral immunization are often given within a 30 day period. It is possible that the timing could affect the immune response. Consideration needs to be given to the administration of a multi-dose oral regimen in coordination with the Expanded Programme on Immunization (EPI). An expanded schedule of immunization (2, 4 and 6 months) was used with the inactivated ETEC-rCTB vaccine in a trial in Egyptian infants [47]. The vaccine or placebo was administered in 6 ml of bicarbonate buffer. Significant IgG and IgA immune responses to ETEC antigens were observed in children vaccinated on the EPI schedule. Moreover, based on immune responses when the ETEC vaccine was given concomitantly with OPV, there was no interference between the two vaccines.

As mentioned above, 6 ml of bicarbonate buffer was administered with the vaccine, but the buffering requirements for oral delivery of vaccine to infants need to be better defined. Many children in DCs have very low levels of acid production and factors such as nutritional level, breast-feeding versus bottle-feeding and age are possible confounding factors. The nature of the antigens critical to vaccine immunogenicity may play a role in determining buffer requirements. The immunogenicity and efficacy of the inactivated whole cell cholera vaccine produced in Vietnam [113] is based on LPS and does not require buffering. The live attenuated cholera vaccine, Peru-15, was found to be more immunogenic when given with a rice-based buffering agent, designated CeraVacx [114]. Similarly the systemic immunogenicity of the prototype live attenuated ETEC vaccine developed by Acambis was improved when the vaccine was given in CeraVacx [63]. This area of vaccine enhancement warrants further investigation. It should be remembered, however, that acidity is only part of the challenge of delivering vaccines orally, as some antigens may also be susceptible to enzymatic degradation.

The decreased intestinal responsiveness described may also be improved by use of alternate mucosal routes, alone or in combination with the oral route. It is possible that the rectal route would be more responsive than the oral route in the infant population of DCs. The rectal mucosa is known to be an effective site for immunization [115–117]. *S. typhi* Ty21a induced similar immune responses in serum or intestinal secretions whether it was administered orally or rectally [118]. Further, rectal delivery of LT was proven to be safe compared to oral delivery and resulted in a strong immune response to this antigen [119]. In spite of all these potential advantages, the rectal route may not be acceptable to parents for infants in DCs. Likewise the nasal route is known to be effective for inducing strong immune responses [120], but this route faces greater safety hurdles than the oral or rectal routes, and may not be practical for children in DCs. That the use of alternate mucosal routes is not fully understood is indicated by a recent study in Sweden where it was shown that neither intranasal nor rectal immunization of adult volunteers with CTB induced any significant mucosal immune responses in the small intestine, at variance with corresponding oral immunization that induced good such responses [121].

Transcutaneous (TC) administration of antigen might be used to circumvent the intestine altogether or to prime or boost responses to intestinally-administered antigens. John et al. [122] studied immune responses in mice given cholera toxin or its B subunit transcutaneously or an attenuated vaccine strain of *V. cholerae* expressing the cholera toxin B subunit. Optimal immunological responses to the B subunit in this study were obtained in mice that were orally primed with the vector expressing the B subunit and transcutaneously boosted with CT. TCI has also shown promise with isolated ETEC fimbrial antigens [91] as well as ETEC CFA/I and CS3 antigens associated with inactivated whole cells of *S. flexneri* [58]. Of importance is the fact that the use of this technology in infants would require substantial modifications in the current approach to eliminate the possibility that toxic LT could be ingested from a dislodged patch.

Limited evaluation of parenteral immunization with purified ETEC fimbriae in combination with oral delivery has been reported [123]. In this study, subcutaneous priming with 50 μg of CFA/I did not induce serum IgG, but strong intestinal anti-CFA secretory activity was induced in 4 out of 8 volunteers when followed by oral boosting with 1 mg of CFA/I in two divided doses in sodium bicarbonate. These four responders were protected against subsequent challenge with CFA/I positive ETEC. Systemic priming followed by oral boosts can induce significant local responses [124].

Another approach to increasing responsiveness of children in DCs to ETEC vaccines may be to use mucosal adjuvants to improve the host response. At present these are still at the pre-clinical stage or have been shown to have some residual level of toxicity in adult volunteers. For example, Kotloff et al. [125] found that 20–25% of volunteers given 25 μg of LTR192G (a less toxic modification of LT [45]) with an inactivated whole cell *Helicobacter pylori* vaccine experienced mild diarrhoea. LT could serve as an adjuvant and an antigen in an ETEC vaccine if its safety can be assured. It is possible that additional mutations to LTR192G could accomplish this.

5. Safety considerations for the use of live and killed bacteria in children in DCs

Since administration of an ETEC vaccine to be used in DCs would begin at less than 6 months of age, it is critical to consider safety of vaccines that may be given to this age group. Many attempts to develop enteric vaccines have involved the use of attenuated organisms with the idea that these provide the best means for mucosal immunization. Achievement of the requisite balance between attenuation and immunogenicity has proven to be a difficult hurdle for some live attenuated vaccine candidates against enteric pathogens, and presents a no less formidable challenge for

groups working on vaccines for children in DCs. Vaccine associated reactogenicity could be a larger problem among children in DCs than in older or healthier individuals, due to malnutrition and underlying diseases occurring in this population. For example, diarrhoeal diseases, including those caused by ETEC, were more severe in malnourished children in India [126]. Morbidity due to diarrhoeal diseases is generally increased in the presence of micronutrient deficiencies which are common in DCs [127,128].

Another problem which could be important in children in DCs is that multiple enteric pathogens will often occur together [129] and could enhance virulence synergistically, such as described recently for ETEC and enteropathogenic *E. coli* [130]. Synergism among pathogens could also be a problem if multiple attenuated organisms are used in a combined-agent enteric vaccine. While these observations do not predict that all attenuated vaccines will be problematic in children in DCs, they do not rule out the possibility that under some circumstances, infectious complications may be present in this population that are not seen in a traveler's population. These problems could present dosing challenges in attempts to overcome the refractoriness to oral immunization of some children living in DCs.

There is some evidence that inactivated whole-cell vaccines can be relatively safe in populations with underlying disease. This is suggested by the apparent safety of the B-subunit whole-cell cholera vaccine when used in a location where there was a high prevalence of HIV in the general population [131]. Nevertheless, the oral formalin inactivated whole cell ETEC vaccine induced vomiting in infants 6–17 months of age which was not seen in older children [132]. In this study, the dose was reduced to avoid adverse effects without any loss in immunogenicity.

Reiter's syndrome describes a triad consisting of arthritis, urethritis and conjunctivitis [133]. A number of invasive enteric pathogens have been associated with this syndrome, including *Shigella*, *Salmonella*, *Yersinia*, and *Campylobacter*. This syndrome has not been noted following ETEC infections. This observation may be important when considering live vectors, such as *Shigella*, for ETEC antigens.

6. Development issues

6.1. Proposed business model: focus and continuity through a business-like approach with adequate resources and funding

For the WHO, the primary target for an ETEC vaccine is infants and young children at risk for severe diarrhoea caused by ETEC in the developing world. To date, most commercial ETEC vaccine development efforts have been driven largely by small companies (biotechs) hoping to meet the needs of the military and travelers who desire highly effective short-term protection against the inconvenience and discomfort of traveler's diarrhoea. The large vaccine manufacturers have examined this potential market in terms of technical feasibility and return on investment and found it to be very challenging indeed: the market is considered to be too small and the multiplicity of agents that cause traveler's diarrhoea too complex to make such a vaccine a commercial success. Consequently, no large vaccine manufacturer has an active ETEC vaccine development program for this target population. Furthermore, because of the inherent technical R&D risks associated with developing an ETEC vaccine and with no guarantee of vaccine uptake/purchase in the long term, a vaccine designed specifically for use in young children in DC has not been considered to be an interesting market opportunity by any vaccine developer, large or small.

It should be recognized that while much of the critical fundamental and clinical research towards an ETEC vaccine for children in DC has been driven by government-sponsored research programs, such a vaccine is unlikely to be developed by the academic sector alone. The full complement of R&D skills and activities that would permit a product to achieve registration and the large scale manufacturing facilities that will be required are available only in the industrial sector. However, if industry is to take on this important challenge there will need to be an alternative development strategy that would involve significant sharing of the costs and risks inherent in such a development program.

There are several successful models upon which such a Product Development Partnership (PDP) could be built, including those currently supporting the development of vaccines for malaria (MVI and EMVI), tuberculosis (Aeras Global TB Vaccine Foundation), HIV (IAVI) and hookworm (HHVI). These organizations differ considerably in structure and operations, but what they all have in common is a single-minded commitment to an agreed goal, appropriate technical expertise, a source of core funding, and sufficient organizational flexibility to allow them to adapt to the specific complementary roles that each member of the partnership is asked to assume.

An ETEC vaccine development PDP with one or more industrial partners would rely on internal and external consultation to reach agreed project objectives and milestones, define the scope of R&D activities, identify roles and responsibilities, and work in a coordinated fashion to accelerate vaccine development at the front-end and to mobilize resources for vaccine purchase and uptake.

6.2. Access and demand

A vaccine can be truly effective only if the populations who need the vaccine have access to it and if the vaccine is in demand by the community. It is therefore critical to understand the major obstacles that could prevent rapid introduction and uptake of an ETEC vaccine. For example, in studies among rural residents in China, Chen et al. [134] noted that perception of vulnerability to the disease and familiarity with the disease are critical factors underlying community interest and readiness to use vaccines against enteric dis-

eases. This will require proactive investment and translational research in parallel with clinical R&D activities.

6.3. Production: manufacture and regulatory issues for an ETEC vaccine

Specific manufacturing and regulatory issues will need to be addressed dependent upon the nature of the candidate ETEC vaccine. Based on the current state-of-the art, it seems that the most likely near-term candidates will involve the use of attenuated or inactivated whole cell vaccines. The degree of attenuation of a live vaccine and the possibility for reversion will need to be addressed. The inactivation process for a killed vaccine must be robust and well defined. For either type of vaccine, a strain history including all manipulations and evidence that the preparation is free of adventitious agents will be important. Dialogue with relevant regulatory organizations should begin as early as possible in the development and trial processes.

In addition, there may be no industrialized world market for an ETEC vaccine designed specifically for very young children in DC and inducing only partial protection against diarrhoea, and therefore it may not be feasible to register such a vaccine with the FDA or EMEA. Registration of an ETEC vaccine for DC infants may therefore have to rely on new regulatory pathways, such as those developed by the WHO, or through Article 58 of the EMEA's Committee for Medicinal Products for Human Use (CHMP), which allows this regulatory agency to provide scientific opinions on medicinal products for human use that are intended exclusively for markets outside of the EU.

6.4. Animal models

The difficulty in identifying a suitable animal model to test immune protection against ETEC-mediated disease has been an impairment to development of a vaccine against this pathogen. While multiple simple animal tests which may correlate with protection in humans are available for the *Shigellae* [135] this has not been the case for ETEC. Recently an adult immunocompetent mouse model of intestinal colonization with human ETEC strains has been reported [136]. When treated with both cimetidine to reduce gastric acidity and streptomycin to eradicate normal flora, CD-1 strain mice can be colonized with ETEC, but none of the animals develop diarrhoea. Whether quantitation of these organisms in intestinal segments can be used to demonstrate protective immunity remains to be seen, but the model may be useful in demonstrating the importance of certain colonization factors.

The mouse has also been used as a model for intranasal challenge with ETEC [137,138]. The applicability of the pulmonary colonization versus intestinal colonization remains to be seen. Further evaluation of this model may be worthwhile, and application of an in vivo imaging system (IVIS) could facilitate use of this approach.

Although not as available as the laboratory mouse, a New World primate, *Aotus nancymae*, is susceptible to disease following oral challenge with 11-log cfu ETEC cells and develops an immune response to intranasally administered ETEC antigens [139]. This animal model was not protected from disease upon re-challenge with an homologous strain of the pathogen, suggesting more development of the model will be necessary if this animal is to be used in vaccine evaluation.

To date, the animal model which has been shown to demonstrate protection against disease following intestinal challenge with ETEC is the removable intestinal tie, adult rabbit diarrhoea (RITARD) model. The original model first described by Spira and associates [140] was modified by using a PBS wash of the small bowel to remove intestinal contents which might interfere with colonization and administering tincture of opium to reduce peristaltic clearance of the pathogen [141]. Although this model is labor intensive, with surgery being required, it is a practical possibility for vaccine testing and could benefit from further evaluation.

6.5. Human challenge model

Human challenge trials remain as perhaps the best near-term option for use in evaluation of ETEC vaccines. Vaccine Testing and Evaluation Units sponsored by NIAID (USA) at a number of US universities or other academic institutions with a long history of enteric vaccine clinical research are available for phase I and possibly phase II evaluation in volunteers. Likewise, the Mahidol University (Bangkok, Thailand) has the experience of challenge studies in human volunteers with enteric pathogens. Although results obtained

Table 3
Experience from ETEC Challenge studies in volunteers following active immunization

Vaccine	Dose	Regimen	Challenge strain dose	Protection	Reference
Attenuated E1392-75-2A (LT^-ST^-)	5×10^{10} cfu	1 oral dose	E24377A/5, 5×10^8 cfu (LT^+ST^+)	Yes	Levine [16,17]
HolaVax (Further Att. E1392-75-2A)	2×10^9 cfu	2 oral doses on days 0 and 10	E24377A/5, 3×10^9 cfu	Mitigation of disease	McKenzie et al. [59], Turner et al. [61,62]
Colicin treated cells	3×10^{10} cfu	2 oral doses on days 0 and 30	H10407, 5×10^9 cfu	Yes	Evans et al. [52,53]
Purified CFA/I, CFA/II	50 μg SC, 500 μg oral	SC prime + 2 oral doses on days 15 and 22	H10407, 4×10^8 cfu	Yes	Evans et al. [123]
LT patch	50 μg/patch	3 TC doses on days 0, 21, 42	E24377A 6×10^8 cfu	Mitigation of disease	McKenzie et al. [27]

with adult individuals may not correlate directly with those observed with children in DCs, they do provide an initial assessment of the safety and immunogenicity potential of a vaccine and can serve as an indicator of the potential protection that may be associated with an ETEC vaccine candidate. Previous work suggests that human challenge models should be able to clearly demonstrate the protection potential for an ETEC vaccine if the vaccine dose relative to the challenge dose is sufficient. This experience is summarized in Table 3.

The utility of the human challenge model for testing ETEC vaccines may benefit from further evaluation and development. A review of challenge studies suggests that the following variables may be important to consider in order to develop a better model: challenge dose, delivery method, and immune status of subjects. In general, high ETEC challenge doses of 5×10^8 to 5×10^9 cfu given in bicarbonate buffer to fasted subjects have been needed to achieve diarrhoea attack rates high enough (70–80%) to evaluate vaccine efficacy in a reasonable number of subjects. These doses are probably much higher than those that may be encountered in nature [28–30]. Attempts to deliver lower ETEC challenges in a standard meal have met with mixed results. Attack rates have varied from 100 to 30% with no clear dose response relationship between dose and outcome being apparent [142]. It is possible that use of more effective buffers could help reduce the challenge dose needed.

One variable that has not been extensively examined in the development of the ETEC challenge model is the immune status of the subjects that have participated in the dose-finding studies needed to set the challenge dose for subsequent immunization and challenge trials. Historically ETEC illness has been thought to be relatively uncommon and that, in contrast to residents of DCs, most U.S. adults have had little to no prior exposure to ETEC pathotypes. However, as ETEC detection methods have improved, it has become clear that ETEC outbreaks in the U.S. may be more common than originally thought [143]. Consequently some subjects volunteering for ETEC trials may have existing immunity to this agent. In support of this hypothesis, anti-CS3 IgA titers have been associated with protection in volunteers participating in a challenge study [84]. In these studies, anti CS3 titers >155 were associated with protection against moderate to severe illness and a significant reduction in median stool volume. When pre-challenge CS3 IgA titers were evaluated with regard to protection induced with an attenuated ETEC vaccine strain, there was a correlation between these titers and milder symptoms post challenge. A retrospective analysis of pre-challenge anti-CS3 serum IgA titers in subjects participating in dose-validation studies for one of the more commonly used ETEC challenge strains, E24377A, indicates that approximately 30% had anti-CS3 titers >155 at the time they were challenged.

Beyond laboratory volunteer studies, particularly if clear-cut successful challenge models can be demonstrated, it is not likely that further studies in traveler's will be very useful in predicting success in infants in DCs. It will therefore be important to establish safety and immunogenicity in this population as quickly as possible following establishment of these two conditions in adult volunteers.

7. Considerations for a way forward

7.1. Research priorities

Although considerable attention has focused on certain colonization antigens and LT for inclusion in an ETEC vaccine, it remains possible that other cellular antigens could also be important in providing effective and broad-spectrum protection. Application of proteomic analyses to ETEC could help identify new antigen candidates for this pathogen.

Further evaluation of ST as a protective antigen should also be undertaken. Although protection has been obtained without this antigen, the fact that 75% of ETEC strains express this toxin would suggest that it could have broad spectrum benefit. Research is needed to determine if fusion peptides containing this toxin can be formulated to produce toxin-neutralizing antibodies and if the toxin component is sufficiently attenuated for human use. Transcutaneous delivery as has been accomplished for LT has the potential to help circumvent the toxicity issue for ST.

Buffers will be important for many vaccines which may be administered orally and studies to improve their utilization will be necessary. The true buffering requirements for infants in DCs should be determined and the composition of these buffers should be developed so as to insure that they resist the adverse effects of chlorinated water up to reasonable concentrations of chlorine. Research is needed to identify the best means for revitalizing dried bacteria and design of buffers to improve this process would be beneficial. Investigations should be carried out to determine the benefits of including micronutrients or other immune modulators in buffers.

Live ETEC and colicin-treated cells have been reported to protect against disease upon re-challenge of volunteers. Although not tested in a volunteer challenge, a formalin-killed ETEC vaccine did not seem to protect as well when used in travelers, which should represent a less severe challenge model. One explanation for this is that critical antigenic sites may have been affected by the inactivation process. The immunogenicity of ETEC cell "ghost" preparations could be evaluated in comparison to formalin-inactivated cells and, if promising, tested in subsequent challenge studies.

The lack of an easily accessible animal model has been a serious impediment to the development of vaccines against ETEC, and this problem is likely to persist for the immediate future. Passive protection of the infant mouse may be useful, although a model for active immunity would be desired. The fact that *Aotus* monkeys develop disease upon challenge with sufficient numbers of ETEC raises the hope that this model can be adapted to demonstrate protection. The possibility of using mouse pulmonary or intestinal clearance as a model for vaccine efficacy also should be investigated further.

Active immunization studies in animals should be used in conjunction with clinical trials to develop possible correlates of protection against disease.

Some attempts to make an ETEC vaccine have used *Shigella*-ETEC hybrids, which are consistent with the concept of making a combined pathogen vaccine. Research needs to continue to demonstrate effective attenuation of the *Shigella* component without loss of immunologic effectiveness. Other hybrid-type ETEC vaccines may be sufficiently attenuated (e.g., CVD 103-HgR) but the immunogenicity of these constructs needs to be evaluated. Further, it must be determined whether expression of the relatively conserved ETEC antigens on a heterologous organism are sufficient for protection.

Even with relatively low numbers of live cells, it will be necessary to gain new insight on the possible risk of Reiter's syndrome if *Shigella* or other organisms associated with this condition are used to deliver ETEC antigens.

Research is needed to determine if 2 or 3 doses of an attenuated vaccine may permit effective immunization of children in DCs with equal or lesser amounts of cells/dose than would be needed with a single dose approach. The number of cells/dose may also be reduced for attenuated organisms if they can eventually be combined with a safe mucosal adjuvant.

Safe and effective mucosal adjuvants could be of benefit to a number of possible vaccine candidates. Work to establish the safety, particularly of the enterotoxin-based adjuvants, is necessary as well as research to define the optimal ways in which adjuvants or combinations of adjuvants might be used. The effectiveness of various routes and regimens for administration in children living in DCs needs to be studied.

Research to identify the optimal formulations for an enteric vaccine for use in infants and children in DCs is needed. For example, if a buffered vaccine is to be given orally, it must be packaged to be both efficacious and simple to administer. Further it should be relatively stable without complex refrigeration requirements. Advances in drying techniques should be studied for their applicability to formulation of enteric vaccines in DCs. While a variety of formulation possibilities are already being developed, a focused effort is needed to optimally apply them to the enteric vaccine needs of children living in DCs.

Although ETEC is a major cause of diarrhoeal disease, it is also one of the most difficult enteric pathogens to recognize, so its contribution to disease may be under appreciated. Thus simple and rapid diagnostic methods should be established for ETEC.

Since clinical trials will be a key component of future vaccine evaluation, the human challenge model needs to be standardized. Previous experience leads to the conclusion that challenge doses of ETEC should not exceed the vaccine dose such that protective immunity is overwhelmed. Mid 10^8 challenge doses have been successfully used before and this dose may possibly be lowered even further if volunteers are screened for pre-existing immunity. Optimizing the buffer system or food vehicle for delivering the challenge dose may also permit use of lower numbers of organisms. While these steps may lead to more sensitive challenge models, they will still be using doses larger than those which might be encountered in natural infections.

7.2. Critical steps

7.2.1. Establishment of an ETEC vaccine program

The most likely chance for successful development of an ETEC vaccine for use in DCs is through establishment of a focused and funded program to do this. A product development partnership model for ETEC vaccine development, as described above, can take several forms. Although differing in their structure, these efforts have strong committed funding to address the problem of DC's and could be adapted to meet the needs of an ETEC Vaccine Program. A recent decision by the Bill and Melinda Gates Foundation to invest in this area of research should provide the funding necessary for such an enterprise.

7.2.2. Identification of appropriate study sites

As 30–40% of child deaths from diarrhoea occur in sub-Saharan Africa, clinical trials for enteric vaccines should be conducted in this region, in addition to Asia and Latin America. A review of what has been learnt concerning important antigens at different geographic sites and immune responses associated with protection as well as what is available at potential sites on different continents would be beneficial towards making sure that the information and infrastructure necessary for an effective ETEC vaccine program is available when needed. In conjunction with this, efforts should begin to centralize reagents and diagnostic services needed for meaningful studies. The reference center approach may improve the quality and comparability of data from trial sites.

7.2.3. Identification of more effective immunization strategies

Mucosal immunization of children in DCs presents a significant challenge even if protective vaccines are available. A review of this particular problem should address not only technical approaches that might work, but also using these approaches in formulations and regimens that are realistic for use in infants in DCs. Health officials from DCs could play an active role here to ensure that immunization approaches are not only scientifically sound, but would meet the sociological, logistical and economic requirements of DCs.

7.2.4. Identification of ETEC vaccine candidates with high potential for near-term development

This white paper constitutes a first but important step in this direction. Although well over a dozen approaches have been pursued in the past 20 years (Table 2), it is possible at this

Table 4
Identification of ETEC vaccine candidates with high potential for near-term development for use in infants

	Presence of toxin and CF antigens in current formulation	Safety and immuno-genicity in Phase 1	Practical and acceptable formulation for infants	Active protection in challenge studies	Readily produced in DC	Potential low costs	Likely compatibility with EPI vaccines	Initiation of clinical trials leading to go-no go decision possible within 2–3 years
Downstream ETEC vaccine candidates								
1. Inactivated ETEC	Yes	Yes, adults and children	Yes	Yes in adults; no in children	Yes	Yes	Yes	Yes
2. Attenuated ETEC	Yes[a]	Yes, in adults	Yes	ND[b]	Yes	Yes	Yes	Yes
4. CVD 1208S Shigella hybrid	Yes	ND	Yes	ND	Yes	Yes	Yes	Yes
5. CVD 103-HgR *Vibrio cholerae*-ETEC hybrid	Yes	ND[c]	Yes	ND	Yes	Yes	Yes	Yes
6. Peru-15 cholera-ETEC hybrid	BS only	ND[c]	Yes	ND	Yes	Yes	Yes	Yes
7. *S. typhi*-ETEC hybrid	BS only	Yes, in adults	Yes	ND	Yes	Yes	Yes	Yes
8. Inactivated Shigella-ETEC hybrid	CF only	ND[c]	Yes	ND	Yes	Yes	Yes	Yes
Upstream ETEC vaccine candidates								
1. LT patch for TCI	Toxin only	Yes, in adults	Unknown[d]	+/−	No?	No	Yes	Yes
2. Plant derived LT	Toxin only	Yes, in adults	Yes	ND	No	Yes	Yes	No
2. Vectored ST constructs	Toxin only	ND	Yes	ND	Yes	Yes	Yes	No
3. DNA/vectored vaccine	CF only	ND	Yes	ND	Yes?	No	Yes	No
4. Fimbrial tip adhesins	CF only	ND	Unknown[e]	ND?	Unknown[e]	Unknown[e]	Unknown[e]	No
5. TC CS6	CF only	Yes, in adults	Unknown[d]	ND	No?	No	Yes	No
6. CF in PLGA microsphere	CF only	Yes, in adults	Yes	No	No?	No	Yes	No
7. Conjugated toxins	Toxin only	ND	Yes	ND	Yes	Yes?	Yes	No

[a] Only candidate currently formulated with all CF and toxin antigens thought to be necessary.
[b] ND designation used because previous challenge study may have used too high a challenge dose relative to the immunization dose.
[c] The vector organism alone has been found safe in children.
[d] The LT patch is practical and safe in adults, but a regimen for its safe and effective application to infants has not been established.
[e] The formulation for these antigens is not yet defined.

Table 5
Target profile for ETEC vaccines

	Target description	Target rationale	Minimum acceptable profile
Target population(s)	Infants <6 months Travelers/military	High disease burden from 6 months High incidence in group; easier target; ready market	Protection starting at 6 months; Work in infants alone Not relevant
Indication(s)	Protects against all diarrhea/dehydration by ETEC	ETEC is a major cause of infantile diarrhea in developing countries	Protects against severe diarrhea caused by vaccine types
Formulation, route, schedule	Single oral dose; cold chain independent; with EPI; palatable liquid in single dose disposable dispenser	To fit with logistic and programmatic use in developing countries	3 doses <6 months; booster at 9 months; 2–8 °C storage; lyophilized
Expected efficacy	.90% of all ETEC diarrhea; 2 year protection	Least morbidity and most lives saved	50% protection against severe disease due to ETEC in less developed country during the first 2 years of life
Storage	Ambient temperature up to 40 °C	Operational aspects; Transport to end user	4–8 °C
Shelf life	Ideally 3–5 years	Flexibility on supply and distribution	2 years
Nature and contents of container	Single dose, squeezable disposable dispenser; liquid	Ease of delivery and storage; safety of device	Multi-dose; Lyophilized and resuspended
Target customers	MOH; international donors; UNICEF vaccine fund; private market; military	Largest possible market	International donor community
Regulatory risk/issues	FDA/EMEA approval; NRA positive assessment; WHO pre-qualification; GMO acceptability	Highest standard; maximum market commitment	WHO pre-qualification; NRA assessment or Article 58; GMO acceptability
Warnings and precautions/pregnancy and lactation	No contra-indication for pregnancy; safe in immunocompromised individuals; no rare AE safety concerns identified	Ease of administration and implementation	Contra-indication in pregnancy acceptable; caution in HIV patients acceptable; minor attributable DVF reaction

time to use past experience to narrow down the field of near-term possibilities for priority evaluation. This down-selection of the approaches listed in Table 4 is based on:

a. Strong rationale for the antigens in the candidate vaccine to be expressed by most prevalent clinical strains.
b. Safety and immunogenicity established in Phase 1 trials in adults or infants or are likely based on the nature of the vaccine.
c. Potential for practical and acceptable formulations for administration to infants in DCs.
d. Demonstrated protection in challenge studies.
e. Feasibility of production in a developing country.
f. Potential for low product and distribution costs.
g. Probable compatibility with other vaccines.
h. Probability of initiation of clinical testing in the next 2–3 years leading to go-no go decisions regarding further clinical evaluation of the product.

Of note in Table 4 is that those vaccines thought to be possible near-term candidates for use in infants are those delivered by attenuated or inactive microorganisms while those thought to be more upstream generally involve acellular fimbrial or toxin preparations. Also, if CFs are needed for a complete vaccine that would be useful in infants in DCs, then many of the near-term candidates will require addition of further antigens once their utility is demonstrated in early clinical trials. Finally, some candidates designated "Upstream", such as the LT patch and the fimbrial tip adhesins, could move reasonably quickly into the "Downstream" category once the formulations and delivery regimens for infants are resolved.

Although the criteria used to down-select vaccine candidates are useful for decisions regarding moving products into clinical trials, it will be necessary that the subsequent trials substantiate the criteria used for their selection and also show that the vaccine candidate fits a target profile for an ETEC vaccine to meet the needs of infants and children. Product target profile criteria are presented in Table 5. These were drafted by the ad hoc committee through discussions held in Baltimore, MD, in June 2006. All of the approaches identified above as near-term candidates have the potential to meet the target profile for an ETEC vaccine. The most critical profile factor that must be met is a minimal requirement for protection against severe disease in the paediatric population.

8. Concluding remarks

The approaches listed above lend encouragement that safe and effective vaccines against ETEC are feasible and some may be selectable for advanced development. Although much of the development work requires mainly scientific and technical input, this effort will only be successful if the input of the health care providers in DCs is also harnessed from the beginning of this endeavor. Their understanding of the needs of the population as well as the realities of the environment will be critical to insuring that the most appropriate products for children in DCs are developed. They will also be essential to building the public acceptance necessary for integration of new enteric vaccines into a public health program.

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