Approaches to Enhance and Evaluate the Immunogenicity of an Oral ETEC Vaccine

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The more I read, the more I acquire, the more certain I am that I know nothing.

Voltaire

ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of childhood diarrhoea in the developing world and the most common cause of travellers' diarrhoea. A new oral multivalent ETEC vaccine (MEV) containing killed recombinant *E. coli* bacteria expressing increased levels of the most prevalent ETEC colonisation factors (CFs), i.e. CFA/I, CS3, CS5 and CS6, and the toxoid LCTB*A*, a hybrid between the binding subunits of ETEC heat labile toxin (LTB) and cholera toxin (CTB), has been developed at the University of Gothenburg. The main aim of this thesis was to analyse immune responses induced by MEV and related vaccines in humans, and to evaluate different approaches to enhance and measure such responses.

The safety and immunogenicity of two oral doses of a prototype of MEV, containing CFA/I over-expressing *E. coli* bacteria and LCTB*A,* were evaluated in a Phase I trial in adult Swedish volunteers. The vaccine was safe and induced significant faecal secretory IgA and intestine-derived antibody-secreting cell (ASC) IgA responses in peripheral blood against CFA/I and LTB, as well as IL-17A and IFNγ T cell responses to LTB. However, detailed studies of the kinetics of ASC responses induced by an oral inactivated model vaccine, the CTB-containing cholera vaccine Dukoral®, indicated that peak ASC responses may have been missed in the prototype ETEC vaccine trial assessing ASC responses 7 days after each vaccine dose. Thus, whereas CTB-specific ASC responses to Dukoral® peaked around 9 days after the first dose, ASC responses to a second or late booster dose (given 6 months - 14 years later) peaked already on day 4-5. The distinct kinetics of ASC responses to primary and booster vaccinations suggests that early peak ASC responses may indicate the presence of mucosal B cell memory.

In preparation for testing MEV with the mucosal adjuvant double-mutant LT (dmLT), we evaluated the effect of dmLT on human T cell responses *in vitro*. dmLT enhanced both IL-17A and IFNγ responses to LTB in cells from ETEC vaccinees and IL-17A responses to mycobacterial antigens in cells from BCG vaccinees; this effect was dependent on IL-1β and IL-23 and could be mediated via monocytes.

We also studied the functional characteristics of the antibody responses induced by MEV. Two oral doses administered ± dmLT to adult Swedish volunteers, as well as a single booster dose administered 13-23 months later, induced cross-reactive mucosal antibody responses to multiple related, non-vaccine CFs. Using a novel assay, we showed that the avidity of both mucosal and serum antibodies to key vaccine antigens increased in response to the late booster dose.

Collectively, our results indicate that MEV can induce mucosal antibodies with the potential to protect against a broad range of ETEC strains. Our demonstration that dmLT can enhance T cell responses indicates that dmLT may promote B cell differentiation and memory development. Our studies of the kinetics of ASC responses have indicated optimal sampling time points for performing such analyses and established a method for memory assessment. These results are important for continued clinical evaluation of the new ETEC vaccine.

Key words: ETEC, vaccine, adjuvant, human, mucosa, antibody, cross-reactivity, avidity, T cell, immunological memory

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Clinical trial to evaluate safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* prototype vaccine containing CFA/I overexpressing bacteria and recombinantly produced LTB/CTB hybrid protein.**
Lundgren A, Leach S, Tobias J, Carlin N, Gustafsson B, Jertborn M, Bourgeois L, Walker R, Holmgren J, Svennerholm AM.
Vaccine. 2013 Feb 6; 31(8):1163-70.

II. **Different kinetics of circulating antibody-secreting cell responses after primary and booster oral immunizations: a tool for assessing immunological memory.**
Leach S, Lundgren A, Svennerholm AM.
Vaccine. 2013 Jun 26; 31(30):3035-8.

III. **The adjuvant double mutant *Escherichia coli* heat labile toxin enhances IL-17A production in human T cells specific for bacterial vaccine antigens.**
Leach S, Clements JD, Kaim J, Lundgren A.
PLoS One. 2012;7(12):e51718.

IV. **Cross-reactivity and avidity of antibody responses induced by an oral, multivalent enterotoxigenic *Escherichia coli* (ETEC) vaccine.**
Leach S, Lundgren A, Carlin N, Löfstrand M, Svennerholm AM.
In manuscript.

CONTENT

ABBREVIATIONS

AE	Adverse Event
AI	Avidity Index
ALS	Antibody in Lymphocyte Supernatant
APC	Antigen-Presenting Cell
ASC	Antibody-Secreting Cell
BCG	Bacillus Calmette-Guérin
CF	Colonisation Factor
CFU	Colony Forming Unit
CT	Cholera Toxin
CTB	Cholera Toxin B-subunit
DC	Dendritic Cell
DALY	Disability-Adjusted Life Years
dmLT	Double-Mutant LT
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunospot
EPI	Expanded Programme on Immunisation
ETEC	Enterotoxigenic *Escherichia coli*
FDC	Follicular Dendritic Cell
GALT	Gut-Associated Lymphoid Tissue
KSCN	Potassium Thiocynate
LPS	Lipopolysaccharide
LT	Heat-Labile Toxin
LTB	Heat-Labile Toxin B-subunit
MEV	Multivalent ETEC Vaccine
mLT	Mutant LT
PBMC	Peripheral Blood Mononuclear Cells
PHA	Phytohaemagglutinin
PPD	Purified Protein Derivative
PV	Prototype ETEC Vaccine
RV	Reference ETEC Vaccine
SIgA	Secretory IgA
ST	Heat-Stable Toxin
Tfh	Follicular Helper T Cell
Th	Helper T Cell
Treg	Regulatory T Cell
WCA	Whole Cell Pneumococcal Vaccine Antigen

INTRODUCTION

Diarrhoea is still a principal cause of child morbidity and mortality in developing countries. Diarrhoeal disease accounts for approximately one in ten of all child deaths, second only to pneumonia after the neonatal period (Fig 1) [1]. Though the global mortality rate for diarrhoeal disease in children under five years is declining [1], the disease incidence is decreasing more modestly [2]. Children with poor health and malnutrition are more vulnerable to serious diarrhoea, and often suffer multiple episodes per year [3]; at the same time, diarrhoea exacerbates poor health and malnutrition, creating a vicious cycle. Repeated diarrhoeal episodes early in life thus convey multiple burdens, including growth stunting and cognitive impairment [4].

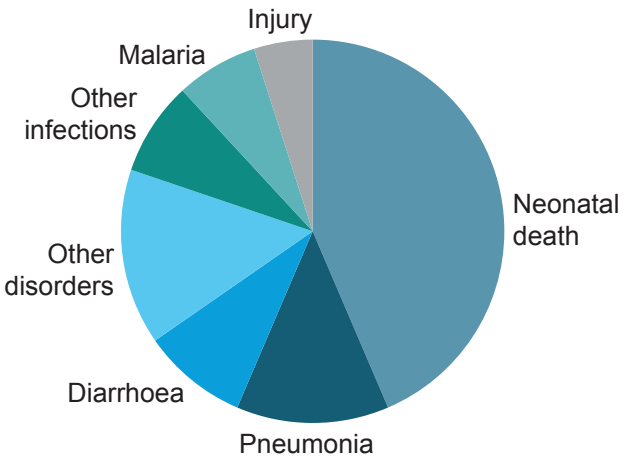

Figure 1. Global causes of death in children under 5 years of age in 2013, adapted from [1].

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhoea in the developing world, especially in children, as well as the most common cause of diarrhoea in travellers to these regions. The development of an ETEC vaccine is therefore considered an important goal in global health. A new oral ETEC vaccine has been developed at the University of Gothenburg in collaboration with Scandinavian Biopharma, Stockholm [5,6]. This vaccine contains killed recombinant *E. coli* bacteria expressing increased levels of the most prevalent ETEC colonisation factors (CFs) and the toxoid

LCTB*A*, a hybrid between the binding subunits of ETEC heat labile toxin (LTB) and cholera toxin (CTB). The main aim of this thesis was to analyse immune responses induced by a prototype and the complete multivalent formulation of this novel ETEC vaccine in human volunteers, and to evaluate approaches to enhance and measure these responses. The main features of ETEC infection, and properties of mucosal immune responses of relevance for protection against ETEC infection, are summarised below. Different approaches for developing and evaluating ETEC vaccines are also reviewed.

ETEC

Epidemiology

The incidence of ETEC disease is difficult to determine, due to lack of widespread diagnostic tools and the similarity of symptoms caused by ETEC and other enteric pathogens. Nevertheless, in the Global Burden of Disease study of 2010, ETEC was estimated to cause approximately 120 000 deaths in all age groups, 8% of all deaths due to diarrhoea that year [7]. Of these deaths, approximately 38 000 were in children under four years of age, with the peak incidence in children 28-364 days old, causing 1% of all deaths in that age group [7]. Apart from the considerable loss of life, ETEC is also a significant cause of global morbidity, estimated to account for 8% of all disability-adjusted life years (DALYs) caused by diarrhoeal disease in 2010 [8].

ETEC infection is particularly a burden in the developing world, and was recently found to be one of the top four causes of moderate-to-severe diarrhoea among children under five years of age in sub-Saharan Africa and South Asia (the other most common pathogens being rotavirus, *Cryptosporidium* and *Shigella* spp.) [3]. Studies from Egypt and Bangladesh show that mortality due to ETEC occurs predominantly in children under two years of age; multiple ETEC diarrhoeal episodes per year are also common in this age group [3,9,10]. Though ETEC mortality decreases with age, ETEC continues to impact health in later childhood and adulthood [11].

ETEC is also the most frequent cause of diarrhoea in travellers to Africa, Asia and Latin America. With an estimated 10 million ETEC episodes occurring in this population per year, this accounts for 20-60% of all travellers' diarrhoea reported in different studies [12,13].

Bacteriology

ETEC bacteria are a very diverse group of *E. coli*, which have the ability to colonise the small intestine via CFs and produce one or both of two enterotoxins: heat-labile toxin (LT) and heat-stable toxin (ST) (Fig 2). It was previously believed that ETEC bacteria were any *E. coli* lineage that could acquire, express and retain plasmids harbouring CFs and/or toxins. However, recent evidence from a whole genome sequencing study of a global ETEC strain collection indicates that ETEC consist of several identifiable stable lineages, with the majority containing consistent virulence profiles [14]. This implies that the plasmids encoding virulence factors were acquired once, and the strains then spread world-wide.

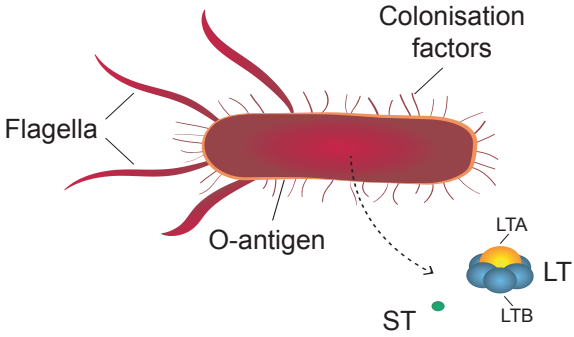

Figure 2. ETEC

Colonisation Factors

ETEC colonises the small intestine by binding to the epithelium via CFs. Over 25 CFs have been identified, of which CFA/I, CS1-7, CS14 and CS17 are the most common [15]. CFs are usually fimbrial or fibrillar in structure, consisting of hundreds of repeating copies of a major subunit with a minor subunit often located at the tip; they may also be nonfimbrial [15]. Both the major and minor subunits have been shown to be important in the binding of bacteria to the epithelium in different studies [16,17]. Individual ETEC isolates may express one, two or three different CFs; however, approximately 30% of all isolates do not express any known CF [18].

Though the exact nature of most of the epithelial receptors for CFs are not known, the binding structures for some of the most prevalent ones have been identified, e.g. CFA/I binds to Lewis blood group and related structures, and CS6 binds to sulphatide [19,20].

Many CFs are closely related and have highly similar major and minor subunits. Thus, CFs can be subdivided into families, e.g. the CFA/I-like family (including CFA/I, CS1, CS2, CS4, CS14 and CS17) and the CS5-like family (including CS5 and CS7) [15,16]. Cross-reactive epitopes have been found within these families, and antibodies induced by infection with CFA/I-expressing ETEC bacteria have been shown to cross-react with the related CS4 and CS14 antigens [21].

LT and ST Enterotoxins

The relative proportions of LT, ST and LT+ST-producing ETEC vary according to geographical area and between patients with ETEC diarrhoea and asymptomatic carriers. Generally, 60% of ETEC isolates express LT, either alone (~30%) or in combination with ST (~30%) [18].

LT is an A-B toxin, which is highly similar in amino acid sequence, 3D-structure and function to the toxin produced by *Vibrio cholerae*, cholera toxin (CT) [22]. The enzymatically active A-subunit (LTA, comprising two chains: LTA1 and LTA2, joined by a proteolytically sensitive peptide) is embedded in a circular LTB pentamer (Fig 2). LTB binds irreversibly to gut epithelial cells via the ganglioside GM1 and other glycoproteins [23,24]. Upon binding and internalisation, the toxic A-subunit dissociates and induces a series of cellular events, resulting in the irreversible activation of adenylate cyclase. This causes levels of intracellular cAMP to increase, which in turn causes an imbalance in electrolyte movement in the epithelial cell. Chloride efflux, together with decreased sodium uptake, causes an osmotic movement of water across the gut wall into the lumen, resulting in watery diarrhoea [22].

ST is a small, 18-19 amino acid peptide which binds reversibly to guanylate cyclase C, triggering an increase in the levels of intracellular cGMP. This causes a similar imbalance in electrolyte movement in the epithelial cells as LT, also resulting in watery diarrhoea [25].

Serotypes

The ETEC serotype is defined by the O-antigen associated with the cell wall lipopolysaccharides (LPS) and the H-antigen of the flagella. The O-antigen is the ETEC antigen with the largest variety, with at least 100 different O groups identified [26]. An extensive meta-analysis of ETEC strains from many countries on most continents found that O6, O78, O8, O128 and O153

are the most common O groups [26]. The H serogroups are considerably fewer than the O groups, though at least 34 have been described [27].

ETEC Infection

ETEC is transmitted via the faecal-oral route and may be contracted by ingesting contaminated food or water. Surface waters have been found to harbour ETEC, hence bathing and using contaminated water in food preparation may also transmit infection [28]. Indeed, ETEC is usually a major cause of diarrhoea in any region where sanitation is inadequate. A high infectious dose of around 10^6 - 10^8 colony forming units (CFUs) is required for infection in human challenge studies [29,30], though this may be lower in the very young, elderly or malnourished. According to these studies, the incubation period is about two days. Mixed infections are common and may be seen in up to 40% of cases; asymptomatic carriage is high in areas of poor sanitation [27].

ETEC bacteria attach to the epithelium via different CFs and do not invade the mucosa. Upon colonisation of the small intestinal epithelium, the bacteria proliferate and release LT and/or ST, causing sudden onset watery diarrhoea and often vomiting, allowing further spread of the bacteria in the environment. The disease severity may range from mild to very severe. ETEC diarrhoea is usually self-limiting, lasting 3-4 days. Micronutrient deficiency (including vitamin A and zinc) may increase the morbidity of diarrhoeal disease [27]. With adequate treatment, including oral rehydration therapy, zinc supplements and continued feeding, the mortality is usually very low [27,31].

Protective Immunity

Several studies have demonstrated that exposure to ETEC results in the development of protective immunity. Epidemiological studies show that the incidence of ETEC diarrhoeal disease in endemic areas decreases with age [10,32]; the incidence also decreases in visitors who reside sufficiently long in endemic areas [33]. Furthermore, experimental ETEC infection of human volunteers has shown that subjects are protected against disease when rechallenged with the same strain [29,30], and natural infection also confers protection against reinfection with homologous strains [9].

Induction of IgA antibodies in the intestinal mucosa is considered essential for the protection against ETEC [5], but less is known about how these responses are initiated and how innate immunity may contribute to protection. General mechanisms of adaptive immune responses relevant for protection against non-invasive pathogens such as ETEC are summarised below.

Mucosal Immune Responses in the Gut

The mucosal surface of the gastrointestinal system, the largest immunological organ in the body, must maintain immunological tolerance to the microbiota, whilst also recognising and eliminating the numerically extremely rare pathogens. Intestinal adaptive immune responses are initiated in organised collections of lymphocytes and antigen-presenting cells (APCs) in close proximity to the epithelium, collectively referred to as the gut-associated lymphoid tissues (GALT), as well as in the gut-draining lymph nodes. The GALT consists of Peyer's patches, found mainly in the distal ileum, and numerous isolated lymphoid follicles, which increase in density distally [34]. These GALT structures resemble those of lymphoid follicles in lymph nodes, but lack afferent lymphatics, as antigens are actively sampled directly from the mucosal surfaces. A summary of how the adaptive immune responses are induced in the intestinal mucosa is provided in Figure 3.

Gut Homing

Effector T and B cells generated in the GALT or the gut-draining lymph nodes are imprinted with gut-homing properties so that they circulate back to the lamina propria. Intestinal dendritic cells (DCs) and epithelial cells express RALDH, the enzyme required for retinoic acid biosynthesis from the dietary vitamin A [34]. Retinoic acid induces B and T cells to express the integrin α4β7 and the chemokine receptor CCR9 [34]. α4β7 binds to the MAdCAM-1 protein expressed on the endothelium in the gut lamina propria and Peyer's patches, and the chemokine ligand CCR9 (CCL25) is expressed by intestinal epithelial cells. Additionally, the chemokine CCL28, more generally expressed by mucosal epithelial cells, attracts IgA+ B cells expressing the chemokine receptor CCR10 [34]. Thus, effector lymphocytes generated in the GALT or draining lymph nodes can home back to mucosal sites.

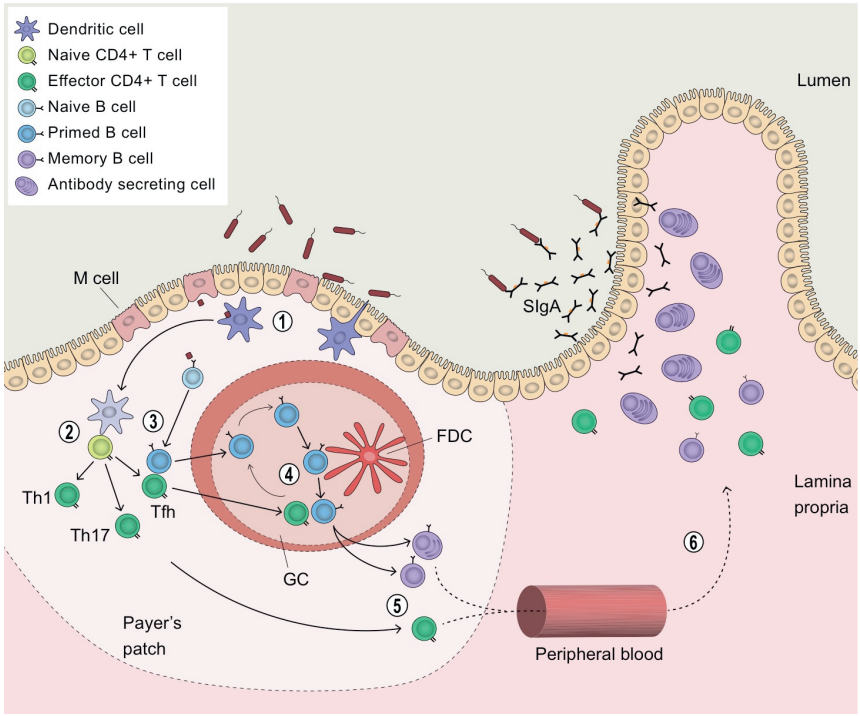

Figure 3. A simplified schematic of mucosal immune responses to non-invasive bacteria. ***1.*** *Peyer's patches are covered by a follicle-associated epithelium containing many M-cells, specialised for effective antigen transfer. Immature dendritic cells (DCs) below the M-cells take up and process antigens and migrate to the interfollicular T cell areas of the Peyer's patch.* ***2.*** *DCs present antigen to naïve T cells, causing them to differentiate into different effector T helper (Th) subsets, including Th1, Th17 and follicular Th (Tfh) cells; the exact factors controlling this polarisation in the human GALT are incompletely understood [35].* ***3.*** *Naïve B cells also take up, process and present antigen to cognate Tfh cells. This induces the B cells to initiate antibody class switching, which is heavily skewed towards IgA in mucosal immune responses, due to several factors including TGFβ, APRIL, BAFF, retinoic acid, as well as various combinations of Th cytokines produced by Tfh and presumably Th1 and Th17 cells [36]. The primed B cells will either enter existing germinal centres or form new ones together with follicular dendritic cells (FDCs) [36].* ***4.*** *The activated B cells will proliferate and undergo somatic hypermutation, during which random mutations in the antigen-binding variable regions of the antibodies are created [36]; the resulting B cell clones have surface antibodies of different affinities. These clones compete for antigen presented on the FDCs and for the limited Tfh help, which is provided via costimulation (e.g. CD40-CD40L and ICOS). Thus, B cell clones with high affinity antibodies will be positively selected [37].Tfh cells also secrete cytokines that promote growth and differentiation (e.g. IL-21, IL-4, IL-10 and TGFβ) [38].* ***5.*** *The surviving B cells may undergo further affinity maturation, or form memory B cells or antibody-secreting cells which may migrate from the GALT via draining lymph nodes with the effector T cells to the peripheral blood. During this stage, the antigen-specific B and T cells may be detected in the peripheral circulation.* ***6.*** *Effector cells migrate back to mucosal effector sites like the lamina propria; the effector B and T cell functions are described separately in the text.*

Humoral Immunity

The majority of plasma cells in the lamina propria produce IgA dimers, joined together by the concurrently produced J chain. These IgA dimers are transported across the epithelium into the lumen via the poly-Ig receptor, expressed by mucosal epithelial cells, which binds to a domain of the J chain, resulting in the transcytosis of the receptor-antibody complexes [34]. On the apical epithelial cell surface, the poly-Ig receptor is proteolytically cleaved so the extracellular domain carrying the IgA is released. The cleaved part of the poly-Ig receptor that remains bound to the IgA is called the secretory component and the complex is called secretory IgA (SIgA). The secretory component protects SIgA from proteolysis by enzymes in the lumen [34]. SIgA has several important antimicrobial effects, including inhibiting bacterial adhesion, neutralising viruses and blocking toxins [36].

T Cell Effector Functions

The majority of T cells in the lamina propria are CD4+ T helper (Th) cells and the most prevalent Th subsets in the intestinal mucosa are Th1, Th17 and regulatory T cells (Tregs) [39]. Th1 cells mainly produce IFNγ, which activates macrophages to kill intracellular microbes, promotes the cytotoxic activity of other cell types, enhances antigen presentation and stimulates the recruitment of other immune cells [40]. Th17 cells produce IL-17 and sometimes also IL-22, and many different cell types have receptors for these cytokines. Thus, IL-17 can promote fibroblasts and epithelial cells to secrete proinflammatory mediators, including IL-8, G-CSF and GM-CSF, which increase the production and release of neutrophils from the bone marrow and their recruitment to the tissue; IL-22 can induce the production of antimicrobial peptides and promote barrier function [41,42]. There is also evidence that Th17 cells may have important effects on humoral immunity. IL-17 can upregulate expression of the poly-Ig receptor on epithelial cells, thus increasing IgA secretion into the lumen [43], and intestinal Th17 cells appear to deviate towards a follicular Th (Tfh) phenotype in Peyer's patches, where they induce germinal centre reactions and the development of IgA responses [44]. Human Tfh cells displaying Th17 properties have also been shown to be potent inducers of IgA production in naïve B cells [45]. On the other hand, Tregs suppress immune responses via several mechanisms, including the production of inhibitory cytokines such as IL-10 and TGFβ, or by IL-2 scavenging [46].

Of note, there is considerable plasticity in the Th subsets, especially in humans: Tregs can convert to Th17 cells in the presence of IL-1β and IL-6, and Th17 cells can give rise to Th1-like cells and Tfh cells [44,47,48]. Tfh cells can also take on Th1, Th17 and Treg properties, including production of the effector cytokines distinguishing these cell subsets [37,38].

Protective Immune Responses to ETEC

Innate Responses

As ETEC bacteria do not invade the intestinal epithelium, ETEC is considered a noninflammatory infection. However, studies in epithelial tissue cultures have shown that IL-8 expression may increase in response to ETEC infection, especially with ST-producing strains [49]. Increased levels of lactoferrin, IL-8, IL-1β and IL-1 receptor antagonist were also found in faecal samples from travellers to India with ETEC infection, indicating a mild innate inflammatory response [50]. IL-8 has a central role in host defence by recruiting neutrophils, and faecal leukocytes were found to be increased in children with symptomatic ETEC infection and asymptomatic carriage [51]. Furthermore, increased faecal IL-8 levels in children with asymptomatic ETEC carriage was associated with reduced infection duration [52], indicating that IL-8 may have a role in ETEC protection.

Humoral Responses

Humoral immune responses, particularly SIgA antibodies, are considered vital for the defence against non-invasive bacterial pathogens like ETEC. In patients infected with ETEC in endemic areas, humoral immune responses determined as specific IgA levels in mucosal samples (faeces and intestinal lavage fluid), antibody-secreting cells (ASCs) in duodenal biopsies and in the circulation, and serum antibodies, are usually induced against the CFs and O-antigen of the infecting strain, as well as against LT after infection with LT-producing ETEC [53-57]. Due to its small size, ST is non-immunogenic in its natural form and hence does not induce antibody responses after infection [25].

When studying the protective effects of antisera directed against different ETEC antigens in ligated small bowel loops in rabbits, anti-LPS, anti-CF and anti-LT antibodies all protected against challenge with bacteria expressing the corresponding antigens [58]. A synergistic effect was seen when anti-CF and anti-LT antibodies were combined. Using the reversible

intestinal tie adult rabbit diarrhoea model, it was shown that previous infection with a CFA/I-expressing ST+LT+ ETEC strain protected against reinfection with not only the homologous strain, but also against a CFA/I-expressing ST+LT+ ETEC strain with different O and H-antigens [59], indicating that anti-CF and anti-LT immune responses are of critical importance for protection.

Many studies have also demonstrated the importance of anti-CF responses in protective immunity against ETEC in humans. In challenge studies, subjects were protected against infection when rechallenged with CF-homologous, but not CF-heterologous, ETEC strains [30]. Also, the oral administration of anti-CFA/I antibodies derived from bovine milk (from cows repeatedly immunised with CFA/I) to adult volunteers, provided protection against challenge with a CFA/I+ ETEC strain [60]. Epidemiological data also support the development of protective immune responses against ETEC infections. In a cohort study of children from birth to 2 years of age in Bangladesh, no children had repeat episodes of diarrhoea with ETEC strains expressing CFA/I, CS1+CS3, CS2+CS3 or CS5+CS6, but had frequent episodes of diarrhoea with CF-heterologous strains [9]. Interestingly, even if the primary infection was asymptomatic, no repeat infections with CF-homologous strains were observed. Thus, anti-CF immunity clearly seems to afford protection against ETEC.

ETEC infection may also induce antibody responses against LT, mainly directed against LTB [5,55,56]. However, repeat infections with LT-producing ETEC are commonly seen in field studies [9,56]. The poor protection induced by natural infection with LT expressing strains may be explained by the relatively low amounts of LT produced during ETEC infection [5]. However, several studies have shown that the relatively high amounts of CTB (which cross-reacts immunologically with LTB) included in the oral cholera vaccine Dukoral®, can induce protective immunity against ETEC [61-63], which will be discussed in more detail later.

T Cell Responses

Very little is known about the cellular immune response to ETEC infection in humans and the potential role of T cells in protective immunity. Though T cells are unlikely to have a direct role in protection, Th cells are crucial for the induction of antibody responses and the establishment of B cell memory. Thus, induction of the Th17 and Tfh lineages would likely be advantageous for protective mucosal immune responses against ETEC. Of note, both CT

and LT have been shown to promote the induction of Th17 responses against different vaccine antigens in mice [64-66]. The possible role of IL-8 in protection against ETEC as described above [49,50] is also noteworthy considering the ability of Th17 cells to promote fibroblasts and epithelial cells to secrete IL-8.

Mucosal Vaccination

Although mucosal surfaces are the main route of entry for infectious pathogens, most licensed vaccines are injected by parenteral routes. There are comparatively few mucosal vaccines routinely administered to humans (against poliovirus, rotavirus, *V. cholerae*, *Salmonella* Typhi and influenza) and of the vaccines recommended by the WHO's Expanded Programme on Immunisation (EPI), only rotavirus and the oral polio vaccines are administered mucosally [67]. There are however many potential benefits of mucosal vs. injectable vaccines, including being easy and logistically simple to administer (hence not requiring trained healthcare professionals), being painless and also running less risk of transmitting infections [68]. Whilst there are parenteral vaccines that can induce protective immune responses against mucosal pathogens, this is mainly due to the invasive nature of the pathogens (e.g. poliovirus, *S.* Typhi) or the relative permeability of the mucosal tissue for serum-derived antibodies. Hence, to protect against non-invasive infections (e.g. ETEC and cholera) at intestinal mucosal surfaces, which are normally poorly accessible to serum antibodies, local mucosal vaccination is most likely advantageous.

When planning the optimal route of administration, consideration must be taken regarding the compartmentalised nature of mucosal immune responses. The strongest immune responses are generally obtained at the site of vaccination and anatomically adjoining sites [69]. For example, oral vaccination induces immune responses mainly in the upper digestive tract and salivary glands, and rectal vaccination induces responses in the large intestine [69].

Challenges of Mucosal Vaccination

There are several challenges in the development of oral vaccines. Unlike parenteral vaccines, where antigen is injected into a normally sterile site, mucosal vaccines must induce immune responses in an environment of commensal bacteria and a highly tolerogenic immune system. At the same

time, whilst there are several adjuvants licensed to enhance immune responses to parenteral immunisation, there is currently no licensed adjuvant for mucosal vaccines. Furthermore, many oral vaccines have been found to be less immunogenic in developing countries compared to when used in developed countries. A striking example of this hyporesponsiveness is the rotavirus vaccine RotaTeq®, which was found to have a protective efficacy of over 90% in high income countries [70,71], yet only 39-49% in low income countries [72,73]. Though the reasons for this underperformance are poorly understood, several factors have been proposed [68,74], including "tropical enteropathy", a subclinical inflammatory gut condition associated with villous blunting and impaired barrier function which may occur in people living in poor sanitary conditions. In addition, interference on vaccine take by maternal breast milk and/or placental antibodies, malnutrition and micronutrient deficiencies and concurrent parasitic infections have also been suggested as contributing factors, and great efforts are currently being made to investigate and try to overcome these obstacles. Encouraging results were seen in a study in Bangladeshi children receiving the oral cholera vaccine Dukoral®, where withholding breast feeding three hours prior to vaccination or zinc supplementation during the three weeks prior to vaccination significantly increased antibacterial antibody responses [75]. Of note, zinc treatment has also been shown to significantly increase IFNγ production of CTB-specific CD4+ T cells in children vaccinated with Dukoral® [76].

Another obstacle for the development and clinical evaluation of mucosal vaccines is the measurement of immunogenicity in human subjects, as described in detail below.

Measuring the Immunogenicity of Mucosal Vaccines in Humans

Serum antibody levels (often measured as enzyme-linked immunosorbent assay (ELISA) titres) are frequently used to assess vaccine immunogenicity in clinical trials, due to the convenience and rapidity of the assays. Indeed, for some parenteral vaccines, the actual quantity of specific IgG antibodies that provides near 100% protection is known, a so-called absolute correlate of protection [77]. However, serum antibodies are seldom a good reflection of mucosal immune responses. Even though analysis of the number of vaccine-specific IgA-secreting plasma cells in mucosal biopsies is likely the best

reflection of the immunogenicity of mucosal vaccination, this method is far too invasive and time-consuming to be used in clinical vaccine trials. As there are few described correlates of protection for mucosal vaccines, surrogate parameters are used, i.e. measurements of immunological responses that are indirectly related to the actual (often unknown) correlate of protection [77]. Three main surrogate parameters are often used to measure the immunogenicity of oral vaccines: serum antibodies (IgA and IgG), faecal antibodies (SIgA) and ASC responses in peripheral blood.

Serum IgA and IgG

Studies have shown that serum IgA and IgG against LT and CFs increase after both ETEC infection and vaccination [30,54,78]. Notably, the magnitudes of anti-CF serum antibody responses are usually lower after ETEC vaccination than after infection, and are much lower after vaccination in non-primed than in primed subjects [21,79,80]. In a study of Egyptian children, serum IgG reciprocal titres >76 against CFA/I were found to be a relative marker of protection against CFA/I-positive ETEC in children under 18 months of age, but not in older children [56]. In the same study, no corresponding serological marker for protection against LT-expressing bacteria was seen. However, it is still unclear how these serum IgG antibodies may be associated with the mucosal immune responses to ETEC infection or vaccination. Serum antibodies are therefore considered a marker of immunogenicity, but not necessarily a reflection of a protective mucosal immune response.

Antibody-Secreting Cells

The quantification of antigen-specific ASCs in peripheral blood after mucosal infection or vaccination is often considered to better reflect the mucosal immune response than analysis of serum antibodies. Primed B cells migrate from the inductive sites (GALT) and enter the circulation to home back and seed the intestinal mucosa (Fig 3). By assessing vaccine-specific ASCs after vaccination (traditionally 7 days after administration of each vaccine dose [81]), the vaccine-specific mucosal B cell responses can be estimated. Antigen-specific IgA ASC responses increase in blood after both ETEC and cholera vaccination and infection, and the large majority of ASCs induced by cholera vaccination have been shown to express the mucosal homing receptor integrin α4β7 [55,82-84]. The ASCs detected in blood

during early ETEC infection correlate with the number of ASCs detected in intestinal biopsy specimens one week later [55]. Vaccine-specific ASC responses after oral ETEC vaccination also reflect vaccine-specific SIgA levels in intestinal lavages [79]. Thus, ASC responses are considered relevant measurements of the local intestinal immune responses. Importantly, the transient nature of ASC responses in peripheral blood make these responses a more sensitive reflection of recent infection or vaccination compared to analysis of serum antibodies, which may remain elevated for a prolonged period (especially IgG) and mask recent immunological challenges.

ASC responses can either be measured by the enzyme-linked immunospot (ELISPOT) or the antibody in lymphocyte supernatant (ALS) assays, both of which are based on the detection of spontaneous secretion of antibodies by ASCs in culture. In the ELISPOT assay, the numbers of antigen-specific ASCs are determined by counting spots developed on a membrane [81], and in the ALS assay, the levels of antigen-specific antibodies spontaneously secreted by the ASCs *in vitro* are measured by ELISA [85,86]. Several studies have shown that results from the ALS assay closely correlate with results from the more traditional ELISPOT assay [85,87]. The ALS assay has many advantages over ELISPOT: it is much less laborious and as the supernatants can be stored and transported, the ALS samples can be analysed (and reanalysed) at convenience.

Faecal IgA Responses

Mucosal antibodies can also be measured directly in either intestinal lavages or extracts from faecal samples. Studies have shown that subjects respond with increased IgA antibody levels against both CFs and CTB in faecal samples after ETEC vaccination, and that these responses correlate well with corresponding IgA levels in lavage samples [79]. However, the sensitivity of the antibody analysis in faecal samples was slightly lower than that in intestinal lavage in this study. A correlation has also been observed between levels of faecal IgA against CFs and the number of CF-specific ASCs in the duodenum of patients after ETEC infection [55]. Although intestinal lavages have been considered the golden standard of assessing intestinal immune responses in humans, this method is labour intensive and unpleasant for the volunteers who have to drink two to four litres of isotonic salt solution on each sampling occasion. The comparative ease of collecting and analysing faecal samples makes this method more suitable in clinical vaccine studies.

Additional Parameters

Apart from the immune response parameters described above, there are other characteristics of mucosal immune responses which might be useful to assess when evaluating the immunogenicity of oral ETEC vaccines, and when comparing different administration regimes and mucosal adjuvants. As not all antibodies are functionally equal, it may be of value to characterise antibody responses beyond mere quantity. One such functional characteristic is antibody avidity, which has been shown to be important in protection against several different infections [88-91]. Avidity is defined as the overall strength of the multivalent interactions between antibodies and their antigens, which develops as B cells undergo the germinal centre reaction. Avidity can be measured using complex biospecific interaction analysis methods, such as surface plasmon resonance, or by simpler, indirect assays such as chaotropic ELISA assays [92-94]. In a chaotropic ELISA, the stability of antigen-antibody complexes is measured in the presence of chaotropic agents like urea, thiocynate or diethylamine, which disrupt the interactions that maintain the complexes (hydrophobic, electrostatic, hydrogen, van der Waals forces etc.). Low-avidity antibodies are more sensitive to the dissociating effect of the chaotrope than high-avidity antibodies, and therefore an avidity index can be calculated. Chaotropic ELISA analysis has been shown to be able to rank the avidities of monoclonal antibodies in the same order as biospecific interaction analysis [95].

Toxin-neutralisation capacity is another functional antibody characteristic, which is used as a primary immunological correlate of protection for many parenteral vaccines against toxin-producing pathogens, e.g. diphtheria and tetanus [96]. The ability of serum antibodies to neutralise LT can be measured using toxin sensitive cell lines (e.g. Y1-adrenal or CHO cells), thus enabling the quantification of anti-LT antibody function [97]. However, these assays are generally performed using serum samples, and their relevance for assessing mucosal immunity is currently unclear.

Although the induction of immunological memory is a cornerstone of vaccination, long-term memory is often not evaluated in clinical trials, due to the lack of simple methods for memory assessment, as well as time constraints. Previous studies of immune memory responses to mucosal vaccination in humans have primarily assessed circulating vaccine-specific memory B cells [98-100]. However, the relation between frequencies of

circulating memory cells and functional mucosal memory is not known at present.

Due to the central role of Th cells in promoting antibody responses and immunological memory, the characterisation of Th cell responses to mucosal vaccination could also be of use for vaccine assessment, especially when evaluating the effects of administering the vaccine together with mucosal adjuvants, which often have strong effects on T cell responses. It has previously been shown that the majority of subjects orally immunised with a first generation ETEC vaccine responded with vaccine-specific T cells [101]. These T cells were capable of producing IFNγ when cultured with the CFs included in the vaccine, indicating a Th1-response to ETEC vaccination. Considering recent advancements in the understanding of mucosal immune responses in humans, assessing the ability of mucosal vaccines to induce Th17 and Tfh responses would be of particular interest.

Vaccines Against ETEC

Cholera Vaccine

The oral cholera vaccine Dukoral® was developed at the University of Gothenburg, and consists of whole-cell formalin-killed *V. cholerae* bacteria + 1 mg recombinant CTB (referred to as rCTB-WC). Considering the extensive immunological cross-reactivity between CT and LT, this vaccine was tested for possible protective capacity against LT-producing ETEC via the rCTB component. Indeed, two doses of rCTB-WC were found to induce a 70% short-term protection (6 months) against LT-producing ETEC in Bangladesh [63]. Similarly, in studies of Finnish travellers to Morocco [61] and North American students travelling to Latin America [62], two doses of rCTB-WC have been shown to afford a significant 50-60% protection against travellers' diarrhoea caused by LT-producing ETEC.

The short-term protection provided by rCTB-WC against LT-producing ETEC has made its use as a travellers' vaccine plausible [12,102]. However, as rCTB-WC only protects against travellers' diarrhoea caused by LT+ ETEC strains, an ETEC vaccine inducing a broader and more long-term protection than rCTB-WC is needed, primarily in endemic, but also traveller populations.

The First Generation Killed Oral ETEC Vaccine

After extensive studies of the virulence factors and protective antigens of ETEC bacteria isolated world-wide, an ETEC vaccine was developed by Ann-Mari Svennerholm et al. at the University of Gothenburg [5]. The adopted vaccine approach was to use killed ETEC bacteria expressing the most common CFs in immunogenic form on the surface, combined with an LT toxoid component, to be given orally. The great variation in O and H-antigens in ETEC made these antigens less attractive vaccine targets, and whilst an ST component would be beneficial for protection, several attempts to make ST immunogenic and non-reactogenic have so far been unsuccessful [25,103,104]. The first generation ETEC vaccine contained 1 mg rCTB plus 10^{11} formalin-killed wild-type ETEC bacteria of five different strains expressing high amounts of the most prevalent CFs, namely CFA/I and CS1-5 (referred to as rCTB-CF ETEC). The vaccine was given in two oral doses in 150 ml of sodium bicarbonate solution. The rCTB-CF ETEC vaccine has been subjected to extensive clinical testing in Phase I-III trials in different parts of the world [5].

In several Phase I studies, rCTB-CF ETEC was found to be safe, and to give rise to mucosal immune responses against the different vaccine antigens in the majority of adult volunteers in Sweden, Egypt and Bangladesh [78-80,105]. In studies in endemic areas where subjects are most likely to be previously primed by natural infection (Egypt and Bangladesh), it was noted that immune responses (as measured by vaccine-specific ASCs in peripheral blood) were lower after the second than after the first vaccine dose [80,105]. Based on previous studies of oral live attenuated *S.* Typhi Ty21a vaccination [106], the hyporesponsiveness to the second dose was believed to be caused by the presence of neutralising antibodies in the gut mucosa. In the primed population, the first vaccine dose would serve as a "booster dose". By then giving a second dose during an active mucosal immune response, the presence of mucosal antibodies capable of neutralising vaccine antigens before uptake would result in lower ASC responses.

In descending-age Phase II trials, the rCTB-CF ETEC vaccine was found to be well-tolerated and safe in Egyptian and Bangladeshi children (ages 18 months - 12 years), and was also immunogenic in this age-group [80,107,108]. However, when rCTB-CF ETEC was tested in infants (6-17 months of age), the full dose of the vaccine induced vomiting and the study was terminated [109]. A tendency towards increased reactogenicity to the

vaccine was also seen in Egyptian children <12 months of age, compared to older children and adults [110]. Notably, there were no differences in vomiting between the groups receiving vaccine or *E. coli* K12 bacteria placebo in the Bangladeshi study [109], suggesting that this side-effect was either caused by the bicarbonate buffer in which the vaccine was given, or by Gram negative bacteria *per se* (which contain significant amounts of somatic antigens, including LPS, which may lead to gastrointestinal upset). In a subsequent Phase II dose-finding trial in Bangladeshi children (2-12 years and 6-17 months of age), a quarter of the full dose of rCTB-CF ETEC was found to be safe [109]. The quarter-dose of rCTB-CF ETEC induced similar responder frequencies to vaccine antigens as the full dose in older children and adults, though the magnitudes of responses were lower.

In a Phase III trial in North American travellers to Mexico or Guatemala [111], rCTB-CF ETEC provided a significant 77% protective efficacy against non-mild ETEC diarrhoeal disease (i.e. symptoms that interfered with daily activities or >5 loose stools per 24 hour period). However, no significant protection was seen against ETEC diarrhoea of all severities, i.e. including mild cases, in this study. In a further Phase III trial assessing the efficacy of rCTB-CF ETEC in Egyptian children (6-18 months of age), only a non-significant 20% protective efficacy was seen against mild/moderate disease [112]. There were mainly mild cases reported in this trial, which could in part have been due to the use of active surveillance, which often entails extensive reporting of mild disease. It has also been established that for many paediatric vaccines, e.g. against rotavirus, the protective efficacy against mild disease is considerably lower than against more severe diarrhoeal disease [113]. Nevertheless, immune responses, particularly against CFs, decreased with age and the youngest children in this study responded with comparatively lower serum antibody responses against CF antigens than the older children and adults in Egypt, as well as compared to adults in Sweden and the USA [5,114].

Collectively, these clinical trials showed that the rCTB-CF ETEC vaccine was safe and immunogenic, providing a 77% protective efficacy against non-mild ETEC diarrhoeal disease in the adult traveller population. However, no significant protection was seen in young children in an endemic setting, and the vaccine also appeared to be less immunogenic and more reactogenic in this key target population. Because of this, further efforts have

more recently been made to develop an improved inactivated oral ETEC vaccine.

The Second Generation Killed Oral ETEC Vaccine

A combination of three main approaches have been taken to increase the immunogenicity of rCTB-CF ETEC and thereby the potential to use reduced doses in infants: over-expression of CFs, inclusion of a novel hybrid LTB/CTB toxoid and administration together with a novel mucosal adjuvant. Based on this, a second generation ETEC vaccine has been developed at University of Gothenburg, in collaboration with Scandinavian Biopharma.

Over-Expression of CFs

E. coli strains were modified with recombinant technology to overexpress key ETEC CFs ≥3-10-fold [115]. Thus, the vaccine formulation would contain an increased amount of CF antigen per dose and hence create the possibility to reduce the dosage for infants, without losing immunogenicity. As CS6+ ETEC strains had been shown to be increasingly prevalent in clinical isolates [111,116], a CS6-overespressing strain was added to the vaccine formulation to potentially increase vaccine coverage [115,117]. In rCTB-CF ETEC, CS6 was expressed, but in low amounts. Also, the killing ETEC bacteria with formalin (as was done with rCTB-CF ETEC) resulted in a complete loss of detectable CS6 antigen, a problem solved by treating the CS6-expressing strain with phenol instead, thereby conserving CS6 in an immunogenic form [117].

Thus, the final formulation of the second generation multivalent ETEC vaccine (referred to as MEV) includes four formalin or phenol-inactivated recombinant *E. coli* strains expressing increased levels of CFA/I, CS3, CS5 and CS6.

LCTB*A*

The second approach to increase the immunogenicity of MEV was to include a more LT-like toxoid than rCTB. A CTB-LTB hybrid toxoid, LCTB*A*, had been developed at the University of Gothenburg with the intention that an increased similarity to LTB would induce antibodies which would provide better protection against ETEC [118]. In LCTB*A*, seven amino acids in a surface-exposed part of CTB have been replaced by the corresponding amino acids of LTB, making it immunologically more similar to LTB than CTB [118]. Indeed, serum from mice vaccinated intraperitoneally with LCTB*A*

had a higher LT-neutralising capacity when compared to serum from mice vaccinated with CTB [118].

dmLT

Thirdly, the possibility of administering the ETEC vaccine with an oral adjuvant was explored. As of yet, there is no licensed adjuvant for mucosal vaccines. Both CT and LT have potent mucosal adjuvant properties in mice, yet their toxicity prevents their use in humans [119]. As little as 5 μg of orally administered CT is sufficient to induce significant diarrhoea in humans, and 25 μg of LT can elicit up to 6 L of fluid secretion, seriously limiting the use of these enterotoxins as adjuvants in humans [119]. There have been several parallel attempts to develop mutants of CT and LT with lower enterotoxicity yet retained adjuvanticity, and more than 50 different mutants have been generated by site-directed mutagenesis [119].

John Clements et al. at Tulane University in the USA developed LT(R192G) (also called mutant LT or mLT) in which the arginine in position 192 is replaced with glycine (in the protease sensitive loop) in the enzymatically active A-subunit of LT [119]. This prevents cleavage of the A-subunit, disrupting the enzymatic and toxic activity of LT. mLT retains potent adjuvant activity in animal models and is well tolerated when administered orally by itself in doses of 2 – 50 μg to adult human subjects; however, 100 μg of mLT induced mild to moderate diarrhoea in approximately 15% of volunteers [120]. When mLT was tested in Phase I clinical trials in combination with oral inactivated *Helicobacter pylori* and *Campylobacter* vaccines, 15-20% of the volunteers experienced diarrhoea [121,122]. In an attempt to further detoxify mLT, an additional mutation was introduced at a putative pepsin-sensitive proteolytic cleavage site [123]. Indeed, the toxoid LT(R192G/L211A) (double-mutant LT or dmLT) exhibited a reduced toxicity compared to mLT, as measured by intestinal fluid accumulation in mice, yet the intracellular cAMP production induced by dmLT in an epithelial cell line was undiminished [123]. dmLT has also been shown to enhance immune responses to tetanus toxoid and whole cell mucosal vaccines against *Streptococcus pneumoniae* and *H. pylori* in mouse models [123-125]. Therefore, dmLT was planned to be tested together with the second generation ETEC vaccine.

Other ETEC Vaccine Approaches

There have been several parallel efforts to develop an effective ETEC vaccine. The candidate vaccines which have reached clinical testing are described briefly below.

Live Attenuated Vaccine

ACE527 is a live attenuated vaccine, composed of three ETEC strains collectively expressing CFA/I, CS1, CS2, CS3, CS5, CS6 and LTB, and genetically modified by having had the toxin genes removed and deletion mutations made in the *aroC*, *ompC* and *ompF* genes [126]. When two doses of ACE527 were given in two different dosages (either 10^{10} or 10^{11} CFUs) in a Phase I trial, the vaccine was found to be safe and immunogenic with significant immune responses (as measured by IgA in ALS specimens) against key antigens [127]. However, in a Phase IIb vaccination/challenge study, testing two doses of 10^{11} CFUs, only a non-significant, 27% protective efficacy against moderate/severe diarrhoea was seen [128]. Also, a significantly increased frequency of vomiting was seen in the vaccinees compared to the placebo group. Further clinical studies of this vaccine have been performed, but the results remain to be published.

LT Patch Vaccine

An intriguing approach to an ETEC vaccine, evaluated in extensive clinical studies, was to deliver native LT transcutaneously via a skin patch, aiming to cause antigen up-take by APCs in the skin [129]. Thus, anti-LT immune responses would be induced, but without the enterotoxicity associated with oral administration. In a Phase II challenge study, three doses of the LT patch, given at three-week intervals, were found to be safe and immunogenic, but failed to protect vaccinees against ETEC [129]. Yet, in a double-blinded, placebo-controlled Phase II trial in adult travellers to Mexico/Guatemala, two doses of the LT patch afforded significant protection against travellers' diarrhoea of any aetiology, but not against LT+ ETEC specifically [130]. However, in a large Phase III trial in adult travellers to Mexico/Guatemala, two doses of the LT patch provided some protection against LT+ ETEC, but failed to protect against travellers' diarrhoea caused by all ETEC (the primary endpoint of the study) or any other pathogen [131]. This vaccine is no longer under development.

CF Subunit Vaccines

As purified CFs are easily degraded by proteolytic enzymes in the gastrointestinal tract, they are less suitable as oral antigens. For example, when three or four doses of purified CS6, given alone or encapsulated in a biodegradable polymer, were given to subjects in a Phase I trial, anti-CS6 ASC and serum IgA responses were low, even when administered together with 2 μg mLT [132].

Another approach is to only use the tip adhesins of the CFs, administered parenterally. Although there is some debate over the relative contribution of the major and minor (tip) subunits of CFs to epithelial binding and therefore pathogenicity, there is evidence that for the CFA/I group of fimbrial antigens, the minor subunit (called CfaE) is the adhesin [16]. CfaE is also highly similar within the CFA/I group [16]. Thus, by inducing antibodies against CfaE, the bacterial adhesion of all CFA/I-expressing strains to intestinal epithelium may be blocked. Clinical trials have been performed where CfaE was injected intradermally, either alone or as a chimera fused with the A2 domain of CT and non-covalently complexed with LTB, in combination with mLT [133]. A Phase II challenge study testing this vaccine has recently been performed, but the results from this trial have not yet been presented.

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Among the different ETEC vaccine candidates, the second generation oral killed ETEC vaccine developed at the University of Gothenburg is currently considered to be the most advanced. The studies included in this thesis describe several important steps in the clinical evaluation process of this second generation ETEC vaccine, including a clinical Phase I trial of a prototype ETEC vaccine, assessment of cross-reactivity and avidity of antibodies induced by a complete multivalent ETEC vaccine, as well as methodological studies using an oral cholera vaccine. For clarity, results from all Phase I clinical trials of the multivalent ETEC vaccines performed so far are also summarised in this thesis.

AIMS

The overall aim of this thesis was to analyse mucosal immune responses induced by novel oral inactivated ETEC vaccines in human volunteers and to evaluate different approaches to enhance and measure such responses.

The specific aims were:

• To evaluate whether a prototype second generation ETEC vaccine was safe and immunogenic in adult Swedish volunteers.

• To establish and optimise assays for evaluation of functional aspects of immune responses to mucosal vaccination in humans, including assessment of antibody avidity, antibody cross-reactivity and immunological memory.

• To study T cell responses to ETEC vaccination and to evaluate whether the novel mucosal adjuvant dmLT can influence T cell responses to ETEC as well as model vaccine antigens *in vitro*.

• To analyse if a multivalent ETEC vaccine induces cross-reactive and high avidity antibodies, and if dmLT influences avidity development.

MATERIALS AND METHODS

Vaccines (Papers I-IV)

In this thesis, the immune responses in humans to several different vaccines were analysed (Fig 4). In papers I and III, a prototype second generation oral ETEC vaccine and a reference vaccine to the first generation ETEC vaccine were studied. In paper III, immune responses to the previously administered parenteral Bacillus Calmette-Guérin (BCG) vaccine against *Mycobacterium tuberculosis* were also studied. In paper II, the oral cholera vaccine Dukoral® was used, and in paper IV, samples from two Phase I trials of the complete second generation oral ETEC vaccine MEV were analysed.

Oral ETEC or cholera vaccines were administered in 150 ml of sodium bicarbonate solution, in a two-dose regime with a two-week interval; subjects were not allowed to eat 1 hour before or after vaccination. In papers II and IV, a single oral booster dose of either vaccine was also administered to previously vaccinated subjects. The BCG vaccine is given intradermally in one dose, usually shortly after birth, in adolescence or later if an adult is considered at high-risk (e.g. during medical training).

ETEC Vaccines

Reference vaccine (RV): killed, whole cell vaccine, containing $3x10^{10}$ ETEC bacteria expressing CFA/I + 1 mg rCTB. This vaccine was comparable to the CFA/I expressing strain in the 1st generation ETEC vaccine.

Prototype ETEC vaccine (PV): killed, whole cell vaccine, containing (at 1x dosage) $3x10^{10}$ recombinant *E. coli* bacteria expressing increased levels of CFA/I + 1 mg LCTB*A*, given at either 1x or 4x dosage.

Multivalent ETEC vaccine (MEV, commercial name ETVAX): killed, whole cell vaccine, containing four recombinant *E. coli* strains ($2x10^{10}$ bacteria per strain) expressing increased levels of CFA/I, CS3, CS5 and CS6 + 1 mg LCTB*A*, given alone, or with 10 or 25 μg dmLT.

All vaccine strains were constructed by researchers the University of Gothenburg in collaboration with Scandinavian Biopharma, Stockholm [5,115,134]. The vaccines were produced by Unitech Biopharma, Sweden.

Cholera Vaccine

Dukoral®: killed, whole cell vaccine, containing 1.25 x 10^{11} *V. cholerae* bacteria + 1 mg rCTB. The licensed vaccine is produced by Crucell.

Volunteers (Papers I-IV)

All subjects included in this thesis (n=179, median age 29 years, range 19-62, 56% females) were healthy, adult volunteers recruited from the Gothenburg region (Table 1).

Table 1. Subject demographics

		Paper				
Characteristics		**I**	**II**	**III**	**IV**	**Total**
Immune status at recruitment		ETEC naïve	Cholera naïve or vaccinated with Dukoral®	ETEC naïve or vaccinated with BCG	ETEC naïve or vaccinated with MEV ± dmLT	
Total n		59	18	26[a]	76	179
Sex	**Male**	29 (49%)	6 (33%)	7 (27%)	36 (47%)	78 (44%)
	Female	30 (51%)	12 (61%)	19 (73%)	40 (53%)	101 (56%)
Age	**Median**	27	33	30	24	29
	Range	19-46	21-62	19-58	20-43	19-62

[a] Plus 20 subjects vaccinated with RV or PVx1 in Paper I

In paper I, 72 subjects were screened for eligibility of which 60 were included in the study. The included subjects were established as healthy by medical history and examination, with clinical chemistry and haematology testing. Subjects who had received the cholera vaccine Dukoral® during the last five years or travelled to ETEC-endemic countries within the last two years were excluded. Additionally, subjects who were pregnant, breast-feeding, immunised with any other vaccine or taking immunomodulating drugs less than four weeks prior to and during study participation, or had gastroenteritis within two weeks prior to study participation, were also excluded.

In paper II, nine subjects not previously vaccinated with Dukoral® or exposed to cholera or ETEC (i.e. never travelled to a country where these infections are endemic), and nine subjects who had previously received at least two doses of Dukoral® six months to 14 years prior, were included.

In paper III, 39 BCG-vaccinated volunteers were screened for *in vitro* responses to tuberculin purified protein derivative (PPD). Of these, 26 subjects with a ≥ two-fold increase in IL-17A production in peripheral blood mononuclear cells (PBMCs) stimulated with PPD compared to non-stimulated cells were included in the study. Samples from an additional 20 subjects vaccinated with PVx1 or RV described in paper I were also used in paper III.

In paper IV, samples from 76 subjects included in a Phase I clinical trial of MEV were used [135]. These subjects were established as healthy as in paper I, with similar exclusion criteria. In this study, ETEC-naivety was established by excluding subjects who had previously received cholera or ETEC vaccines, travelled to ETEC-endemic countries within the last three years, or been brought up in, or resided for more than two months during the last 10 years, in such areas. Additionally, samples from 34 subjects included in a follow-up study of MEV, in which a single oral dose of MEV was given 13-23 months after primary vaccinations to a randomly selected subgroup of subjects from the initial vaccination study [Lundgren et al., submitted], were also used.

All studies were approved by the Ethical Review Board for Human Research of the Gothenburg Region and written informed consent was obtained from each volunteer before participation. The clinical ETEC vaccine trials were also approved by the Swedish Medical Product Agency.

Study Design and Endpoints of the Phase I Clinical Trial (Paper I)

The clinical trial comparing PV and RV was a three-armed, randomised, double-blind, comparator-controlled Phase I trial. The primary safety endpoint was that the adverse events (AEs) caused by PV should be non-severe in nature and not significantly exceed those of RV. The primary immunogenicity endpoint was that intestinal or intestine-derived IgA immune responses (i.e. ASC responses) against CFA/I and/or LTB should be significantly higher following immunisation with PV, at any of the two dosage levels tested, than after immunisation with RV.

The study was performed at the Department of Microbiology and Immunology at the University of Gothenburg between the 12th of May and the 15th of December 2010, and all clinical work and immunological analyses were performed by employees at the department. The study was monitored

by Gothia Forum at the Sahlgrenska University Hospital, Gothenburg, Sweden.

Safety Procedures (Paper I)

The volunteers underwent physical medical examinations at screening and the last follow up visit (day 42-49). Clinical chemistry (electrolytes, kidney and liver function tests) and haematology testing (complete blood count) was performed at screening and one week after each vaccine dose.

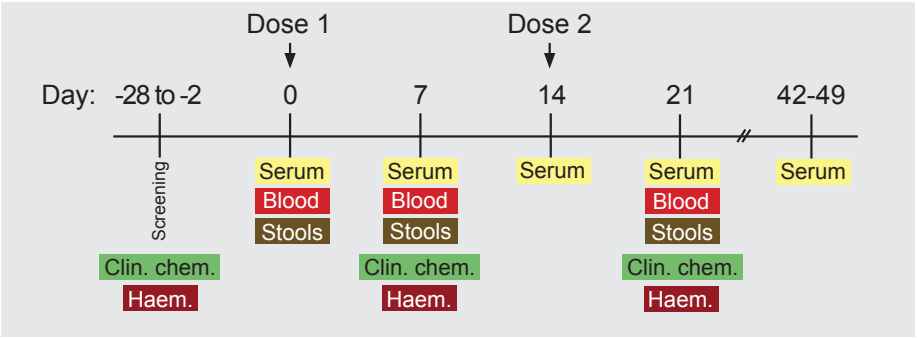
The volunteers recorded all AEs and medications in diaries daily during the seven days after each vaccination, and thereafter if and when they occurred until the last follow-up visit. Solicited AEs (i.e. AEs actively asked for) were gastrointestinal symptoms such as abdominal cramps, nausea, vomiting, diarrhoea/loose stools and fever. An independent safety committee evaluated all safety data collected between days 0 and 21 in each vaccine cohort as satisfactory and benign, in order for immunisation of the next cohort to be allowed to proceed. For the first dosing cohorts where PVx1 or PVx4 were given, each subject was observed for at least 60 minutes after each immunisation before the next subject was allowed to be vaccinated and a maximum of two subjects were vaccinated per day. The vaccine cohorts were organised in such a way that the 4x dosage of PV was only administered in the later cohorts. This dose escalation strategy ensured that no serious or severe AEs were seen at the 1x dose of PV before the 4x dosage was administered.

Sample Collection (Papers I-IV)

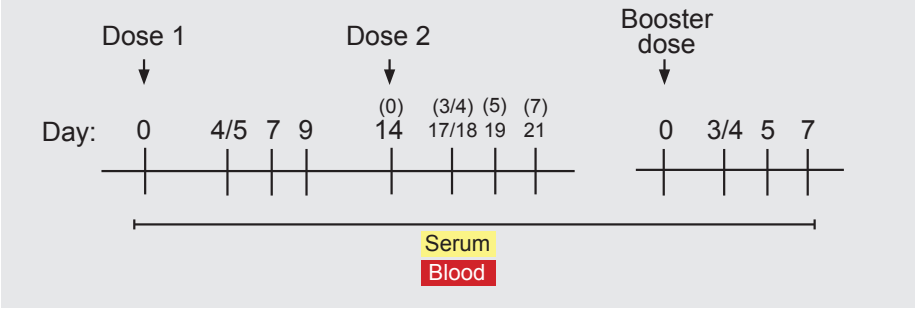
In the clinical trial of PV (paper I), serum samples were collected on days 0 (before administration of the first vaccine dose), 7, 14, 21 and 42 (Fig 4A). Heparinised blood, for isolation of PBMCs, and stool samples were collected on days 0, 7 and 21. Stool samples were immediately frozen at -20^{o}C by the volunteers. Blood was also sampled at screening and on days 7 and 21 for clinical chemistry and haematology testing. PBMCs from a subgroup of subjects from this trial were also used in paper III.

To determine the kinetics of the immune responses to cholera vaccination (paper II), heparinised blood and serum were collected pre-vaccination (day 0) and on days 4/5, 7, 9 and 14 after the first dose, and on days 3/4, 5 and 7 after the second or late booster dose (Fig 4B).

A Paper I and III: PV and RV (ETEC Vaccines)



B Paper II: Dukoral® (Cholera Vaccine)



C Paper IV: MEV ± dmLT (ETEC Vaccine)

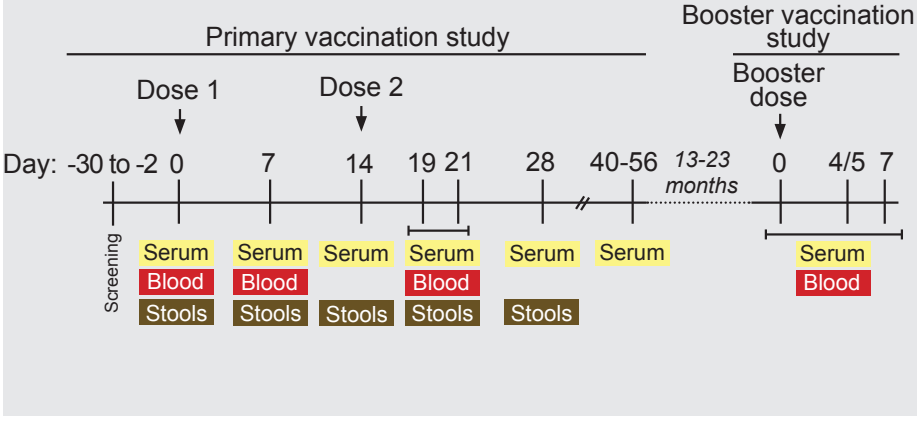


Figure 4. Vaccination and sampling time points.

To analyse the *in vitro* effect of dmLT (paper III), heparinised blood was collected from subjects previously vaccinated with BCG for isolation of PBMCs.

To analyse the cross-reactivity and avidity of antibodies induced by MEV (paper IV), samples from two clinical trials of MEV were used (Fig 4C). In the primary vaccination trial [135], serum samples were collected on days 0, 7, 14, 19, 21, 28 and 40-56. Heparinised blood was collected on days 0, 7, 19 and 21, and stool samples were collected on days 0, 7, 14, 19, 21 and 28. In the booster vaccination trial [Lundgren et al., submitted], serum and heparinised blood were collected on days 0, 4/5 and 7.

Determination of ASC Responses (Papers I, II and IV)

As previously described, antigen-specific ASCs induced by mucosal vaccination can be detected when they are recirculating in the blood from the inductive sites in the mucosa. To detect ASCs, PBMCs were immediately isolated from heparinised blood by gradient centrifugation on Ficoll-Paque and the ASC responses were determined using two methods: ALS and ELISPOT.

In the ALS assay, PBMCs were cultured for 72 hours, after which the supernatants were collected and centrifuged as previously described [87]. Protease inhibitors were added and the supernatants stored at -70^{o}C until analysis of specific antibodies using ELISA.

In the ELISPOT assay, the numbers of antigen-specific ASCs were determined as described [136]. Briefly, 96-well ELISPOT plates were coated with 10 µg/ml purified CFA/I, or GM1 followed by 2.5–5 µg/ml LTB or CTB and the PBMCs were incubated on these plates overnight. Antibodies secreted by individual ASCs were detected as spots on the culture well membrane bottom, using anti-human IgA-HRP or IgG-AP and enzyme chromogen substrates, and the numbers of antigen-specific ASCs per total PBMCs were determined.

Measurement of Antibody Levels in Serum, ALS and Faecal Samples (Papers I, II and IV)

Serum was immediately separated from blood by centrifugation and stored at -20^{o}C until analysis. For analysis of faecal samples, antibodies were extracted [79] and stored at -70^{o}C until analysis. Specific IgA and IgG antibody

responses to CFs, LTB and CTB in ALS, faecal and serum samples were measured by ELISA, as described [78]. Briefly, LTB and CTB-specific antibodies were analysed by coating low-binding plates with GM1 followed by 0.5-1 μg/ml LTB or CTB; CF-specific antibodies were analysed by coating high-binding plates with 0.3-1 μg/ml CFA/I, CS1, CS5, CS7, CS14 or CS17. Samples were added in serial dilutions and anti-human IgA- or IgG-HRP was added after incubation. The plates were developed using H_2O_2-OPD and read in a spectrophotometer. Endpoint antibody titres were determined as the reciprocal interpolated dilutions giving an absorbance of 0.2 above the background at 450 nm (mucosal samples) or 0.4 above the background at 490 nm (sera). The faecal antibody levels were determined as the antigen-specific SIgA titre (Paper I), or the antigen-specific SIgA titre divided by the total SIgA concentration of each sample (Paper IV) [79]. The magnitudes of immune responses (fold rises) were calculated as the post-immunisation divided by pre-immunisation antibody levels.

Determination of Antibody Avidity (Paper IV)

Antibody avidity was measured using two methods: potassium thiocynate (KSCN) elution and a limiting antigen dilution assay.

The KSCN elution assay is based on a traditional ELISA, with the addition of a KSCN wash, as previously described [93,94]. KSCN is a mild chaotropic agent which dissociates low-avidity antibodies. Serum samples were incubated on GM1+LTB coated microtitre plates at a 1:100 dilution, after which two-fold dilutions of KSCN, ranging from 7.5 M to 0.117 M, were added. The antibody avidity index (AI) was defined as the concentration of KSCN needed to displace 50% of bound antibodies.

The limiting antigen dilution assay is based on how effectively antibodies bind to decreasing levels of immobilised antigen in an ELISA. Using microtitre plates coated with two different concentrations of antigen, serum or ALS samples were incubated in serial dilutions. The AI was defined as the ratio of antibody titres at these two antigen concentrations (AI= titre at lower coating concentration / titre at higher coating concentration).

Determination of Toxin Neutralisation (Paper IV)

LT toxin neutralisation titres were determined using the Y1 adrenal cell assay, as previously described [97]. Briefly, serum samples mixed with trypsin-treated LT were added to flat bottom microtitre plates with Y1 cells

cultured to confluence. The neutralisation titre was defined as the reciprocal serum dilution at which <50% of the adrenal cells were affected by toxin (seen by microscopy as a rounding of the cells).

Determination of T Cell Responses (Paper III)

PBMCs were isolated from heparinised blood immediately after collection. For experiments using specific cell populations (CD4+ T cells and CD14+ monocytes), positive isolation and negative depletions were performed using immunomagnetic beads. The purity of the cell fractions were confirmed by flow cytometric analysis.

PBMCs were stimulated with PPD (SSI, Denmark), the whole cell pneumococcal vaccine antigen WCA (kindly provided by R. Malley, USA [125]), LTB or phytohaemagglutinin (PHA). Additionally, increasing concentrations of the toxins/toxin derivatives CT, LT, mLT, dmLT and LTB were added (kindly provided by J. Clements, USA). Supernatants were collected after 72 (PHA stimulation) or 120 (antigen stimulation) hours and stored at -70^{o}C until analysis of IL-17A, IL-13 and IFNγ, using commercial ELISA kits (eBioscience and R&D, USA).

For cytokine neutralisation, antibodies against IL-1β, IL-6, IL-23, as well as isotype controls, were added to the cell cultures. To assess the effect of dmLT on APCs, CD14+ monocytes were stimulated with antigen ± dmLT for 24 hours. After washing, autologous CD4+ T cells were added and supernatants were collected after 120 hours of co-culture.

Cell proliferation was determined by measuring incorporation of radioactive thymidine.

Statistical Analyses

Antibody titres (log-transformed) were evaluated using *t*-tests with Bonferroni correction. Antibody avidity, neutralisation titres and cytokine responses were evaluated using the Wilcoxon signed rank test, Mann-Whitney test or Friedman test with Dunn's multiple comparison post test, as applicable.

RESULTS AND DISCUSSION

Safety and Immunogenicity of PV (Paper I)

The first Phase I trial of the second generation ETEC vaccine aimed to evaluate the safety and immunogenicity of a prototype of the complete vaccine, containing 3 x 10^{10} CFA/I-overexpressing *E. coli* bacteria plus 1 mg LCTB*A* (at x1 dosage), given in two oral doses at a two-week interval. This was done by comparing the prototype vaccine (PV) at two different dosages (x1 and x4) with the first generation ETEC vaccine strain expressing CFA/I (reference vaccine, RV). Since the complete multivalent second generation ETEC vaccine (MEV) would consist of four CF over-expressing *E. coli* strains, the x4 dosage of PV (i.e. 1.2 x 10^{11} bacteria) was included in the trial to mimic the bacterial content of the complete vaccine.

As this was a comparator-controlled study, where the potential superiority of the CF over-expressing bacteria over the wild-type ETEC strains was studied, no placebo was included. Since the complete first generation ETEC vaccine had undergone extensive safety testing in both adults and children, using both bicarbonate buffer alone [78] and heat-killed *E. coli* K12 bacteria [107,109,136] as placebo, testing the safety of PV against RV was considered satisfactory. Nevertheless, due to the fact that two new pharmaceutical products were to be tested in the PV trial (the CFA/I over-expressing bacteria and LCTB*A*) the safety procedures required by the regulatory authorities were stringent, as described in the methods section.

Safety Analyses

Subjects reported AEs in diaries during the whole trial period (day 0-42). The subjects self-assessed the AE intensity as mild (does not interfere with normal activities), moderate (uncomfortable but not hazardous, may interfere but not prevent normal activities) or severe (incapacitating or hazardous). These diaries were then monitored by the investigators at each visit. The relationship of the AEs to vaccination was judged by the study physician as probably, possibly or unlikely to be associated with vaccination, based on knowledge of reactions of a similar nature previously observed with related vaccines and the temporal association (within 72 hours) with vaccination [111].

As expected, based on previous studies with active surveillance via study diaries, a relatively high proportion of subjects reported at least one AE (78%). However, of the total 119 AEs reported, the vast majority were mild (77%) or moderate (19%). Most of the AEs were deemed not associated with vaccination (72%), such as back pain and nasopharyngitis, and the 4% of AEs classified as severe belonged to this group. None of the AEs were serious (defined as life-threatening, or resulting in a considerable disruption to the performance of normal living functions, a congenital defect, hospitalisation or death), no AEs occurred during the 60 minute observation period directly after vaccination, and no volunteers displayed any discomfort or changes in vital parameters during this observation time.

Of the 33 AEs (28% of all reported AEs) which were considered possibly or probably associated with vaccination, 82% were of mild intensity, 18% were of moderate intensity and none were severe. The majority of these AEs were of gastrointestinal origin, such as nausea, loose stools or abdominal pain (Table 2). No volunteer had diarrhoea during the trial (defined as at least three loose stools within a 24-hour period), and only one subject vomited after having received the second dose of PV at the x4 dosage. No significant differences in frequency or severity of AEs were seen between the different study groups. The few deviations in clinical chemistry and haematology seen were not deemed clinically relevant.

Table 2. Number of subjects reporting solicited AEs deemed to have a possible or probable relation to vaccination.

Adverse event	RV (n=20)	PVx1 (n=20)	PVx4 (n=19)
Nausea	3 (15%)[a]	2 (10%)	4 (21%)
Vomiting	0	0	1 (5%)
Abdominal pain	1 (5%)	1 (5%)	1 (5%)
Loose stools	3 (15%)	1 (5%)	1 (5%)
Diarrhoea	0	0	0

[a] Number (%) of subjects who reported the specified AE during the trial.

Thus, RV and PV were both very well tolerated with a good safety profile, and the primary safety endpoint of the trial was met. The AE pattern was similar to that observed in several previous clinical trials of the first generation ETEC vaccine [78,80,105]. As AEs recorded in those trials

occurred at a similar rate in the groups receiving vaccine or placebo (either bicarbonate buffer alone or killed *E. coli* K12 bacteria), the gastrointestinal symptoms seen in this trial are likely to have been at least partially caused by the bicarbonate buffer in which the vaccine was administered. The few mild gastrointestinal AEs seen after vaccination with PV are also put into perspective when compared to the AEs reported after vaccination with the live attenuated ETEC vaccine ACE527, where 39% of volunteers in the vaccine group reported diarrhoea or loose stools and 19% reported vomiting [128].

Immunogenicity Analyses

Three parameters were used to study vaccine immunogenicity in this trial: serum antibodies (IgA and IgG), faecal antibodies (SIgA) and ASC responses in peripheral blood (ALS IgA).

Responses to LCTB*A*

All immunisations (RV, PVx1 and PVx4) induced significantly increased levels of serum and ALS IgA and faecal SIgA antibodies against LTB.

When comparing the different vaccines, immunisation with RV resulted in significant serum IgA responses to LTB in 80% of subjects, and PV in 95%. The strongest serum IgA responses were seen on day 21 and the post vaccination titres that day were significantly higher in subjects vaccinated with PVx1 (two-fold) and PVx4 (three-fold) compared to subjects vaccinated with RV (Fig 5A). IgA serum responses had started to decline on day 42 after all immunisations. Similar trends were seen for anti-LTB serum IgG responses, but the IgG titres remained high on day 42.

Significant anti-LTB IgA responses in ALS samples were observed in 95-100% of subjects in all vaccine groups. Responses to both RV and PV increased substantially already after one dose (day 7), and increased further after the second dose on day 21 (Fig 5B). Although the magnitudes of anti-LTB ALS responses were on average two-fold higher in subjects immunised with PVx1 or PVx4 compared to RV, there were no significant differences in either magnitudes or frequencies of responders in ALS responses to LTB among the groups.

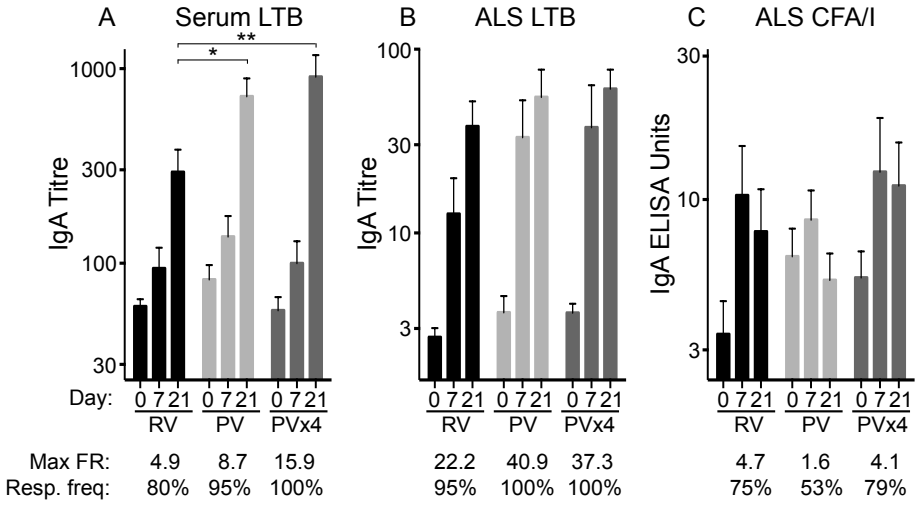


Figure 5. Antibody responses in serum and ALS samples after vaccination with reference vaccine (RV), or prototype vaccine (PV) at two different dosages. *Subjects were given 2 vaccine doses, on days 0 and 14. IgA antibody levels (GM + SEM) against LTB in serum (A) and ALS samples (B), and against CFA/I in ALS samples (C) were analysed before immunisation (day 0) and on days 7 and 21 after the first immunisation. Maximum GM fold rises (Max FR) and cumulative responder frequencies are indicated. Statistical analyses were performed using an unpaired t-test with Bonferroni correction. *P<0.05, **P<0.01.*

Significant anti-LTB IgA responses in faecal extracts were observed in 47% and 63% of subjects immunised with RV or PVx1. Although the anti-LTB SIgA responder frequencies and magnitudes of responses in subjects vaccinated with PV were slightly higher, these responses did not differ significantly to responses in subjects vaccinated with RV. There were however significantly higher and more frequent (95%) SIgA responses in subject vaccinated with PVx4 compared to RV.

LCTB*A* was as effective as CTB at inducing anti-CTB responses in serum, ALS and faecal specimens. Thus, the antibody responses to LCTB*A* were highly cross-reactive to CTB and LTB, and could potentially provide considerable cross-protection against both LT and CT.

Collectively, our results show that LCTB*A* was a safe and a potent immunogen. LCTB*A* induced higher anti-LTB serum IgA responses than a corresponding dose of CTB and was equal to CTB at inducing anti-CTB responses. Therefore, the attempt to make a more LTB-like toxoid was deemed successful. Based on these data, it was decided that LCTB*A* would be included in the complete second generation ETEC vaccine.

Responses to CFA/I

As expected, the anti-CFA/I serum responses to all immunisations were low, consistent with results from other vaccine trials in adults in non-endemic countries of both the first generation ETEC vaccine [78], as well as anti-CF serum responses to the live attenuated vaccine [127]. Thus, vaccination with RV resulted in serum IgA responses to CFA/I in 25% of subjects, PVx1 in 11% and PVx4 in 21%; there were no significant differences among the groups.

In ALS samples, significant anti-CFA/I IgA responses were detected in 75% of subjects vaccinated with RV, 53% of subjects vaccinated with PVx1 and 79% of subjects vaccinated with PVx4. Responses to both RV and PV at both dosages were highest on day 7, and actually decreased slightly after the second vaccine dose on day 21 (Fig 5C). Comparable results were obtained using ALS and ELISPOT assays, demonstrating that the relatively low responses to the second vaccine dose were not a result of the method used. These results were consistent with lower anti-CF ASC responses seen after the second dose of the first generation ETEC vaccine [82,136]. There were no significant differences in anti-CFA/I IgA ALS responses between the different vaccine groups.

When analysing faecal extracts, the frequency of subjects who responded with significantly increased anti-CFA/I SIgA to PVx1 was slightly higher (56%) than those who responded to RV (40%). The 79% responder frequency observed after vaccination with PVx4 was significantly higher than after vaccination with RV and the magnitude of the SIgA responses to PVx4 were also significantly higher than to RV. The significantly stronger responses observed after vaccination with PVx4 compared to RV supported the notion that the mucosal CFA/I responses were dose-dependent.

Collectively, these analyses showed that the new CFA/I over-expressing vaccine strain + LCTB*A* induced significant IgA responses to LTB and CFA/I at both dosage levels tested and that PVx4 induced significantly higher faecal SIgA responses to LTB and CFA/I than RV. The primary immunogenicity endpoint was therefore met. Based on these results, preparations for a second Phase I trial of the complete, multivalent ETEC vaccine including LCTB*A* and four CF over-expressing strains, were initiated. However, although analysis of faecal and serum samples showed that the responses to CFA/I were clearly dose-dependent and there was a

tendency for increased responder frequency in faecal samples collected from subjects immunised with PV compared to RV, there were no significant differences in responses to CFA/I induced by comparable doses of PV and RV. We speculated that this may have been at least partly due to the methodology used for analysis. The low responses detected after the second vaccine dose in ALS samples indicated that we may have missed peak responses. Furthermore, although previous studies have shown good correlations between analysis of vaccine-specific antibody responses in faecal and lavage specimens, faecal analyses may be slightly less sensitive than lavage analyses [79], and a few faecal specimens also needed to be excluded from the analyses due to too low total SIgA levels. Thus, although the data from the trial clearly demonstrated the immunogenicity of the new vaccine components, further methodological studies, particularly of ALS kinetics, were needed in order to prepare for the next clinical trial. It was also deemed important to include more sampling time points for collection of faecal specimens in order to obtain samples of good quality from all volunteers. Efforts were also initiated to evaluate if the ALS method could be used for assessing mucosal memory responses induced by oral vaccination.

Kinetics of Immune Response to Oral Cholera Vaccination (Paper II)

Humoral memory comprises two lines of defence – a constitutive memory composed of pre-existing antibodies secreted by long-lived plasma cells, and a reactive memory composed of memory B cells which may rapidly be reactivated to produce protective antibodies [137]. The constitutive memory is easily quantified by measuring antibodies in serum, though measuring antibodies in mucosal samples is often more technically demanding. The reactive memory is however much more difficult to measure, but nevertheless very important when evaluating vaccine candidates. In parallel with the clinical development of the second generation ETEC vaccine, our group had an interest in establishing methods to analyse memory responses to oral vaccination in humans. Tengvall et al. had shown that when PBMCs from subjects vaccinated with Dukoral® were stimulated *in vitro* with CpG-DNA, BAFF and IL-15, memory IgA B cells differentiated into IgA-secreting cells, allowing quantification of circulating vaccine-specific memory B cells by ELISPOT [138]. However, this and other methods for measuring immune

memory responses to vaccination are complex and not well-suited for clinical trials, and the relationship between circulating memory B cells and mucosal memory remains unclear. Therefore, we sought to develop a simpler assay with the capacity to differentiate between mucosal primary and recall responses.

The rapid development of anamnestic immune responses to booster vaccination in primed individuals, compared with primary immune responses, is a hallmark of immune memory. A few previous studies of both oral *S.* Typhi and cholera vaccination had shown that recall ASC responses to vaccination (as measured by ELISPOT) came earlier than after primary vaccination [139,140]. However, these findings have not been considered when selecting sampling time points in many previous trials of oral vaccines, as it has been the general belief that peak ASC responses are obtained seven days after oral vaccination, irrespective of prior exposure [81].

To determine if ASC responses to vaccination could be used as a simple measurement of functional mucosal memory, and to get a better understanding of the kinetics of ASC responses to primary and secondary vaccination to select optimal sampling time points for coming trials, we comprehensively studied the kinetics of ASC responses, using the ALS assay, after primary and booster oral vaccination. For comparison, serum responses were also analysed.

Immune Responses to Primary and Booster Cholera Vaccination

Since all human studies of unregistered pharmaceutical products must be done within the confines of a clinical trial (requiring stringent safety monitoring and extensive documentation), it would have been highly impractical and expensive to study the kinetics of immune responses to oral vaccination using the ETEC vaccines in development. Instead, we used the licensed cholera vaccine Dukoral® as a model oral vaccine for these studies, since the CTB component of Dukoral® is very similar to the LCTB*A* toxoid in the second generation ETEC vaccines. Two groups of subjects were recruited in this study: 1) a naïve group who had not been vaccinated against cholera or ETEC or ever travelled to a country where these infections are endemic; these subjects were given two oral doses of Dukoral® in bicarbonate buffer at a two-week interval, and 2) a booster group who had previously received both primary vaccinations and 0-4 more booster doses

(average two doses), either six months to four years prior (early booster group) or 9-14 years prior (late booster group); these subjects were given one oral booster dose of Dukoral®.

When analysing serum anti-CTB IgA responses to Dukoral® in the naïve group, the IgA levels increased marginally after the first dose, peaking at a mean two-fold increase on day 9 compared to pre-vaccination levels (Fig 6A). After the second dose, the serum IgA responses continued to steadily rise to reach highest levels on day 21 (the last day of testing) with a 14-fold titre increase. After a single booster dose was given to the booster group (both early and late), the serum IgA levels rose rapidly and high response levels were reached already on day 7, with a six-fold mean increase (Fig 6B).

The ALS responses against CTB were much more transient than the serum responses. In the naïve group, the anti-CTB IgA levels in ALS samples increased marginally after the first dose, peaking at a three-fold increase on day 9 (Fig 6C). After the second dose, the ALS responses increased sharply, peaking on day 18/19 (day 4/5 after the second dose) with a 43-fold increase compared to pre-vaccination levels. Importantly, the responses then fell rapidly, to only 11-fold increased responses on day 21 compared to prevaccination levels. This clearly shows that ASC responses are better measured on 4/5 days rather than 7 days after the second vaccine dose.

After a single booster dose was given to the booster group (both early and late), the peak ALS IgA responses to CTB were reached already on day 5 with a 120-fold increase compared to pre-vaccination and rapidly declined to only 27-fold on day 7 (Fig 6D). Thus, this response pattern clearly differentiates the booster group from the naïve group, supporting that ALS kinetics can be used for evaluation of mucosal B cell memory. The ALS responses to a membrane protein preparation of *V. cholerae* followed a similar pattern: the responses to primary vaccination were low, but a rapid increase in anti-MP IgA on day 5 was observed after vaccination of the booster group, and the responses decreased again by day 7. This confirmed that the differing response pattern after primary and booster vaccination was not restricted to the CTB antigen, but also observed for cellular bacterial antigens.

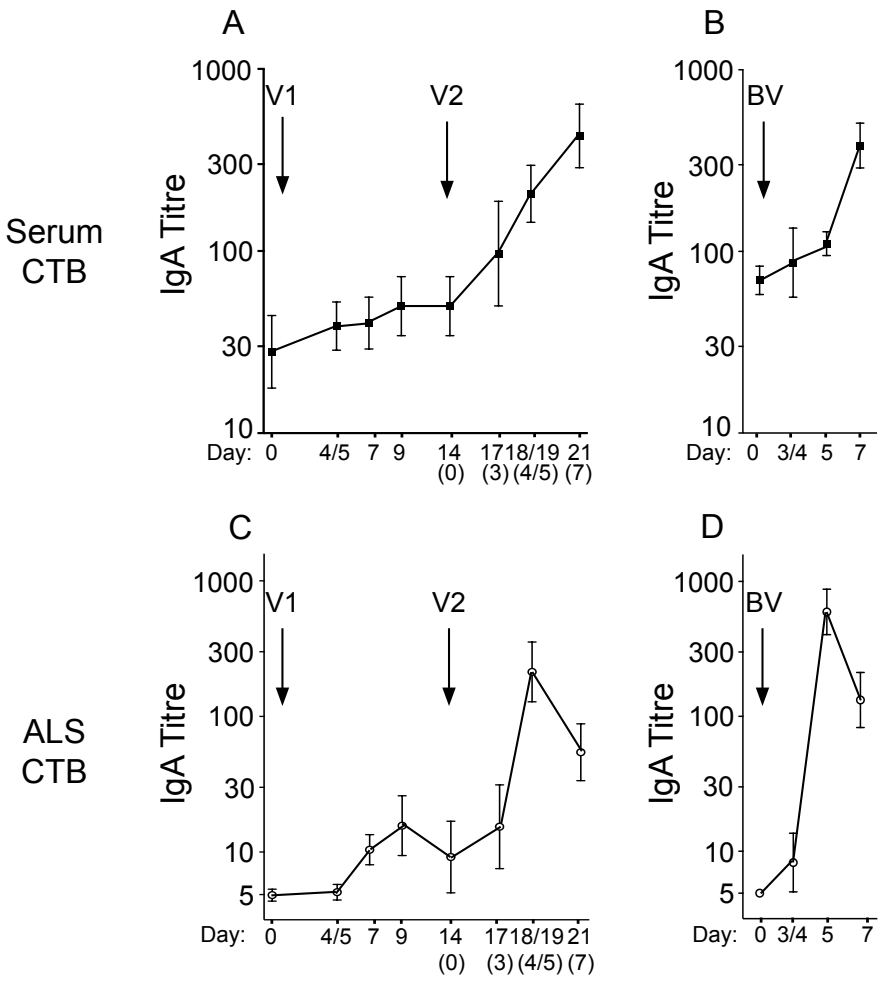


Figure 6. Kinetics of antibody responses to primary and booster oral cholera vaccination. *CTB-specific IgA titres (GM+SEM) were analysed in serum (A and B) and ALS (C and D) samples. (A and C) Naïve volunteers were vaccinated with two doses of oral cholera vaccine at a two week interval (V1 and V2), and IgA titres were analysed before the first immunisation (day 0) and at the different time points indicated after the first dose (numbers within parentheses indicate day after the second dose). (B and D) Volunteers previously immunised with ≥2 doses oral cholera vaccine, six months to 14 years prior, were given a single booster dose (BV), and IgA titres were analysed before the booster vaccination (day 0) and at the time points indicated.*

To be able to compare our results with previous studies, where the ELISPOT assay had been used to evaluate ASC responses, we compared the kinetics of the ASC responses to CTB using the ALS and ELISPOT assays. The same pattern of peak responses on day 4/5 after the second vaccine dose, with a sharp decline thereafter, was seen using both methods. This demonstrated that the differing kinetics of ASC responses to primary and booster vaccination were not dependent on the analysis method used.

Taken together, our results from this study showed that ASC responses to oral vaccination increased much more quickly and peaked earlier after the second vaccine dose than after the first. This was in marked contrast to serum responses, which steadily rose after each vaccine dose. Quicker ASC kinetics to a single booster dose could be also be observed in subjects who had received primary vaccinations up to 14 years prior, indicating very long-lasting mucosal memory, though we cannot exclude that CTB-responses may have been boosted by exposure to ETEC or cholera during the intermediate time. The detection of peak ASC responses 4-5 days after a single oral vaccine dose in previously primed, but not in naïve subjects suggested that analysis of ALS kinetics could be used as a simple method to differentiate primary and recall mucosal responses to vaccination. Based on the results, a study investigating the capacity of MEV to induce mucosal memory using these methods was later performed.

These results also had important implications for the interpretation of our ALS results from the clinical trial of PV (paper I). That trial, as well as previous studies of the first generation ETEC vaccine in Swedish adults, had shown a decrease in anti-CF ASC responses after the second compared to the first vaccine dose [82,136]. In fact, in later trials of the first generation vaccine in children and adults in endemic areas, this effect was even more pronounced [80,105,107,108]. The differing kinetics of primary and recall ASC responses seen after oral cholera vaccination in this study suggest that in the clinical trial of PV, as well as the earlier trials of the first generation ETEC vaccine, the ASC responses had been drastically underestimated after the second vaccine dose as a result of belated sampling. Hence, the results comparing the effect of over-expressing CFA/I in PV to the wild-type strain in RV, as measured by ASC responses, had to be considered inconclusive. Furthermore, it is possible that the differences between LCTB*A* and CTB in

inducing IgA responses revealed by serum analysis would also have been seen in ALS samples, had a more optimal sampling time point been used.

Since clinical trials are expensive and laborious, immunogenicity measurements are often limited to only a few set time points after each vaccine dose, irrespective of assay, number of doses, incorporation of an adjuvant, study population etc. However, the results from this study highlighted the fact that it is imperative that sampling time points are optimised to properly assess the immunogenicity of oral vaccines in clinical trials. Fortunately, preliminary data from this cholera vaccination study was obtained before the initiation of the first Phase I trial of MEV. Hence, the clinical trial protocol for the MEV study was amended just in time to allow testing of ASC responses both on days 5 and 7 after administration of the second dose of MEV.

Adjuvant Effects of dmLT on Human Immune Cells (Paper III)

At this stage of the development of the second generation ETEC vaccine, the novel mucosal adjuvant dmLT, developed by Clements et al. [123], had been shown to enhance immune responses to several mucosal vaccines in mouse models [124,125]. However, dmLT had only been evaluated in mice and no human studies had yet been performed. Since dmLT was being considered for clinical testing together with MEV, we evaluated the *in vitro* effects of dmLT on human T cell responses to gain understanding of the potential for and mechanism of adjuvant function in humans.

Although antibody responses to ETEC vaccination are considered central for protection against ETEC infection, the affinity maturation, differentiation and isotype switching of B cells, as well as memory development, results from their cognate interaction with antigen-specific activated CD4+ Th cells in lymphoid tissues. Considering the vital role of T cells in the induction of antibody responses and memory development, and since many adjuvants influence immune responses via effects on T cells, we analysed the *in vitro* effect of dmLT on T cell responses to both ETEC antigens and to other model vaccine antigens. We were especially interested in analysing Th17 responses, since CT, LT and dmLT had all been shown to induce Th17 cell responses in murine studies [65,66,125] and Th17 cells are known to be important for mucosal immune responses [42].

Effect of dmLT on T cell Responses to Different Vaccine Antigens

To establish an *in vitro* system in which we could evaluate the effect on Th1 and Th17 responses to different vaccine antigens including ETEC LTB, we first analysed T cell responses to LTB in ETEC vaccinees. To analyse if PV or RV (containing 1 mg LCTB*A* or CTB) induced LTB-specific T cell responses which could be detected in peripheral circulation, we stimulated PBMCs isolated from subjects before and after vaccination with PVx1 or RV with LTB. Encouragingly, the production of IL-17A and IFNγ in samples collected on day 21 was significantly higher compared to samples collected on day 0 (Fig 7); comparable responses to LTB were induced by PV and RV. These results indicated that the ETEC vaccines induced both Th17 and Th1 type responses.

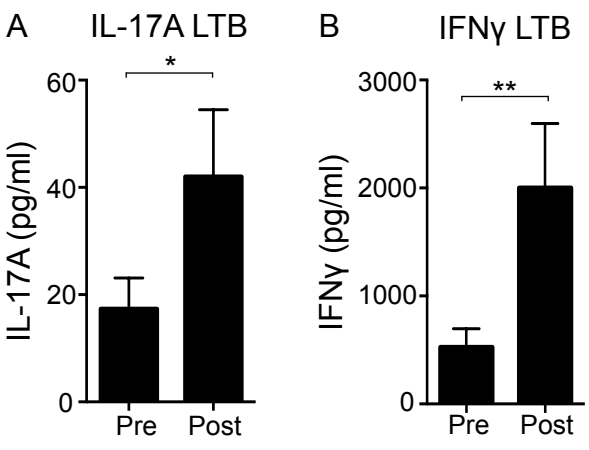


Figure 7. IL-17A and IFNγ responses to LTB in subjects immunised with the ETEC vaccines PVx1 or RV. *PBMCs from 20 volunteers, collected pre and post-vaccination, were stimulated with LTB and the resulting (A) IL-17A and (B) IFNγ production was determined. Statistical analysis was performed using the Wilcoxon signed rank test. *P<0.05, **P<0.01.*

We also investigated *in vitro* T cell responses to other vaccine antigens where Th17-responses are considered important, i.e. PPD (in subjects previously vaccinated with BCG) and the whole cell pneumococcal vaccine antigen WCA (in subjects naturally exposed to *S. pneumoniae*). Our initial studies showed that a majority of medical students vaccinated with BCG had *in vitro* IL-17A and IFNγ responses to PPD. Previous human studies at our lab had shown IL-17A and IFNγ responses to WCA in a majority of tested adult Swedish subjects, interpreted as a result of natural exposure to *S. pneumoniae* [141].

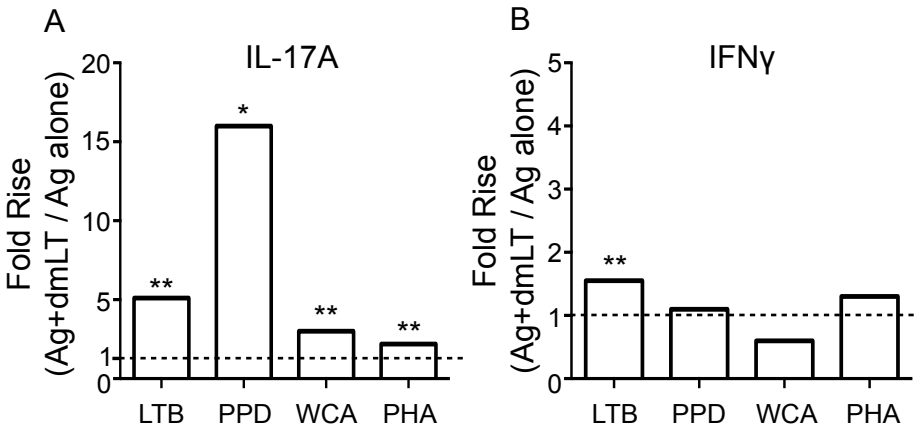


Figure 8. Influence of dmLT on in vitro T cell cytokine responses to different vaccine antigens and a mitogen. *PBMCs from subjects vaccinated with the oral ETEC prototype vaccine or the BCG vaccine, were stimulated in vitro with the antigens LTB, PPD or pneumococcal WCA or were polyclonally stimulated with PHA, with and without 1 μg/ml dmLT. The median fold rises in (A) IL-17A and (B) IFNγ production with the addition of dmLT are shown. Statistical analysis was performed using the Wilcoxon signed rank test. *P<0.05, **P<0.01; compared to cells stimulated with antigen alone.*

We thus had an *in vitro* model system where we could study Th1 and Th17 responses to three different vaccine antigens and evaluate the effect of dmLT on these responses.

When 1 μg/ml dmLT was added to the PBMC cultures of subjects vaccinated with PV or RV, both the IL-17A and IFNγ production in response to LTB stimulation were significantly increased compared to stimulation with LTB alone (Fig 8). However, when PBMCs were stimulated with WCA or PPD + 1 μg/ml dmLT, we found that the IL-17A production, but not the IFNγ production, increased significantly (Fig 8). The potentiation of IL-17A but not IFNγ responses was also seen with PBMCs stimulated with the mitogen PHA (Fig 8); interestingly, the Th2-associated cytokine IL-13 also increased significantly with the addition of 1 μg/ml dmLT to PHA-stimulated PBMCs. When higher concentrations of dmLT (10 μg/ml) were added to the PPD or PHA-stimulated PBMCs, the IL-17A production increased further, indicating a dose-response relationship. Notably, dmLT did not (at any concentration) influence cell proliferation in response to any of the antigens tested. We confirmed that the IL-17A produced in our *in vitro* cultures was indeed mainly derived from CD4+ T cells by depleting these cells from PBMCs.

Thus, when PBMCs isolated from subjects vaccinated with BCG were depleted of CD4+ T cells prior to stimulation with PPD or PHA + 10 μg/ml dmLT, there was no detectable IL-17A production.

Together, these results suggested that dmLT had a variable enhancing effect on Th1 type responses to different antigens, but that the Th17 potentiating effect was consistent for all antigens tested. We further investigated the effects of dmLT and other LT-derived adjuvants on PBMCs isolated from subjects vaccinated with BCG, as these subjects were readily available.

Comparison of the IL-17A Potentiating Effects of Different ADP-ribosylating Toxins, Toxoids and Toxin Subunits

Considering that both CT and LT are excellent mucosal adjuvants in mice [119], and that the dmLT predecessor mLT (with only one of the two mutations introduced to LT in dmLT) has been shown to have adjuvant properties in human vaccine studies [121,122], we compared the effect of dmLT, mLT, LT, CT and LTB on the IL-17A production in response to PPD.

The addition of 1 μg/ml mLT to PPD-stimulated PBMCs from BCG-vaccinated subjects, had a similar IL-17A potentiating effect as dmLT (Fig 9). The fact that dmLT, which is more rapidly degraded than mLT *in vivo* [123], had equivalent IL-17A potentiating ability, was very encouraging. The addition of LT or CT had an approximately three-fold stronger potentiating effect than mLT or dmLT at the same concentration. At 10 μg/ml, dmLT, mLT and LT all significantly enhanced the IL-17A production in a dose-dependent manner. The IL-17A production however decreased when 10 μg/ml of CT was added, suggesting a toxic effect at this concentration. This effect is also seen in murine cells, but at much lower CT concentrations [142]. As expected, LTB had no influence on the IL-17A production at any concentration tested, confirming that ADP-ribosylating activity was vital for the observed adjuvant effect.

Thus, dmLT had the same potency as mLT, but was less potent than the native toxins CT and LT, at enhancing Th17-responses in human immune cells.

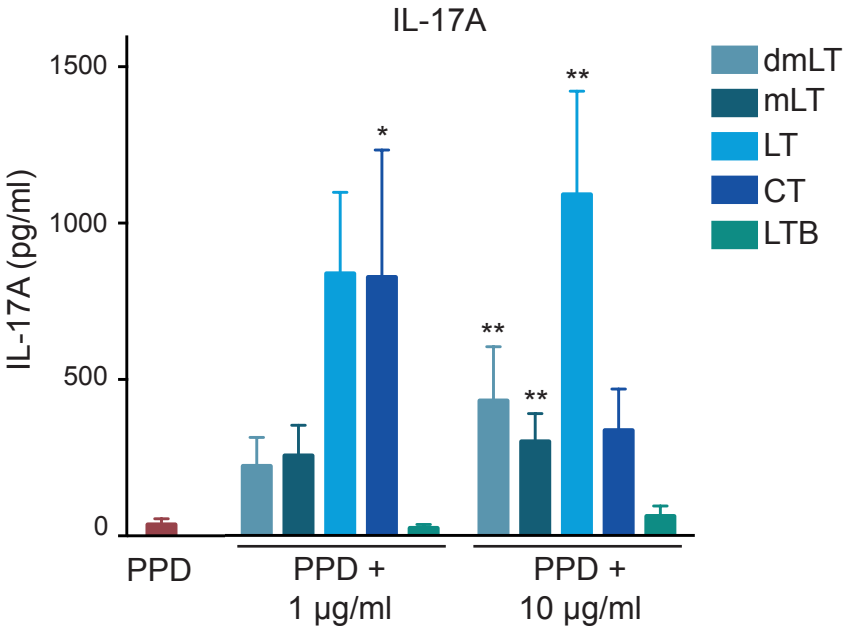


Figure 9. IL-17A production in response to PPD and increasing concentrations of ADP-ribosylating toxins, detoxified mutants or subunits. *PBMCs from subjects vaccinated with BCG were stimulated with PPD alone or together with 1 or 10 µg/ml of dmLT, mLT, LT, CT or LTB, and the resulting IL-17A production was determined. Statistical analysis was performed using the Friedman test with Dunn's multiple comparison post test. *P<0.05, **P<0.01; compared to cells stimulated with PPD alone.*

Mechanisms of the Th17-Enhancing Effect of dmLT

In the *in vitro* cultures described above, dmLT could potentially influence any of the lymphocytes, monocytes or DCs present in PBMCs. We therefore investigated the possible mechanisms behind the Th17-potentiating effect of dmLT in a more defined cell culture system. Since several adjuvants, including CT and LT, are known to enhance immune responses via effects on APCs [143], we focused on monocytes as they are present in relative abundance in PBMCs (approximately 10%) compared to DCs (1-2%), and have been shown to be able to orchestrate CD4+ T cell differentiation [144].

The Role of Monocytes

To see if the Th17-potentiating effect of dmLT could be achieved via monocytes, we stimulated CD14+ monocytes from BCG-vaccinated subjects with PPD ± dmLT for 24 hours. After washing, autologous CD4+ T cells

were added to the monocytes and the IL-17A production analysed 5 days later. In all subjects tested, the IL-17A production was increased in cultures where the monocytes had been stimulated with PPD + dmLT compared to PPD alone. Thus, the IL-17A potentiating effect of dmLT could be conveyed via monocytes.

The Role of Soluble Factors

Though monocytes were clearly important, it was still unclear if the adjuvant effect was cell-mediated or if secreted factors were sufficient. As the cytokines IL-1β, IL-6 and IL-23 (predominantly produced by APCs) had been shown to be of importance in Th17 induction and sustainment [144,145], we blocked these cytokines with neutralising antibodies. Indeed, IL-17A production in response to PPD was significantly abrogated when IL-1β or IL-23 were neutralised (Fig 10), indicating a vital role of these APC-derived cytokines in our system.

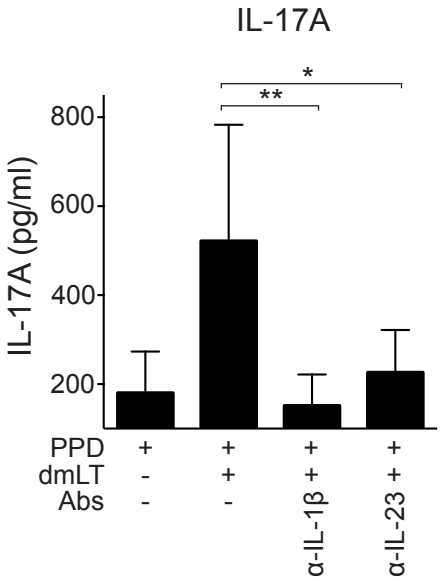


Figure 10. Effect of neutralising IL-1β or IL-23 on IL-17 production in response to PPD and dmLT. *PBMCs from BCG-vaccinated subjects were stimulated with PPD and dmLT in the presence and absence of neutralising antibodies (Abs) against IL-1β or IL-23, and the resulting IL-17A production was determined. Statistical analysis was performed using the Friedman test with Dunn's multiple comparison post test. *P<0.05, **P<0.01.*

Taken together, these results suggest that dmLT primarily acts on monocytes in our PBMC cultures, and potentiates Th17-type responses via IL-1β and IL-23.

Subsequent preclinical evaluation of MEV ± dmLT in mice showed that dmLT increased anti-CF and anti-LTB IgA levels in faecal, jejunal and serum samples in response to MEV [134]. Strikingly, when lower dosages of vaccine were tested in this study, the adjuvant effect of dmLT was especially

strong, suggesting that dmLT has a dose-sparing effect. dmLT was also tested alone in a Phase I clinical trial in the USA and found to be safe and minimally reactogenic at doses up to 100 µg [146]. dmLT was also found to be immunogenic in itself, with the highest levels of systemic and mucosal dmLT-specific immune responses seen at the 50 µg dose; the responses did however not increase at 100 µg dmLT.

Collectively, the results from these parallel studies of dmLT showed that dmLT had an adjuvant effect on human monocytes and T cells *in vitro*, was safe and immunogenic in humans, and had a dose-sparing effect in mice. Based on these results, the clinical development process advanced to the next step, i.e. testing the complete ETEC vaccine with and without dmLT in adult Swedish volunteers, which was the first evaluation of dmLT in combination with any vaccine in humans.

Summary of Results from Two Clinical Trials of MEV (Background to Paper IV)

Since samples collected in two clinical trials of MEV are used in paper IV, a summary of the main results of these trials is included below.

Phase I Clinical Trial of MEV (Primary Vaccination Study)

MEV, consisting of four recombinant *E. coli* strains expressing CFA/I, CS3, CS5 and CS6 + 1 mg LCTB*A*, was tested for safety and immunogenicity in a double-blinded, placebo-controlled Phase I study in 129 Swedish adult volunteers [135]. MEV was given in two doses at a two-week interval alone (n=35) or together with 10 µg (n=30) or 25 µg (n=30) dmLT. The placebo group (n=34) received bicarbonate buffer alone. As previously described, immunogenicity sampling time points had been amended after the first trial of PV to include both days 5 and 7 after the second vaccine dose (Fig 4C).

MEV was found to be safe and well tolerated. AEs were recorded at a similar frequency as in the clinical trial of PV (paper I), and no significant differences in frequency or intensity of AEs were observed among subjects receiving placebo, vaccine or vaccine + dmLT at either dosage. This supported that dmLT was indeed more attenuated than mLT, which had been

found to induce unacceptable gastrointestinal reactions at comparable doses when given together with *H. pylori* and *Campylobacter* vaccines [121,122].

The vaccine was also immunogenic, with significant increases in anti-LTB serum IgA and IgG as well as toxin-neutralising capacity, seen in all vaccine groups when compared to placebo. Also, significant increases in mucosal immune responses, measured by IgA in ALS samples and SIgA in faecal samples, were seen against all the primary vaccine antigens (i.e. LTB, CFA/I, CS3, CS5 and CS6) in all vaccine groups. As expected from the results from the ALS kinetics study (paper II), ALS responses to all vaccine antigens peaked 5 days after the second vaccine dose and decreased significantly until day 7, as illustrated by the anti-LTB and anti-CFA/I responses depicted in Fig 11. Had the day 19 sampling time point not been added and ALS responses had only been evaluated on day 21, as in the PV study (paper I), the CFA/I responder frequency would have been 42% instead of 66%. When considering the anti-LTB responses, the responder frequency would have been similarly high independently of sampling time point (89% on day 19 vs. 86% on day 21), yet the magnitudes of responses an average four-fold lower. These results confirmed the findings from paper II - ASC responses to oral vaccination increase much more quickly and peak earlier after the second vaccine dose than after the first, demonstrating the importance of selection of correct time points for optimal immunogenicity evaluation.

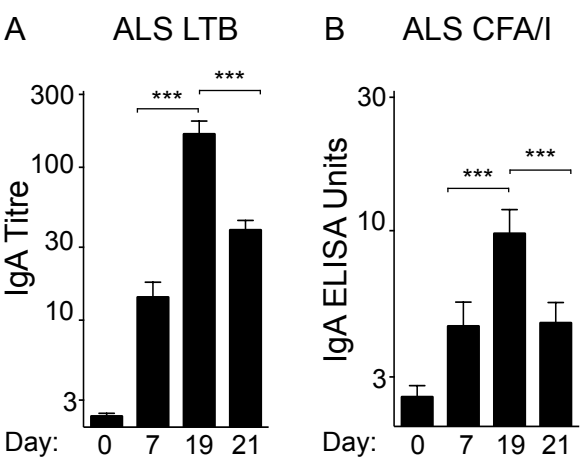


Figure 11. Kinetics of ALS antibody responses after vaccination with MEV. *IgA antibody levels (GM + SEM) against (A) LTB and (B) CFA/I in ALS samples from subjects immunised with MEV ± 10 or 25 µg/ml dmLT on days 0 and 14 (n=84) were analysed before immunisation (day 0) and on days 7, 19 and 21 after the first immunisation. Statistical analysis was performed using a paired t-test with Bonferroni correction. ***P<0.001.*

Interestingly, significantly higher anti-CS6 IgA ALS responses were seen in the group receiving vaccine + 10 μg dmLT compared to the group receiving vaccine alone; there was also a trend of higher ALS responses against CFA/I and CS5 in this group. As CS6 was the antigen present in the lowest amount in the vaccine, this supported the notion that dmLT provides a dose-sparing effect, as seen in the murine studies [134]. Similarly to the results from the trial assessing a single, oral dose of dmLT, where 100 μg dmLT was less immunogenic than 50 μg [146], the addition of 25 μg dmLT to MEV resulted in less enhancement of responses than the addition of 10 μg. The reason for this is not currently known.

Thus, MEV ± dmLT was found to be safe and broadly immunogenic and dmLT increased immune responses to the CF antigen present in lowest amounts in the vaccine. However, immune responses were only investigated short-term after vaccination. To determine if MEV induced long-term memory responses, and to evaluate the potential influence of dmLT on memory induction, a follow-up trial was also performed using the ALS kinetics method established in paper II as the primary method for memory evaluation.

Follow-up Trial of Memory Responses Induced by MEV (Booster Vaccination Study)

To evaluate the mucosal and systemic immunological memory induced by the primary vaccinations with MEV, a single booster dose of MEV was administered to a subset of subjects from the first trial, 13-23 months after the primary vaccinations [Lundgren et al., submitted]. Subjects were randomly selected from amongst the groups who had received either MEV alone (n=17) or MEV + 10 μg dmLT (n=18); the 10 μg dmLT group was selected because this group had significantly higher anti-CS6 ALS responses than the group receiving MEV alone. A group of non-immunised naïve control subjects (n=20) was also recruited. ALS and serum responses were measured on days 0, 4/5 and 7 after vaccination (Fig 4C).

ALS responses to all CFs and LTB were considerably higher and significantly more frequent after administration of the single dose of MEV in the previously immunised subjects, than in the naïve subjects. Peak ALS responses against all vaccine antigens were observed on days 4/5 in most of the previously immunised subjects and the responses declined markedly by day 7, whereas only a few of the previously non-immunised individuals

responded before day 7. The same pattern was seen in paper II, and these recent data from the booster vaccination study support the notion that analysis of ALS kinetics is a useful approach to differentiate primary and recall responses. There were no differences in ALS responses to the single booster dose of MEV between subjects previously immunised with vaccine alone or vaccine plus dmLT adjuvant.

Thus, MEV induced significant IgA memory responses to all primary vaccine antigens which persisted for at least two years after primary vaccination. However, our previous results indicate that mucosal memory may remain for longer periods, since memory responses to CTB could be detected as late as 14 years after primary vaccination with Dukoral® in paper II. No differences in memory responses were seen in the groups receiving primary immunisations with or without dmLT.

Functional Characteristics of Antibodies Induced by ETEC Vaccination (Paper IV)

We used samples from both the primary vaccination trial and the booster/memory study to explore the functional characteristics of the antibody responses to MEV, i.e. cross-reactivity, avidity and toxin neutralisation capacity, and to evaluate if antibody function was influenced by dmLT.

Antibody Cross-Reactivity

As antibodies that cross-react with several CFs could potentially broaden the CF coverage of MEV, we investigated whether MEV had the ability to induce such cross-reactive antibodies in adult Swedish subjects. We focused on the CFA/I family (comprising CFA/I, CS1, CS14 and CS17) and the CS5 family (comprising CS5 and CS7). Since anti-CF responses in serum were low and infrequent in response to MEV [135], and ALS and faecal antibodies better reflect mucosal immune responses, we studied the cross-reactivity of antibodies in ALS and faecal samples.

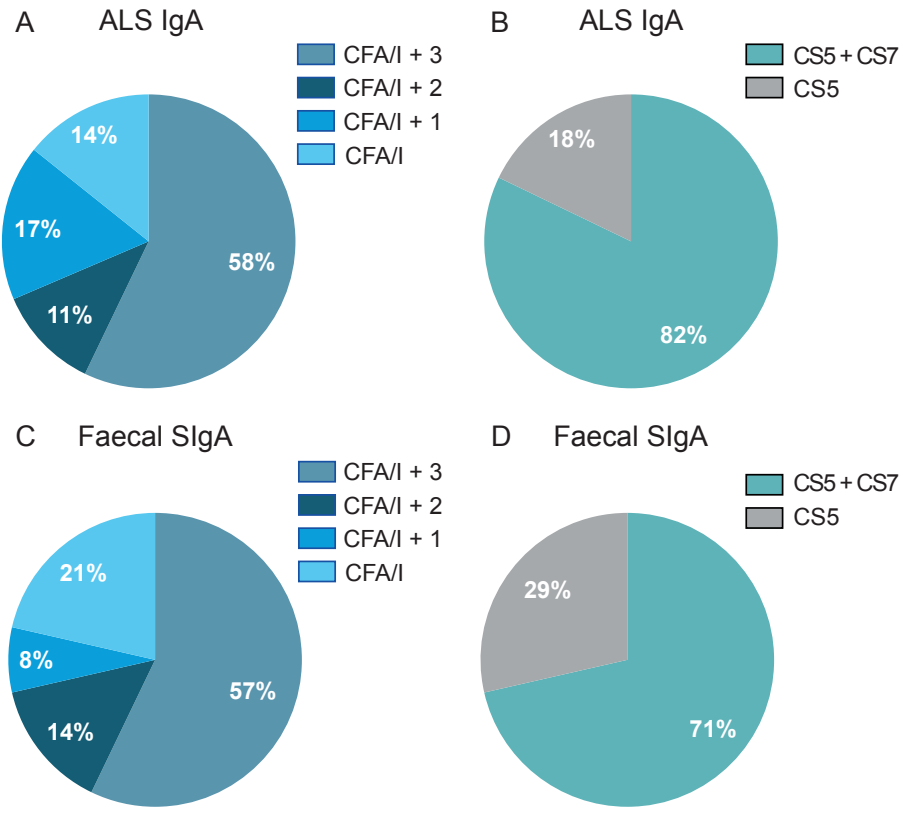


Figure 12. Mucosal antibody responses to vaccine and related, non-vaccine CFs after immunisation with MEV. *IgA responses in ALS samples (A and B) and SIgA responses in faecal samples (C and D) after immunisation with MEV ± dmLT in the primary vaccination study or MEV alone in the booster vaccination study. The proportion of subjects who had responses against the vaccine antigens CFA/I (A and C) and CS5 (B and D) and one or more related, non-vaccine CFs are indicated.*

We randomly selected ALS samples from subjects in both the primary and booster vaccination trials of MEV, and faecal samples from subjects in the primary vaccination trial, who had developed significant responses to CFA/I or CS5. Indeed, among the CFA/I ALS responders, 58-86% had antibodies cross-reacting to 1-3 of the related, non-vaccine CFs tested; just 14% had responded to CFA/I only (Fig 12A). Also, among the CS5 ALS responders, 82% had antibodies cross-reacting to CS7 (Fig 12B). The same pattern of antibody cross-reactivity was seen in faecal samples (Fig 12C-D). There were no differences in the cross-reactivity of antibody responses among the groups receiving MEV, MEV + 10 μg/ml dmLT and MEV + 25

μg/ml dmLT. However, the sample size was small and further studies may be required to fully evaluate the effect of dmLT on cross-reactivity.

Our results thus showed that MEV has the ability to induce cross-reactive mucosal antibodies to related, non-vaccine CFs, both in non-primed and primed adult subjects. The CFs expressed by the bacterial strains included in MEV account for approximately 45% of all clinical ETEC isolates; if the related CFs included in this study are taken into account, the potential CF coverage may be increased by up to 20% [18,147,148]. When also considering the included LT component, MEV could afford a potential coverage of more than 85% of clinical ETEC isolates [18,147,148].

Antibody Avidity

Even if a vaccine can induce high antibody levels, not all of these antibodies necessarily have the capacity to bind and neutralise the pathogen or toxin effectively *in vivo*. Antibody avidity, the overall strength of the multivalent interaction between antibodies and their antigens, has also been shown to be an important characteristic of protective immune responses [88-91]. Several adjuvants have been shown to influence the development of antibody avidity [88,149,150]. Hence, we investigated whether MEV had the ability to induce high-avidity antibodies and if dmLT influenced avidity development.

Though there are many different ways of measuring antibody avidity (discussed in the introduction), we needed an assay that used low amounts of sample and did not require high antibody titres, so that ALS samples could be analysed; hence chaotropic ELISA assays were not suitable. We also wanted a simple assay, which could potentially be used in a clinical trial setting. Based on these considerations, we developed the limiting antigen dilution assay for antibody avidity measurement.

Development of the Limiting Antigen Dilution Assay

The limiting antigen dilution assay is based on how effectively antibodies bind to decreasing levels of immobilised antigen detected by ELISA. When antibodies are incubated on plates coated with decreasing concentrations of antigen, high-avidity antibodies require lower concentrations of antigen than low-avidity antibodies to obtain equivalent binding (Fig 13A). After initial titration experiments, two coating concentrations for each antigen were chosen for good discrimination in antibody binding, and an avidity index was calculated (AI = titre at lower coating concentration / titre at higher coating concentration).

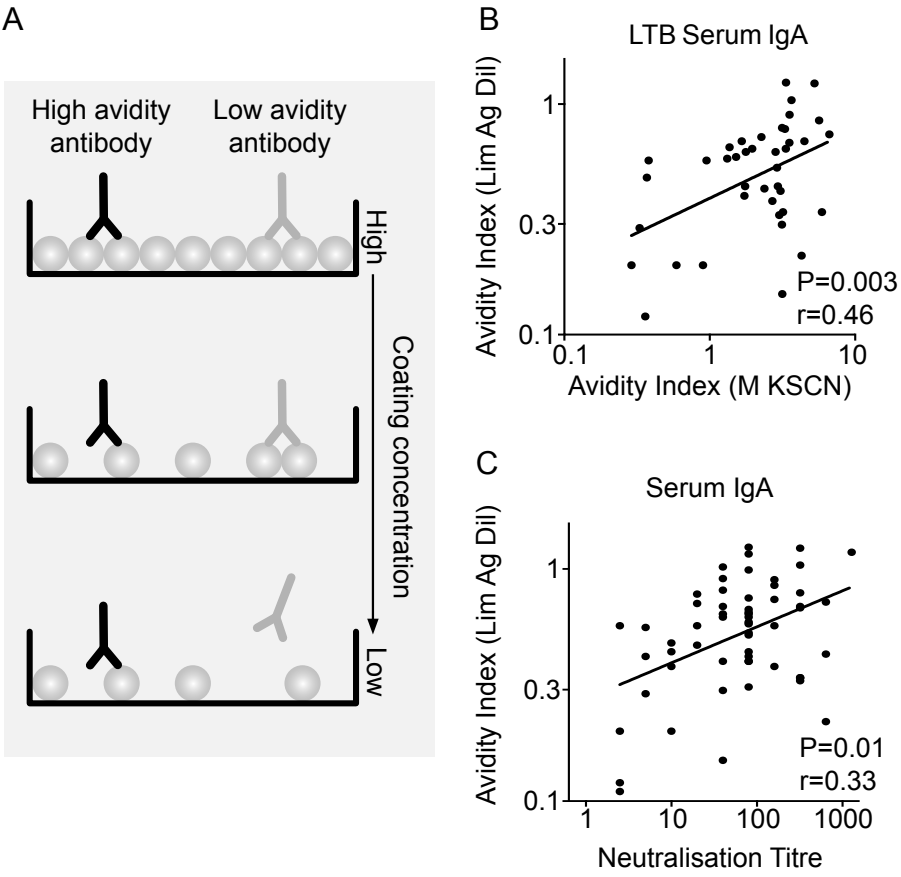


Figure 13. Methods for evaluation of antibody avidity after immunisation with MEV. *(A) Schematic representation of the principle underlying the limiting antigen dilution avidity assay, adapted from [151]. (B and C) Serum samples from subjects immunised with MEV ± dmLT in the primary vaccination study or with MEV alone in the booster vaccination study were analysed. (B) The avidity of LTB-specific IgA antibodies was analysed using both the limiting antigen dilution assay (y-axis) and KSCN elution (x-axis). (C) The antibody avidity as measured by limiting antigen dilution (y-axis) was also compared with the LT-neutralising titre of the serum samples (x-axis). Statistical analyses were performed using the Spearman correlation test.*

We validated this method by analysing the avidity of anti-LTB serum antibodies with both limiting antigen dilution and a chaotropic ELISA using potassium thiocynate (KSCN). There was a significant correlation between the results from the two methods, suggesting that the AI obtained using the limiting antigen dilution method indeed reflected antibody avidity (Fig 13B).

Effect of dmLT on Antibody Avidity

We used the limiting antigen dilution assay to measure possible differences in anti-LTB antibody avidity in serum and ALS samples among the groups receiving MEV, MEV + 10 μg/ml dmLT and MEV + 25 μg/ml dmLT. We found no significant differences among the subjects receiving MEV alone or MEV + dmLT at any concentration. Unfortunately, due to the lower magnitudes of responses to CFs than LTB, we were unable to analyse avidity differences in responses to CFs among the vaccine groups in a large enough number of subjects. In future studies of MEV in children, who will likely receive a lower vaccine dose due to reactogenicity and when dmLT may have increased potential to influence responses, it will be interesting to see if dmLT does have an effect on the antibody avidity.

Antibody Avidity after Primary and Booster Vaccinations

As the immune responses to the first dose of MEV were low and infrequent, we were unable to analyse differences in antibody avidity after one and two doses. To investigate whether repeat doses of MEV induced antibodies with higher avidity, we compared avidity after the second dose of the primary vaccinations and a late booster dose, given 13-23 months later.

We found that the avidity of anti-LTB IgA and IgG antibodies in serum did indeed increase significantly after a late booster dose, even though the magnitudes of responses did not significantly increase (Table 3). Similarly, the avidity of anti-LTB IgA antibodies in ALS samples increased without parallel titre increases. In contrast, anti-CS3 IgA in ALS samples increased significantly in both magnitude and avidity. These observations that antibody avidity increased both with and without parallel titre increases support that the avidity differences seen after booster vaccination with MEV were not dependent on antibody titres, but indeed reflected increased binding strength.

Antibody avidity has been used as a surrogate marker for immune memory in a number of studies of different infections and vaccines [152-154]. According to current understanding (Fig 3), B cell clones with higher affinity surface antibodies may capture more of the limited amount of vaccine antigen on the surface of FDCs in germinal centres and therefore compete more successfully for the limited help from Tfh cells by providing higher numbers of antigen peptide-MHC II complexes [38]. Subsequently, B cells that produce high-affinity antibodies and receive Tfh help differentiate into long-lived memory B cells or plasma cells that seed the mucosa. The

significant increase in antibody avidity observed after booster vaccination compared to after primary vaccination supports the notion that avidity reflects memory; the relationship between avidity and memory will be further explored in future studies.

Table 3. Magnitudes and avidity of antibody responses in serum and ALS samples after primary and booster vaccination with MEV.

Antigen	Fold Rise [a]		Avidity Index [b]	
Sample	**Primary [c]**	**Booster [d]**	**Primary**	**Booster**
LTB				
Serum IgA	10	11	0.48	0.67***
Serum IgG	7	5	0.39	0.58**
ALS IgA	121	131	0.67	0.86*
CS3				
ALS IgA	22	108*	0.56	0.87**

[a] Magnitudes were expressed as GM fold rises of antibody titres.
[b] Avidities were expressed as median avidity indexes as determined by the limiting antigen dilution assay.
[c] Primary vaccination responses were determined by comparing antibody levels in serum and ALS after administration of two doses of MEV ± dmLT with levels in samples collected on the day of the first vaccination (day 0).
[d] Booster vaccination responses were determined by comparing antibody levels in serum and ALS after administration of a booster dose of MEV with levels in samples collected on the day of booster vaccination (day 0).
Statistical analyses were performed using the Wilcoxon signed rank test for avidity indexes and the paired *t*-test for antibody titres. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared to after primary vaccinations.

Toxin Neutralisation

To assess actual antibody function, we used an LT-toxin neutralisation assay to test the capacity of the anti-LTB antibodies induced by MEV to neutralise LT. In the primary vaccination study of MEV, the LT-neutralising responses were seen to closely mirror the serum anti-LTB IgG and IgA titre increases determined by ELISA, indicating that these antibodies were highly functional [135]. To investigate whether repeat doses of MEV could induce antibodies with a higher LT-neutralising capacity, we tested serum samples from subjects in both the primary and booster vaccine studies.

We found that the LT-neutralising capacity of serum collected after the late booster dose was significantly higher than after primary vaccinations,

even though the IgA and IgG titres were not significantly increased. Interestingly, the LT-neutralising titres were significantly correlated with the avidity of anti-LTB antibodies, as measured by the limiting antigen dilution assay (Fig 13C). Unfortunately, we were unable to test ALS samples in the LT-neutralising assay due to too small sample volumes. However, the significant correlation between avidity and toxin neutralising capacity is very encouraging, suggesting that the increase in antibody avidity after repeat vaccinations, which we also found in mucosal ALS samples, may be of functional importance.

Collectively, these results showed that vaccination with MEV induces cross-reactive antibody responses to multiple related, non-vaccine CFs, thereby potentially broadening the protection against different ETEC strains. After late booster vaccination with MEV, the avidity of both mucosal and serum antibodies to key vaccine antigens increased, as well as the LT-neutralising capacity of serum antibodies. Thus, repeat vaccinations with MEV may result in vaccine-specific antibodies with enhanced functional characteristics.

CONCLUDING REMARKS

The studies included in this thesis have been important steps in the clinical evaluation of the second generation ETEC vaccine developed at the University of Gothenburg in collaboration with Scandinavian Biopharma, and have provided new insights into human immune responses to oral vaccines and adjuvants. Firstly, we showed that a prototype of the second generation ETEC vaccine was safe and immunogenic, inducing significant mucosal IgA responses to both the CFA/I over-expressing vaccine strain and the LCTB*A* toxoid. Based on these results, the complete multivalent ETEC vaccine (MEV) progressed to clinical evaluation [135]. However, subsequent detailed studies of ASC kinetics, using the model oral cholera vaccine, showed that the sampling time point after the second vaccine dose was too late to capture peak ASC responses. Consequently, the results from the comparison of the CFA/I over-expressing prototype strain and the first generation ETEC reference vaccine strain were not conclusive. Thanks to the lessons learnt in these initial studies, sampling time points were adjusted in subsequent clinical trials of the complete multivalent ETEC vaccine. The detailed analysis of ASC response kinetics to cholera and ETEC vaccines thus suggest that the lower immune responses to a second compared to a primary oral vaccine dose, as seen in many previous clinical trials of ETEC vaccines, particularly in primed populations, was primarily a result of suboptimal sampling rather than the presence of neutralising antibodies in the gut mucosa. Though persisting neutralising antibodies have been seen to abrogate immune responses to booster vaccination with the oral live attenuated *S.* Typhi Ty21a vaccine [106], the kinetics of mucosal immune responses probably differ between the persisting antigen exposure provided by a live vaccine and the short antigen exposure of a killed vaccine.

Based on the positive safety and immunogenicity results from the clinical trials of the prototype and complete vaccines, MEV is today considered to be one of the most promising ETEC vaccines currently undergoing active clinical development [133]. Our demonstration that a high proportion of subjects vaccinated with MEV developed antibodies that bound to related, non-vaccine CFs is also encouraging, suggesting that MEV has the potential to provide broad protection against ETEC. The next important step is now to establish safety and immunogenicity of MEV in the paediatric population. A large Phase I/II, descending-age trial has recently been initiated

in Bangladesh, where MEV will be given in escalating doses, with and without dmLT, to adults, toddlers and infants. If the results from this trial are satisfactory, studies are planned to move MEV into Phase III trials assessing the protective efficacy of the vaccine. A Phase II study testing the efficacy of MEV in adult travellers is also planned.

Several novel approaches to evaluate immune responses to mucosal vaccination in humans have also been developed as a part of this thesis, which may support the further clinical evaluation of MEV, as well as other oral vaccines. Our studies of ASC kinetics not only led to the identification of optimal sampling time points, but also to the demonstration that functional mucosal memory induced by oral vaccination can be assessed by analysis of ASC response kinetics. Thus, whilst peak ASC responses to the first dose of oral cholera vaccine were seen after around 9 days, peak responses to the second or late booster dose were seen after only 4/5 days, indicating a functional mucosal memory. Using this approach, we showed that mucosal memory to oral cholera vaccination can be detected up to 14 years after primary vaccinations, which may have implications for how frequently cholera booster vaccinations should be recommended. These results were later confirmed in subjects vaccinated with MEV [Lundgren et al., submitted]. In the MEV study, circulating vaccine-specific memory IgA B cells, detected by polyclonal stimulation of PBMCs and subsequent ELISPOT analysis [138,155], were undetectable 13-23 months after primary vaccination with MEV. Unlike the circulating memory B cell population, which is clearly not a good reflection of long-lasting mucosal memory, we believe that analysis of ASC kinetics is a simple and more reliable method to measure functional mucosal memory following oral vaccination. In future studies, it would be of interest to further examine the memory responses to oral vaccination in humans by analysing the vaccine specific ASC population induced by booster vaccination for additional functional and phenotypic characteristics, including expression of gut homing integrins.

In addition to the memory assay, a simple method to assess antibody avidity was developed, based on limiting antigen dilution. Importantly, this method could also be used with ALS samples. Using the limiting antigen method, we showed that a late booster dose of MEV induced vaccine specific antibodies with higher avidity than those induced after primary vaccinations. We also showed that anti-LT serum antibody avidity was associated with LT-neutralising capacity. These results suggest that an immunisation schedule of

two vaccine doses of MEV with an additional booster dose 12-18 months later may be ideal to induce highly functional antibodies. However, these results should be confirmed in a naturally primed populations, and especially in children. It is also important to consider how this dosing regimen may fit into the current EPI schedule.

In this thesis, the influence of dmLT on T cells and antibody avidity was also evaluated. We showed that both Th1 and Th17 responses induced by ETEC vaccination can be enhanced *in vitro* by dmLT, supporting the notion that this adjuvant may influence human immune responses. Using *in vitro* PPD responses in BCG-vaccinated subjects as a model system to evaluate the effects of dmLT further, we showed that dmLT primarily acts on monocytes and potentiates Th17-type responses via IL-1β and IL-23. The results from this study were confirmed and extended in subsequently more detailed investigations into the mechanisms of the IL-17A potentiating function of dmLT in human cells at our laboratory. Larena et al. recently showed that the adjuvant action of dmLT, as well as CT, on T cell responses induced by the superantigen staphylococcal enterotoxin B was dependent on caspase-1/inflammasome-mediated IL-1 signalling and production of cAMP [156]. However, as DCs are the professional APCs with the best competence to activate naïve T cells, investigating the adjuvant effect of dmLT on DCs, as well as the effect of dmLT on other cell types likely to be affected by dmLT *in vivo*, such as epithelial cells, will be important in future studies. Whilst our preliminary analyses indicated that dmLT had no major effects on IL-21 production from CD4+ T cells, on-going studies in our laboratory are also investigating the specific effects of dmLT on Tfh cells (the main producers of IL-21), which may be the Th subset with the greatest potential to influence antibody production, affinity maturation and memory B cell development.

In the clinical trial of MEV, coadministration of the vaccine with dmLT had little or no detectable effect on the magnitudes of responses or responder frequencies induced by LTB or most of the vaccine CFs [135]. However, anti-CS6 IgA ALS responses were significantly higher in the group receiving vaccine + 10 μg dmLT compared to the group receiving vaccine alone. As CS6 was the antigen present in the lowest amounts in MEV, this indicates that dmLT may have a dose-sparing effect. Unfortunately, we were unable to measure the avidity of anti-CS6 antibodies in ALS samples due to low titres. However, neither the avidity nor the LT-neutralising capacity of

LT-specific antibodies was increased in the vaccine + adjuvant group compared to the vaccine alone group.

Notably, interesting results have recently been presented regarding the adjuvant effect of dmLT on protection induced by the live, attenuated ETEC vaccine ACE527 [157]. Preliminary results suggest that subjects immunised with ACE527 + dmLT were significantly protected against subsequent ETEC challenge, whilst those who had received vaccine alone were not. However, all measured immune responses were comparable in subjects receiving vaccine ± dmLT. Although these results need to be confirmed in further studies, since numbers of volunteers were small in the study described, the results support that dmLT may have important adjuvant effects in humans, although the mechanisms are yet to be determined. It will therefore be important to evaluate the capacity of dmLT to influence both the magnitudes and functional characteristics of immune responses, as well as try to find additional biomarkers of the effect of dmLT, in the continued clinical evaluation of MEV in children and infants. It will be of particular interest to determine if dmLT affords a dose-sparing effect, or influences antibody avidity or memory development in infants, who are likely to receive lower vaccine doses to avoid potential side-effects. On-going studies are also investigating the potential effects of dmLT on the induction of Th1, Th17 and Tfh responses to MEV *in vivo* in adults, which may provide further insights into how dmLT may influence the induction of adaptive immune responses.

In conclusion, the results presented in this thesis are important for and have already facilitated the clinical evaluation of the new ETEC vaccine. We now know the optimal time sampling points for analysing ASC responses induced by oral ETEC vaccines, and can measure mucosal immunological memory and antibody avidity using simple methods. Our demonstration that MEV induces cross-reactive antibodies is also encouraging, indicating that MEV may have the potential to provide broad protection against many different ETEC strains. Hopefully, the methods established through this work will facilitate further studies of human immune responses to mucosal vaccines and adjuvants in different age groups in the future.

SAMMANFATTNING PÅ SVENSKA

Infektion med enterotoxinbildande *E. coli* bakterier (ETEC) är en av de vanligaste orsakerna till diarré hos barn i u-länder och s.k. turistdiarré, men ännu finns inget vaccin mot denna infektion. ETEC-bakterierna fäster till tarmslemhinnan med hjälp av speciella ytproteiner, s.k. kolonisationsfaktorer, och producerar gifter, toxiner, som orsakar diarré. Ett nytt drickbart ETEC-vaccin (multivalent ETEC-vaccin; MEV) som har utvecklats vid Göteborgs Universitet består av döda bakterier som på sin yta har en stor mängd kolonisationsfaktorer, samt en icke-giftig toxoidkomponent. Tidigare studier har visat att antikroppar kan skydda mot ETEC genom att blockera bindning av bakteriernas kolonisationsfaktorer och toxin till slemhinnan. Huvudsyftet för denna avhandling var att analysera immunsvar mot det nya ETEC-vaccinet hos vuxna svenskar, samt att utvärdera olika sätt att förstärka och mäta dessa immunsvar.

Vi fann att två doser av ett prototypvaccin av MEV gav mycket få biverkningar och gav upphov till produktion av antikroppar som kunde binda till bakteriens kolonisationsfaktorer och toxin. Studier av hur immunsvaret mot det närbesläktade koleravaccinet Dukoral® utvecklades över tid tydde dock på att vi hade mätt immunsvaren något för sent i ETEC-vaccinstudien och därmed missat de starkaste svaren. Därför ändrades tidpunkterna för immunanalyser i senare studier av MEV, vilket ledde till säkrare resultat. Våra observationer tydde även på att vi genom att studera den tidpunkt då immunceller från slemhinnan vandrar via blodbanan tillbaka till tarmen efter vaccination kan mäta om ett vaccin ger upphov till immunologiskt minne, dvs förmågan hos immunsystemet att reagera snabbare och effektivare mot en infektion som har påträffats tidigare och därmed skydda mot infektion under lång tid.

Eftersom MEV senare skulle testas tillsammans med en ny immunförstärkande substans, ett s.k. adjuvans (dmLT), undersökte vi även hur dmLT kunde påverka immunceller. Vi fann att dmLT kunde förstärka funktionen hos T-celler från personer vaccinerade både mot ETEC och andra vacciner. Eftersom T-celler kan påverka bildningen av antikroppar och immunologiskt minne kan detta ha betydelse för hur vaccinet kan skydda mot ETEC-infektion. Vi undersökte även funktionen hos antikroppar som producerats efter vaccination med MEV med och utan dmLT-adjuvans. Vi

fann att antikropparna kunde binda både till de kolonisationsfaktorer som fanns med i vaccinet och till närbesläktade kolonisationsfaktorer. Detta tyder på att MEV kan ge ett brett skydd mot olika typer av ETEC-bakterier. Vi fann även, med hjälp av en ny analysmetod, att en tredje vaccindos som gavs 1-2 år efter de första två doserna gav upphov till antikroppar som band starkare än de antikroppar som bildades efter de första två doserna. Vi kunde dock inte se någon effekt av dmLT-adjuvans på bindningsstyrkan hos antikropparna.

Sammanfattningsvis har dessa studier på flera sätt bidragit till den kliniska utvärderingen av det nya ETEC-vaccinet. Den första vaccinstudien var ett viktigt steg för att kunna utföra ytterligare prövningar av vaccinet. Vi vet nu även när vi ska mäta immunsvar efter ETEC-vaccination och vi har bättre möjlighet att analysera immunologiskt minne och bindningsstyrka hos antikroppar. Det är vår förhoppning att dessa metoder och resultat ska underlätta fortsatta studier av immunsvar mot ETEC-vaccin och adjuvans hos både vuxna och barn i framtiden.

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