VACCINATION AGAINST CHOLERA AND ETEC DIARRHEA AND INTERVENTIONS TO IMPROVE VACCINE IMMUNE RESPONSES

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Sweden 2009
The pictures on the cover page show a hospitalized child with diarrhea, a child receiving oral cholera vaccine at the field clinic and a child receiving zinc supplementation.

Printed by Geson Hylte Tryck
Gothenburg, Sweden, 2009
Dedication

This thesis is dedicated
-to my late father Amjad and to my wonderful mother, Fahmida, who have raised me to be the person I am today and always, supported my endeavors
-to my beloved wife, Chuty, who inspires me to be all that I can be
-and my inspiration, of course, to my two kids, Ariana and Tanisha, who are my constant companions, delights, and irritants

“The world is my country, all mankind are my brethren,
and to do good is my religion”

-Thomas Paine
Vaccination against cholera and ETEC diarrhea and interventions to improve vaccine immune responses

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Department of Microbiology and Immunology, Institute of Biomedicine at the Sahlgrenska Academy, University of Gothenburg

Abstract

*Vibrio cholerae* O1 and enterotoxigenic *Escherichia coli* (ETEC) together account for the majority of bacterial causes of acute dehydrating diarrhea in children in Bangladesh. Vaccines should be considered as an important public health tool for prevention of these diarrheal diseases. However, a limitation for the use of vaccines in developing countries is that the efficacy and immunogenicity of vaccines, especially oral enteric vaccines, are lower in these countries than in the industrialized world. The main objectives of the thesis were to study the safety and immunogenicity of oral cholera toxin B subunit (CTB) containing inactivated whole cell ETEC and cholera vaccines in young children in a developing country and to identify possible immune modulating factors, e.g. vaccine dose, different buffer formulations, effects of breast milk withholding and zinc supplementation.

For determining optimal doses of the ETEC vaccine, we immunized 6 months to 12 year old children with full, half and quarter doses of the ETEC vaccine. Safety and immunogenicity of different vaccine doses were compared. All doses of the ETEC vaccine were found to be equally immunogenic in the older children. However, a quarter dose, although giving somewhat lower antibacterial responses than a full dose, was required for children 6-18 months to avoid reactogenicity.

For determining the safety and immunogenicity of the cholera vaccine in young children and the effect of different interventions to try to enhance immune responses, children 6-18 months of age were given two doses of the vaccine according to the standard protocol or with different modifications. In addition to analyzing antibacterial and antitoxic B-cell responses, T-cell responses were determined using a new flowcytometric technique, FASCIA. The vaccine was found to be safe and to induce both antibody and Th1 type T-cell responses. Vibriocidal antibody responses were improved by temporarily withholding breast-feeding for three hours before immunization as well as by giving 20 mg of zinc from 3 weeks prior to and one week after the second dose of vaccine. Zinc supplementation also enhanced IFN-γ responses to CTB.

Further objectives of this thesis were to analyze the immune responses to one of the most prevalent ETEC colonization factors (CFs), i.e. CS6, in patients infected with CS6-positive ETEC and to evaluate if there is an association between expression of certain Lewis blood group antigens of the host and infection by ETEC expressing different CFs. Natural infection with CS6 ETEC was found to induce robust systemic and mucosal immune responses in 70-90% of adults and children with diarrhea caused by CS6 positive ETEC strains, suggesting that CS6 could be an important immunogenic component of a new ETEC vaccine. We could also show that individuals with Le (a+b-) blood group had increased susceptibility to infection with ETEC expressing CFA/I group fimbriae.

The results of these studies give important background information regarding the possibility of inducing effective immune responses to oral inactivated enteric vaccines in young children in developing countries.

**Keywords:** *Vibrio cholerae*, ETEC, oral vaccine, CS6, CFA/I, Lewis blood group, zinc, breast feeding, T cell, B cell

**ISBN 978-91-628-7789-7**
Original Papers

This thesis is based on the following papers referred to in the text by the given Roman numerals:


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<th>Description</th>
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<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>ALS</td>
<td>Antibody in lymphocyte supernatants</td>
</tr>
<tr>
<td>ASC</td>
<td>Antibody secreting cell</td>
</tr>
<tr>
<td>BC</td>
<td>Birth cohort</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin B subunit</td>
</tr>
<tr>
<td>CF</td>
<td>Colonization factor</td>
</tr>
<tr>
<td>CFA</td>
<td>Colonization factor antigen</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>chMP</td>
<td><em>Vibrio cholerae O1</em> membrane protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CS</td>
<td>Coli surface</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme linked immunospot</td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
</tr>
<tr>
<td>FASCIA</td>
<td>Flow cytometric assay of specific cell-mediated immune response in activated whole blood</td>
</tr>
<tr>
<td>Fuc</td>
<td>L-Fucose</td>
</tr>
<tr>
<td>FUT</td>
<td>Fucosyl transferase</td>
</tr>
<tr>
<td>Gal</td>
<td>D-Galactose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GM1</td>
<td>Ganglioside monosialic acid 1</td>
</tr>
<tr>
<td>GMT</td>
<td>Geometric mean titer</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>ICDDR,B</td>
<td>International Centre for Diarrhoeal Disease Research, Bangladesh</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>Heat labile toxin</td>
</tr>
<tr>
<td>Le</td>
<td>Lewis</td>
</tr>
<tr>
<td>mCTB</td>
<td>Mutant/modified CTB</td>
</tr>
<tr>
<td>MSHA</td>
<td>Mannose-sensitive haemagglutinin</td>
</tr>
<tr>
<td>NCHS</td>
<td>National center for health statistics</td>
</tr>
<tr>
<td>n.t.</td>
<td>Not tested</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>rCTB</td>
<td>Recombinant CTB</td>
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<tr>
<td>RF</td>
<td>Responder frequency</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
</tr>
<tr>
<td>ST</td>
<td>Heat stable toxin</td>
</tr>
<tr>
<td>TCP</td>
<td>Toxin-coregulated pilus</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Vacc</td>
<td>Vaccine</td>
</tr>
<tr>
<td>VCO1</td>
<td><em>Vibrio cholerae O1</em></td>
</tr>
<tr>
<td>WC</td>
<td>Whole cell</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZnDef</td>
<td>Zinc deficient</td>
</tr>
<tr>
<td>ZnSuf</td>
<td>Zinc sufficient</td>
</tr>
<tr>
<td>ZnVacc</td>
<td>Zinc plus vaccine</td>
</tr>
</tbody>
</table>
INTRODUCTION

The noninvasive diarrheal pathogens *Vibrio cholerae* O1 and enterotoxigenic *Escherichia coli* (ETEC) together account for the majority of bacterial causes of acute diarrhea in hospitalized and community based settings in children in Bangladesh. Overall, these two pathogens cause about 35% of the hospitalization due to diarrhea in children up to 5 years of age. The two pathogens share many clinical and epidemiological features. Peak rises in rates are seen twice a year, once in the spring and then again in the post-monsoon season with additional peaks during natural disasters (Figure 1).

![Graph showing the percentage of isolated pathogens over the month of isolation](image)

**Figure 1.** Isolation of enterotoxigenic *E. coli* (ETEC) and *V. cholerae* O1 (VCO1) from diarrheal stools of under-5 children obtained from the 2% systematic sampling at International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) Dhaka Hospital during the period of 2002-2007.
Both ETEC and *V. cholerae* O1 cause dehydrating diseases in adults and children. Cholera can cause severe disease in both children and adults while ETEC diarrhea is often more severe in adults (128). Both pathogens induce mucosal and systemic antitoxic as well as antibacterial immune responses in patients (124, 181) and effective vaccines should stimulate such responses. Immunity in these diseases is dependent on the stimulation of the mucosal immune system and generation of secretory IgA (sIgA) antibodies in the gut associated lymphoid tissue (72, 96), and antibodies present on the mucosal surfaces of the gut as well as memory B cells can protect against subsequent disease.

The control of diarrheal diseases has made progress over the past decade. However, even now about 2.0 million children die each year from diarrheal diseases that are potentially vaccine preventable. If effective vaccines could be made available against *V. cholerae* and ETEC, a large proportion of the diarrheal disease burden would be decreased. Additionally, the prevention of disease in children during the first 5 years of life could also reduce mortality. The World Health Organization and other international agencies have given high priority to the control of cholera and ETEC diarrhea through vaccination, since effective vaccines appear to be the most appropriate preventive interventions for the developing world.

The development of candidate vaccines for children in developing countries is however associated with substantial problems, since these children often fail to mount strong immune responses to different vaccines. Effective vaccination strategies require to be optimized to overcome the hyporesponsiveness and studies to determine the role of undernutrition, including micronutrient deficiency, environmental factors, breast feeding patterns and the influence of genetic factors would be important to improve immunogenicity as well as the effect of different doses of vaccine and the role of adjuvants.

A whole cell killed cholera vaccine containing B subunit of cholera toxin (CTB) is licensed in many countries of the world, while an oral inactivated ETEC vaccine with a similar formulation as that of the cholera vaccine has been tested in Phase III studies in
large groups of both adults and children (139, 145). Both of these vaccines have proved efficacious when tested in adults but particularly the ETEC vaccine has been found to be less effective in children in resource poor settings, e.g. in Egypt and Bangladesh (116, 139, 145). To make vaccines effective for infants and young children in such settings, there is a need for improved composition of the candidate vaccines and/or modified immunization regimens. The issues relevant to the composition of the candidate vaccines need attention, but equally important are other factors that may affect vaccine responses, e.g. the nutritional status of the vaccinees, environmental factors and genetic diversity.

CHOLERA

Cholera Epidemiology

_V. cholerae_ O1 is a major diarrheal pathogen (35) causing millions of cases and at least 200,000 deaths in adults and children each year (35, 91, 93). It is assumed that there are at least 300,000 severe cases and 1.2 million infections in people in Bangladesh alone. The rate of cholera varies from around 1 to 8 per 1000 people and the highest attack rate is in children 2 to 9-year of age (124). Cholera is now also being documented in very young children (35, 148). After colonizing the proximal small intestine, the bacteria produce cholera toxin (CT), the major virulence factor for all toxigenic strains of _V. cholerae_. CT is a heterodimeric exotoxin which consists of a single, enzymatically active A subunit non-covalently associated with five identically-sized B subunits responsible for binding to ganglioside monosialic acid 1 (GM1) receptors on epithelial cells (50). CT activates adenylate cyclase in the mucosal epithelium causing a profuse secretory diarrhea, which is a characteristic feature of cholera disease.

Natural protection against cholera

Studies to-date in patients with cholera suggest that different components of the immune system, both humoral and cell mediated, innate as well as adaptive, are activated in response to natural infection (8, 119, 125). The best studied responses are the humoral immune responses and both mucosal and systemic antibody responses have been found to be related to protection (70, 155, 158). The serological responses such as the complement mediated vibriocidal antibody response, antibody responses to lipopolysaccharide (LPS)
and CT as well as to protein antigens have been found to be significantly increased in response to clinical cholera (26, 70, 158). The antibacterial responses include, in addition to LPS, responses to the toxin-coregulated pilus (TCP), which is a colonization factor and potentially protective antigen (9, 165, 177), as well as to the mannose sensitive haemagglutinin (MSHA), a type IV pilus antigen (76) which is also immunogenic and gives rise to antibody secreting cell (ASC) responses and fecal as well as plasma antibodies in patients (123) (Table 1). SIgA antibodies to the major protective antigens have been detected in mucosal secretions of patients, e.g. in intestinal lavages, feces as well as in breast milk and saliva specimens. Of these, fecal extracts have been found useful due to the ease of collection, and relatively satisfactory mucosal responses have been estimated in patients and vaccinees using these samples (70, 72, 147, 155). There is however a need for more sensitive analytical methods and appropriate clinical specimens to better gauge the mucosal response.

Table 1. Immune responses to specific protective antigens of Vibrio cholerae O1 in response to natural infection.

<table>
<thead>
<tr>
<th>Antibody responses in</th>
<th>Serum</th>
<th>Stool</th>
<th>Saliva</th>
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<tr>
<td>CTB</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>LPS</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TCP</td>
<td>+</td>
<td>+</td>
<td>n.t.¹</td>
</tr>
<tr>
<td>MSHA</td>
<td>++</td>
<td>+</td>
<td>n.t.</td>
</tr>
<tr>
<td>Vibriocidal</td>
<td>+++</td>
<td>-</td>
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¹ n.t. stands for ‘not tested’

Cholera vaccines

Vaccines which reduce the rates of cholera will provide an overall health benefit for children and adults who are at risk of disease. There are currently three oral cholera vaccines that are licensed in different parts of the world. The first, Dukoral®, has been developed at the University of Gothenburg and is commercially produced by SBL Vaccin, Stockholm, Sweden. This vaccine contains recombinant CTB plus heat and
formalin killed *V. cholerae* organisms thus stimulating both anti-bacterial and anti-toxic immunity (Box 1).

**Box 1.** Composition of the cholera vaccine used in the studies.

<table>
<thead>
<tr>
<th>WC-CTB-Cholera Vaccine (Dukoral)¹</th>
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<tr>
<td>Consists of the following <em>V. cholerae</em> O1 components (1x10¹¹ bacteria/dose):</td>
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<tr>
<td>Formalin-killed El Tor Inaba (strain Phil 6973)</td>
</tr>
<tr>
<td>Heat-killed Classical Inaba (strain Cairo 48)</td>
</tr>
<tr>
<td>Heat-killed Classical Ogawa (strain Cairo 50)</td>
</tr>
<tr>
<td>Formalin-killed Classical Ogawa (strain Cairo 50)</td>
</tr>
<tr>
<td>plus 1 mg of rCTB</td>
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¹WC stands for whole cell

This cholera vaccine should be given as two doses to individuals ≥6 years, and as three doses to children aged 2-6 year, at 1–6 week intervals between doses, with a buffer to protect CTB against stomach acidity. Before being licensed, this vaccine was extensively tested in both adults and children in large field trials in cholera endemic areas (24, 28, 85, 94) and it is now licensed in over 50 countries of the world, including Sweden and Bangladesh. The vaccine provided a very high degree of short term protection in all age-groups, 85-90% (26), but a more lasting protection in adults (~60% during 3 years) than in children in a field trial carried out in Matlab in Bangladesh (26). Subsequent analyses of data from the field trial in Bangladesh showed that a greater than 90% reduction in cholera disease burden could be achieved by this vaccine through herd protection, even when the level of coverage was only moderate (~50% - 60%) (5, 6, 91). The vaccine gives rise to intestinal sIgA responses directed against CTB as well as against *V. cholerae* LPS, which are thought to synergistically contribute to the protection afforded by the vaccine (118, 125, 147, 155, 156) (Table 1). The vaccine enhances serum vibriocidal antibody responses, which is known to be the best available indirect correlate of
protection after oral immunization or infection (105, 106); it also induces systemic antibody responses against CTB and LPS (71, 125, 155). However, less is known about the T-cell responses induced after immunization with this cholera vaccine. In mice, T-cell responses to CT are strictly dependent on the presence of CD4+ T cells (39, 97, 98). Studies also suggest that humans mount CTB-specific T-cell responses to the oral cholera vaccine (21, 87).

The cholera vaccine has mostly been tested in adults and children ≥2 years, but the disease is also seen in infants and under 2 year old children (148, 153). Therefore, it is important to test the vaccine in younger children down to 6 months of age where the disease is prevalent, especially when maternal antibody protection wanes and weaning from breast feeding is generally initiated (53, 54, 104).

The second licensed oral cholera vaccine, CVD 103HgR or Orochol® that was previously produced by Berna/Crucell, is a single-dose, live attenuated vaccine. It was derived from the classical Inaba 569B strain with 94% deletion of the enzymatically active A-subunit of the cholera toxin leaving only the immunologically active B-subunit (29). This vaccine was shown to be safe and immunogenic in various trials in North America (81), Switzerland (30), Peru (55), Indonesia (149, 152) and in HIV seropositive individuals in Mali (110) and was also protective in challenge studies in the US (164). However, a large field trial with more than 67,000 subjects in Indonesia failed to show protective efficacy (133). Production of this vaccine was stopped several years ago (93).

Another killed oral whole cell cholera vaccine is available which is produced in Vietnam by the local manufacturer Vabiotech following technology transfer from Sweden. This vaccine consists of killed *V. cholerae* O1/O139 whole cells (WC) and has been shown to be safe and immunogenic in subjects aged 1 year and older (171) and to have 50% long term effectiveness in Vietnam (168). This vaccine was initially only licensed in Vietnam but has very recently also been licensed in India. In order to expand the use of this vaccine globally, the vaccine has been reformulated, and is currently under trial in Kolkata, India (99); production is now being conducted by a WHO-prequalified vaccine manufacturer in India (Shanta Biotech, India).
Several other live and killed candidate vaccines have been developed or are currently in development. Among them, Peru-15 (80, 120, 121, 166), *V. cholerae* 638 (48), CVD 111 (163, 167) and a combined B-subunit bivalent O1/O139 vaccine (70) should be mentioned.

**ETEC**

**ETEC epidemiology**

It has been estimated that diarrhea due to ETEC alone causes 650 million episodes of diarrhea and over 380,000 deaths annually in children less than five years of age (13, 15), but ETEC diarrhea are also frequent in adults in endemic countries (184) as well as in travelers to these regions (14, 73). The clinical symptoms of the disease include watery diarrhea often accompanied with abdominal cramps, malaise, and low grade fever. The disease may last from 3-7 days and symptoms range from mild diarrhea to dehydrating cholera like disease, which is seen in about 5% of cases and mostly in adults (128).

**Pathogenesis and mechanisms of immunity against ETEC diarrhea**

The pathogenicity of ETEC is due to the ability of the bacteria to colonize the small intestine and produce one or both of two types of toxins, the heat-stable (ST) and/or heat-labile (LT) enterotoxin (6, 13, 128, 141, 160). The bacteria also possess a variety of surface located adhesins, termed colonization factors (CFs) that attach them to intestinal mucosal receptors (41, 45, 172). The LT toxin has a similar structure as CT, whereas ST is a small non-immunogenic protein. After colonization, toxin secretion increases intracellular cAMP or cGMP which leads to hypersecretion of water and electrolytes into the bowel lumen in a similar way as CT.

Natural ETEC infections are protective with an age related decrease in infection starting from 5 years of age (10, 92). Antibodies that can be induced locally in the gut are believed to be protective and antibodies directed against the CFs have been shown to cooperate synergistically with antibodies to LT in providing protection (3, 160). Studies in animal models and human volunteer studies also suggest that ETEC infections can protect against reinfections (86, 127, 131, 162).
ETEC express a large number of CFs, of which the most common and best characterized ones are CFA/I, and the coli surface (CS) antigens CS1, CS2, and CS3 (collectively designated as CFA/II), CS4, CS5, and CS6 (previously collectively designated as CFA/IV) (46). There are also different related fimbriae, e.g. within the CFA/I and CS5 families (7); within each of these families there are cross-reactive epitopes that have been considered as protective antigens for candidate vaccine development (7, 114, 136).

The CS6 colonization factor of ETEC is seen increasingly in clinical ETEC isolates (138, 146, 187). Most CS6-expressing ETEC strains express ST (LT/ST or only ST). CS6 is a non-fimbrial polymeric protein (3, 128, 131, 135, 189) and has been shown to promote binding of ETEC to rabbit and human enterocytes but not to cultured intestinal cells and other human-derived tissue (61, 62). Very recently, CS6 was shown to bind strongly to sulfatide or sulfatide structures that are present in high concentration in rabbit or human enterocytes (66). The CS6 antigen is present either alone or in association with CS4 or CS5 on ETEC strains producing either ST or both enterotoxin types (46, 128, 187). Little is known about the capacity of CS6 to induce immune responses in humans compared to the other ETEC CFs (63) and it is not clear if anti-CS6 responses may protect against reinfection, since detailed studies of immune response to CS6 have not been carried out in ETEC patients (63). Such information is important for understanding the requirements for and the design of an effective vaccine to protect against CS6-expressing ETEC.

**ETEC vaccines**

Efforts have recently been intensified to develop vaccines for protection against ETEC diarrhea (161, 180). Since both anti-CF and anti-toxic immunity are essential for protection, both types of antigens have been targeted for inclusion in candidate vaccines. Based on the epidemiological and clinical data on ETEC, it is believed that a vaccine suitable for all settings and regions will be one with a multivalent composition containing the major CF antigens as well as an LT toxoid. The ST toxin, although being a potent virulence factor, has not yet been included in vaccine formulations since it is not immunogenic in its native form and efforts to prepare immunogenic conjugates have failed so far (161). A vaccine containing the most prevalent CFs and an LT toxoid has
the potential to provide protection against over 80% ETEC strains all over the world (157, 160).

**Box 2.** Composition of the ETEC vaccine used in the studies.

<table>
<thead>
<tr>
<th><strong>CF-CTB-ETEC Vaccine</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Consists of 5 formalin-inactivated strains of ETEC (1x10^{11} bacteria/dose) expressing:</td>
</tr>
<tr>
<td>CFA/I</td>
</tr>
<tr>
<td>CS1</td>
</tr>
<tr>
<td>CS2</td>
</tr>
<tr>
<td>CS3</td>
</tr>
<tr>
<td>CS4</td>
</tr>
<tr>
<td>CS5</td>
</tr>
<tr>
<td>plus 1 mg of rCTB</td>
</tr>
</tbody>
</table>

Several groups have conducted work to construct inactivated and live vaccine candidates to prevent ETEC diarrhea (161, 184). For one vaccine, the oral CF-CTB-ETEC vaccine, the same concept as used for development of Dukoral has been applied. This ETEC vaccine is composed of inactivated ETEC strains expressing CFA/I and five of the most prevalent CFs (CS1, CS2, CS3, CS4, and CS5) as well as recombinantly produced CTB (rCTB), which is antigenically related to LT (Box 2). This vaccine has been tested extensively in ETEC endemic countries like Egypt and Bangladesh as well as in Swedish volunteers and travelers from the US to Guatemala and Mexico over the last 15 years (2, 57, 69, 117, 129, 139, 144, 145, 161, 179). The vaccine has protected travelers from more severe ETEC disease, whereas it did not afford any significant protection in children in Egypt (161, 180, 184). In Bangladesh, phase I/II studies showed that the vaccine was safe and immunogenic in adults as well as in children down to 18 months of age (117, 129). Since ETEC is most prevalent in infants and young children in developing
countries, causing not only mortality and morbidity but also growth retardation and growth faltering, the vaccine has been tested in children with decreasing age, who are at risk developing of ETEC diarrhea (15, 16, 59).

Based on the high prevalence of CS6-positive ETEC, this CF is now considered an important antigen to incorporate in an ETEC vaccine. Efforts have been made to administer CS6 by different immunization routes, including the oral (42, 79, 180), transcutaneous (56, 189), and intranasal routes in mice (19, 34). Strategies for designing CS6 containing ETEC vaccines for use in humans has included the development of an oral inactivated vaccine (161), oral live attenuated strains expressing CS6 (172, 173) or recombinant CS6 antigen. Efforts to express CS6 in high amounts on ETEC strains (170) is one strategy to optimally deliver the antigen in oral or live vaccine preparations. Another CF antigen, CS7, may also be considered for incorporation in an effective ETEC vaccine, since recent data suggest that it is becoming the most prevalent ETEC in some regions (59) and particularly in children (122).

FACTORS INFLUENCING THE IMMUNE RESPONSES TO ORAL VACCINES

Hyporesponsiveness of vaccines in children in developing countries
The efficacy and immunogenicity of oral mucosal vaccines in children are generally lower in children in developing than in developed countries (138). This has been found to be the case for cholera (52, 133), rotavirus (89, 90, 132), ETEC (160, 161, 180), typhoid vaccines (150) and also for oral polio vaccine (75). There are a number of factors that may contribute to such decreased vaccine “take rates” in children in these settings. These factors may include frequent breast feeding behavior, poor nutritional status, maternal malnutrition and low birth weight of the child. It is believed that maternal trans-placental antibodies and breast milk antibodies as well as non-immunoglobulin factors in breast milk might limit stimulation by the vaccine antigens in the gut and adversely influence the immune responses (138). These effects may be more pronounced in developing countries where breast feeding is more frequent during the first 24 months of life and breast milk may contain higher levels of antibodies against specific pathogens compared
to in developed countries. E.g. breast feeding has been shown to interfere with the serum immune responses to oral rotavirus vaccine, although this effect could be overcome by administering three rather than one dose of the vaccine (132).

The number of doses of vaccine required for a subject in a developed versus in developing countries may be different as has been shown e.g. for the dosage required for oral polio vaccine. The need for higher doses of the live oral cholera vaccine to be immunogenic was seen for children in Indonesia (81, 133) and Bangladesh compared to e.g. in the USA (120). In addition, general malnutrition and specific micronutrient deficiencies can also lead to immune suppression e.g. by inducing villous atrophy which leads to poor absorption of the vaccine components through the intestinal mucosa.

**Interventions to overcome hyporesponsiveness**

There have been several potential suggestions to overcome the problems of hyporesponsiveness such as delaying the vaccine schedule, to lessen the impact of maternal antibodies by separating vaccination from breast feeding to avoid the neutralization of antigen and inhibition by factors in breast milk, and by providing micronutrients e.g. zinc to boost immune responses (4). However, factors which may contribute to lowered immunogenicity of vaccines have not been well studied. Thus, although it is well established that zinc has an influence on multiple aspects of the immune system, including the normal development, differentiation, and function of cells belonging to both innate and acquired immunity (101, 134, 183), the mechanisms responsible for the positive effects of zinc treatment observed after vaccination as well as in diseases such as diarrhea, pneumonia and shigellosis have not been elucidated. Studies have also shown that zinc supplementation may increase the immunogenicity of Dukoral in older children in Bangladesh (4) as well as in Norwegian adults (77), and Bangladeshi infants showed a serotype specific increase in response to a pneumococcal conjugate vaccine when given zinc (107). However, it is still unclear if zinc only promotes immune responses in zinc deficient individuals. Since zinc supplementation is now recommended for all the children with diarrhea in developing countries, it is particularly important to analyze the effects of zinc in children in relation to their individual zinc status.
Influence of the genetic diversity of the host on natural infection

Expression of different ABO histo-blood group types has been shown to be associated with different risks of enteric infections (17, 18, 51, 58, 60, 65, 127, 137), presumably through differential expression of cell surface glycoconjugates that are used as receptors for pathogens infecting the intestinal mucosa. Blood group antigens are also expressed in the intestinal mucosa and in the meconium (78). Our recent study showed that ETEC diarrheal episodes were more common in children with blood group AB and A than in blood group O individuals (127). A predisposition for dehydrating cholera has been seen in blood group O individuals (25, 51, 60, 175).

In addition to the interaction with the ABO blood groups, interest has also been focused on the Lewis blood group antigens which are present in mucosal secretions, on mucosal epithelial cells and naturally adsorbed on erythrocyte membranes (64, 82, 83, 88, 103). In the intestinal mucosa, the Lewis antigens are synthesized through a group of glycosyltransferases, which insert fucose residues in type 1 and type 2 oligosaccharide precursors (102, 182). The synthesis of Lewis antigens is dependent on the fucosyl transferase 2 and 3 genes (FUT2 and FUT 3) (Figure 2). If both genes are functional, the phenotype of the Lewis antigen is Le (a-b+), whereas individuals in whom the FUT2 gene is not expressed are Le (a+b-). Failure to express both FUT2 and FUT3 will result in the less prevalent Le (a-b-) variant. The Lewis a-b+ phenotype is termed as secretor positive, while the Lewis (a+b-) is termed as the non-secretor status (33).

Recent studies have shown that CFA/I expressed by ETEC binds to glycosphingolipids that are associated with Lewis a antigen (67). The glycosphingolipid binding capacity of CFA/I fimbriae resides in the major CfaB subunit protein and similar binding to glycosphingolipids has been demonstrated for CS1 and CS4 (12, 25, 67). However, whether children having specific Lewis blood group antigen phenotypes have different susceptibilities to diarrhea caused by ETEC expressing major colonization factors has not previously been investigated.
Figure 2. Biosynthesis pathways of the human Lewis histo-blood group antigens based on the type 1 and 2 precursors (Fuc, L-fucose; Gal, D-galactose; GlcNAc, N-acetylglucosamine).

A holistic approach to increase the understanding of vaccine related interventions to decrease disease burden from the two major bacterial pathogens causing acute diarrhea in
children is needed. The major aims of this thesis were therefore to determine the immune responses against natural ETEC disease, to examine the influence of host genetic factors on susceptibility to ETEC infections and to identify immune modulating factors on ETEC and cholera vaccine specific humoral and cellular immune responses, including dosing regimens, zinc supplementation and brief breast milk withdrawal.
AIMS

The overall objective of this thesis was to identify different factors and vaccine administration regimens that may influence the immunogenicity of oral inactivated ETEC and cholera vaccines in young children and infants in developing countries.

This includes:

1. To examine the safety and immunogenicity of different doses of a prototype ETEC vaccine in Bangladeshi infants less than 2 years.

2. To investigate the mucosal and systemic immune responses to one of the most common colonization factors, CS6, in patients with ETEC diarrhea.

3. To determine the influence of Lewis blood group phenotypes of the host on the susceptibility to diarrhea with ETEC expressing different colonization factors.

4. To study the safety and immunogenicity of, and different interventions that may improve antibody responses to, the oral inactivated cholera vaccine Dukoral in Bangladeshi children less than 2 years of age.

5. To analyze cholera vaccine specific T-cell responses in Bangladeshi infants and the influence of zinc supplementation on these responses.
MATERIALS AND METHODS

Study sites
Studies were either performed with participants from the ICDDR,B hospital in Dhaka, or in the Mirpur field area. The ICDDR,B is the only international research centre for enteric diseases located in a developing country. Mirpur is located in the urban metropolitan area of Dhaka city around 6-7 km from the ICDDR,B (Figure 3). The area of Mirpur is around 90 sq km and is a densely populated area with 2.5 million inhabitants, corresponding to about 20% of the population in Dhaka City. We chose the Mirpur site for our studies since it is representative of a middle to low-income community, where we had experience in carrying out a large number of field and laboratory based studies over the last 15 years. Our field clinic is located at the centre of sections 10-12 of the Mirpur area. These sections cover about 10 sq km and have a population of around 0.3 million. The safety and immunogenicity studies of vaccines, as well as studies to determine the impact of interventions to improve the immune responses to cholera and ETEC vaccines in young children, were conducted in this study area (Paper I, IV & V). A birth cohort study has previously been performed in Mirpur (127), and was followed up in the present study to determine the relationship between infections with CFA/I-ETEC and Lewis blood group antigen expression by the host (Paper III).

In addition, we also enrolled patients with ETEC diarrhea from the Dhaka Hospital at ICDDR,B to study immune responses against natural ETEC infection (Paper II). The majority of the immunological work was carried out at the immunology unit of the ICDDR,B, e.g. studies utilizing ELISA, enzyme linked immunospot (ELISPOT) and flow cytometric assays (FACS). Additional laboratory work, e.g. FACS and radioactive thymidine uptake assays for measuring T-cell proliferation, was also carried out at the Department of Microbiology and Immunology, the Sahlgrenska Academy at the University of Gothenburg, Sweden.
Figure 3. Study sites
Study participants

Vaccination studies (Paper I, IV, V): For the ETEC and cholera vaccination studies, healthy male and female children aged from 6 months to 12 years were enrolled (Table 2). Around 1200 subjects were screened and those with a history of gastrointestinal disorder, diarrheal illness in the past 2 weeks, febrile illness in the preceding week or antibiotic treatment at least 7 days prior to enrollment as well as children, weight-for-length $<-2SD$ of the median value of the National Centre of Health Statistics (NCHS) were excluded from the study. Also children found to be asymptomatically positive for any bacterial enteric pathogen, including ETEC or \textit{V. cholerae}, were not included in the study. Finally, a total of 668 participants were enrolled in the different studies. The general health status of the children at the time of inclusion in the study was assessed by a study physician.

Table 2: Characteristics of the different studies.

<table>
<thead>
<tr>
<th>Papers</th>
<th>Number of subjects</th>
<th>Type of study</th>
<th>Study site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper I</td>
<td>268</td>
<td>Clinical trial: open and blinded</td>
<td>Community: Mirpur</td>
</tr>
<tr>
<td>Paper II</td>
<td>46</td>
<td>Prospective: CS6-ETEC patients</td>
<td>Hospital: ICDDR,B</td>
</tr>
<tr>
<td>Paper III</td>
<td>462</td>
<td>Prospective and cross sectional: birth cohort</td>
<td>Community: Mirpur</td>
</tr>
<tr>
<td>Paper IV</td>
<td>340</td>
<td>Clinical trial: open</td>
<td>Community: Mirpur</td>
</tr>
<tr>
<td>Paper V</td>
<td>60</td>
<td>Clinical trial: open</td>
<td>Community: Mirpur</td>
</tr>
</tbody>
</table>

For establishment of methods for analysis of T-cell responses to the oral cholera vaccine, six healthy Swedish adults (mean age $34.7\pm5.3$ years, 2 males) were also recruited at the University of Gothenburg (Paper V).
*Lewis blood group study (Paper III):* One hundred and seventy nine children, who had previously participated in a prospective community based birth cohort study (BC) on ETEC diarrhea (127), were enrolled again about 2 years later for determining their Lewis blood groups. To evaluate if children below two years of age had similar distribution of Lewis blood group phenotypes as the older children over four years of age, we also analyzed the distribution of Lewis antigens in a new group of 112 children less than two years of age from the same study area. To compare the distribution of Lewis blood group phenotypes in children and adults, we also studied specimens available from 171 mothers of the BC children.

*CS6 study (Paper II):* To determine the mucosal and systemic immune responses to CS6 expressing ETEC diarrhea, patients with acute watery diarrhea caused by ETEC as the only enteric pathogen were identified at the Dhaka hospital of the ICDDR,B. From 324 ETEC positive patients, 46 patients with diarrhea caused by ETEC expressing CS6 or CS5 plus CS6 were recruited. In addition, apparently healthy age-matched adults and children, living in similar socioeconomic background were included as endemic controls.

Written informed consent was obtained from the adult participants as well as from the parent or guardian of each child before screening and/or enrollment into the study. Assent was also taken from the children who were more ≥8 years of age. The studies were approved by the Research Review Committee (RRC) and Ethical Review Committee (ERC) of ICDDR,B. Ethical permission was also obtained from the Ethical Committee for Human Research at the University of Gothenburg.

**ETEC and V. cholerae antigens and strains used for the studies**

Purified CFs were prepared from disintegrated CFA-positive bacteria using standard ETEC reference strains (40) (Table 3). The purity and concentration of the preparations were determined by spectrophotometry and inhibition ELISA (136). In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were carried out (136). Recombinant CS6 was obtained from Dr. Fredrick Cassels at the Walter Reed Army Research. It was prepared from a bacterial strain *Escherichia coli* (HB101) and a plasmid containing the four-gene operon necessary for CS6 expression was inserted by
recombinant techniques. The CS6 genes were cloned from ETEC strain E8875 (188). Purified CTB was obtained from SBL Vaccin, Stockholm, Sweden; it was highly pure and free of other antigens and bacterial products. A modified CTB molecule with a single amino acid substitution causing reduced binding to GM1 was also produced by recombinant techniques at the University of Gothenburg (74, 84, 140). The ETEC and *V. cholerae* strains used for purification of the antigens used in the studies are showed below (Table 3).

Table 3. ETEC and *V. cholerae* strains used for antigen preparation and/or immunological analyses in studies

<table>
<thead>
<tr>
<th>Strains</th>
<th>Antigens</th>
<th>Toxin types</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>325542-1</td>
<td>CFA/I</td>
<td>ST</td>
</tr>
<tr>
<td>258909-3</td>
<td>CFA/I</td>
<td>ST/LT</td>
</tr>
<tr>
<td>H10407</td>
<td>CFA/I</td>
<td>ST/LT</td>
</tr>
<tr>
<td>E11881A</td>
<td>CS4+CS6</td>
<td>ST/LT</td>
</tr>
<tr>
<td>E1392-79</td>
<td>CS1+CS3</td>
<td>ST/LT</td>
</tr>
<tr>
<td>278485-2</td>
<td>CS2+CS3</td>
<td>ST/LT</td>
</tr>
<tr>
<td>E17018A</td>
<td>CS5+CS6</td>
<td>ST/LT</td>
</tr>
<tr>
<td>VM75688</td>
<td>CS5+CS6</td>
<td>ST/LT</td>
</tr>
<tr>
<td>334A/E29101A</td>
<td>CS7</td>
<td>ST/LT</td>
</tr>
<tr>
<td>E8875/HB101</td>
<td>rCS6</td>
<td>ST</td>
</tr>
<tr>
<td>E7476A</td>
<td>CS14</td>
<td>ST</td>
</tr>
<tr>
<td>E20738 A</td>
<td>CS17</td>
<td>LT</td>
</tr>
<tr>
<td>286C2</td>
<td></td>
<td>LT</td>
</tr>
<tr>
<td><em>V. cholerae</em> O1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ogawa/X25049</td>
<td>LPS</td>
<td>CTB</td>
</tr>
<tr>
<td>Ogawa/X25049</td>
<td>MP</td>
<td>CTB</td>
</tr>
<tr>
<td>569B</td>
<td>rCTB</td>
<td>CTB</td>
</tr>
<tr>
<td>569B</td>
<td>mCTB</td>
<td>CTB</td>
</tr>
</tbody>
</table>
Standard vaccination protocols (Paper I, IV & V)

Both ETEC and cholera (Dukoral) vaccines were obtained from SBL Vaccin, Stockholm, Sweden. The ETEC vaccine (CF-CTB-ETEC) was composed of a total $\sim 1 \times 10^{11}$ CFU of five strains of ETEC. A full 6-ml dose contained 1 mg of rCTB plus $\sim 10^{11}$ formalin-inactivated bacteria of altogether five different ETEC strains producing CFA/I, CS1, CS2, CS3, CS4, CS5 (Box 2). The placebo used in the ETEC vaccination study (Paper I) consisted of $\sim 1 \times 10^{11}$ CFU of heat killed *E. coli* K-12 bacteria. Different volumes of the ETEC vaccine or placebo were formulated in buffer to prepare the different doses. A sachet containing 2.8 g of standard bicarbonate buffer (SBL) was diluted with 150 ml of water. Children over 6 years of age were administered the ETEC vaccine in 75 ml of buffer while those 2–5 years were administered vaccine with 50 ml of buffer and infants 6–17 month were administered the vaccine in 15 ml of buffer.

The cholera vaccine (Dukoral) consists of $\sim 1 \times 10^{11}$ inactivated *Vibrio cholerae* O1 bacteria plus 1 mg of rCTB (Box 1). Immediately before use, each dose of Dukoral was mixed with 20 ml of standard bicarbonate buffer.

Each dose of two-dose regimens of either ETEC or cholera vaccines was given at intervals of 2 weeks. Both vaccines were given orally using a teaspoon to children 6-18 month old. The study children were not allowed to eat 1 h before and 1 h after vaccination and were observed for 1 h in the field clinic after vaccination. Post vaccination surveillance for reactogenicity was carried out for 3 days after each vaccination. The guardians were requested to return to the health clinic at the field site with the children in an event of adverse events, in cases in which they needed clinical support. Each type of reaction was scored as mild (noticeable), moderate (affecting normal daily activities) or severe (suspending normal daily activities) as defined in an earlier study (117). All loose stools were tested for enteric pathogens including bacterial and common parasites.
Dose finding study for ETEC vaccine (Paper I)

For the dose finding ETEC immunization protocol, we initiated an open pilot study in children 6 months to 12 years of age. The study was carried out in decreasing age groups, starting with 6–12-year old children followed by 2–5-year old and finally 6–17 month old children. The children aged 2 years and above received either a full, half or a quarter dose. Thereafter, 6–17 month old infants received a half or quarter dose of the vaccine in two different concentrations of bicarbonate buffer (half and full strength buffer) or a quarter dose of placebo in full strength buffer.

Enhancement of cholera vaccine specific immune responses (Paper IV and V)

To identify factors that may enhance the immunogenicity of the oral inactivated whole cell cholera vaccine (Dukoral) in young children and infants in Bangladesh, we studied the effects of different interventions, i.e. breast milk withholding for 3 h prior to and 1 hour after immunization (Paper IV) and zinc supplementation starting 3 weeks before administration of the first dose of vaccine until 1 week after the second dose (Figure 4) (Paper IV and V) on the immune responses induced by the vaccine. We also compared immune responses induced by the vaccine when given it with (i) the standard bicarbonate buffer (SBL Vaccin, AB), (ii) the same volume of water or (iii) without any additional fluid (Paper IV).

![Figure 4. Vaccination and zinc intervention schedule. ‘Vacc’ stands for vaccine, ‘ZnVacc’ stands for zinc plus vaccine and ‘Zn’ stands for zinc only groups; in addition ‘D’ stands for day.](image-url)
Collection of clinical samples (Paper I-V)

For the vaccination studies, both stool (5 g) and venous blood (1.5-3 ml) were collected from each subject prior to immunization and then 7 days after the first and 7 days after the second dose of vaccination (Paper I, IV & V). Baseline samples were also collected prior to initiation of as well as at the end of the zinc supplementation (Paper IV & V). In addition, to determine cholera vaccine specific T-cell proliferation, 50 ml of blood was collected from adult Swedish volunteers before and 7 and 14 days after the second dose of vaccine for validating the novel flow cytometric technique with traditional radioactive thymidine incorporation methods (Paper V).

To determine the immune responses to CS6 expressing ETEC diarrhea, stool samples (5 g) as well as venous blood (5-10 ml) were collected from the children and adult patients at the acute stage (~day 2) as well as at different time points (days 7 and 21) after onset of infection (Paper II). Blood and stool samples were also collected once from healthy age matched control subjects.

For determining the relation between Lewis blood groups and ETEC infection, venous blood (3 ml) and saliva samples (500 μl) were collected from children of a previous birth cohort (BC) study (127), who were 4-6 year of age at the time for the renewed sample collection, and from newly recruited children from the same area, who were less than 2 years of age, as well as from the mothers of the BC children (Paper III).

Identification of ETEC and other enteric pathogens in stool (Paper I-V)

The monthly as well as diarrheal stool samples collected from the participants in the BC and CS6 studies were analyzed for ETEC as previously described using GM1-ELISA for LT and ST expression and dot blot assays for analysis of CFs including CS6 (127, 151) (Paper II and III). The stool samples were also cultured for other enteric pathogens, e.g. *Vibrio cholerae* O1/O139, *Salmonella*, *Shigella* and *Campylobacter* spp., as well as analyzed for rotavirus by ELISA (186) and tested by direct microscopy to detect cyst and vegetative forms of parasites and ova of helminthes (186). Stools from healthy children were similarly screened, and those subjects that were found to be negative for enteric pathogens were recruited as controls for CS6 studies.
Determination of antibody responses in serum or plasma (Paper I, II, IV & V)
Serum separated from blood, or plasma samples collected from the top of the Ficoll gradient, were stored in aliquots at -20°C until ELISA was performed. Specific IgA and IgG antibody response to CFs and rCTB were measured by ELISA (68, 143). To determine immune response to Dukoral, plasma samples were tested for vibriocidal antibodies using a *V. cholerae* O1 El Tor Ogawa strain, X25049 as the target bacteria (125) (Paper IV & V). Plasma samples were also analyzed for LPS specific antibodies of both IgA and IgG isotypes (126). Antibody titers were calculated using the computer-based program MULTI (DataTree Inc., USA).

Determination of T-cell responses (Paper V)
For determining the T-cell responses against cholera vaccine in young children by T-cell proliferation assays, we adopted a new flow cytometric T-cell response assay, the flow cytometric assay of specific cell-mediated immune response in activated whole blood (FASCIA) (154) (Figure 5). This assay allows analysis of T-cell proliferation in response to stimulation with specific antigens using small volumes of whole blood. Briefly, after dilution of heparinized blood, cells were cultured at 37°C in the presence or absence of the following antigens: mCTB (10 µg/ml), cholera membrane proteins (chMP, 10 µg/ml and 1 µg/ml), and positive control antigen phytohaemagglutinin (PHA, 1 µg/ml, Remel, USA). After 6 days, cell culture supernatants were collected and the cells were stained with fluorescent tagged antibodies (anti-CD3-APC, anti-CD4-PerCP and anti-CD8-FITC; BD, USA). After lysing the red blood cells, samples were washed and fixed in paraformaldehyde and were analyzed using a FACSCalibur machine (BD, USA) and the FlowJo analysis software (Tree Star Inc., USA). The numbers of blast forming CD3+CD4+ T cells acquired in each sample during 120 seconds were determined and the results were expressed as the numbers of CD4+ T-cell blasts/100 µl of sample. In addition, we compared and validated the FASCIA technique with a standard thymidine incorporation assay (95) in initial setup experiments on vaccinated Swedish volunteers. The concentrations of different cytokines, e.g. IFN-γ and IL-13 were measured in culture supernatants using ELISA as previously described (95), and the levels of IL-4, IL-5, IL-2,
IL-10 and TNF-α by the cytometric bead array (BD Pharmingen) as recommended by the manufacturer.

**Figure 5.** Schematic diagram describing the steps of the FASCIA assay for detection of T-cell responses.
Determination of mucosal antibody responses (Paper I, II & IV)

Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation on Ficoll-Isopaque (Pharmacia, Sweden) from heparinized venous blood for determining the specific antibody responses by antibody-secreting cells (ASC) and antibody in lymphocyte supernatant (ALS) at different time points for patients (Paper II) as well as for vaccinees (Paper I, IV). For determining anti-CS6 fecal IgA responses, fecal extracts were prepared and aliquots were frozen at -70°C until ELISA was conducted (117).

To assess ASC responses, PBMCs were assayed for total and ETEC-specific numbers of ASC by the two-color enzyme-linked immunospot technique (ELISPOT) (31, 69, 115). Cells secreting antibodies of the IgA isotype against CFA/I antigen and rCTB (Paper I) as well as CS6 (Paper II) were determined as described (31, 69, 115). Numbers of antibody secreting cells (per 10^7 PBMC) against the different antigens were determined; a post dosing value of ≥10 ASC/10^6 was considered as a significant response (144).

ALS responses were determined for CFs as well as CTB and LPS (Paper II & IV). PBMC (10^7 cells per ml) from patients and healthy controls and also from children of the vaccination study were cultured in 24-well tissue culture plates for 48 h in 5% CO₂, and supernatants of the cultures were stored at -70°C and tested for antibody responses by ELISA (20, 100, 116, 126). Pooled human sera from previous studies on ETEC vaccinees and cholera patients were used as controls to adjust for inter-assay variations.

To assess fecal IgA antibody responses, the total IgA content in fecal samples was determined by ELISA, using pooled human Bangladeshi milk with a known IgA concentration (1 mg/ml) as the standard (2, 185). Specific IgA responses were determined by using the conventional ELISA technique as described (2, 185). The fecal antigen-specific IgA responses were expressed as the interpolated IgA ELISA titer per μg total IgA; specimens with total IgA contents of <10 μg/ml, acute and convalescent specimens whose total IgA contents varied more than 10-fold, and acute and convalescent specimens with specific IgA titers of <1 were not included in the analyses (126, 128).
Determination of Lewis blood group phenotypes (Paper III)

Lewis blood groups were typed using fresh whole blood in an agglutination test assay according to the manufacturers’ instruction (Figure 5) as well as in saliva samples by a dot-blot immunoassay as described previously (111) (Figure 6).

**Figure 6.** Schematic diagram showing the steps of Lewis blood group determination using whole blood and salivary samples. RBC stands for red blood cells.
**Determination of zinc levels (Paper IV and V)**

Serum zinc levels were determined at the baseline for all vaccinated children and at the end of the study period for children given zinc only or zinc plus vaccine. Serum zinc levels were measured by atomic absorption spectrophotometry. Zinc deficiency was defined as values ≤0.7 mg/L (49).

**Statistical analysis**

Data analyses were carried out using the SigmaStat 3.1 program (SPSS Systat Software, Inc.). Children with ≥2 fold rises in serum or mucosal antibody levels to CFs, CT or LPS in ELISA and ≥4 fold increase of vibriocidal antibodies in serum after vaccination as compared to before immunization were considered as responders (27, 70, 71). For determining the T-cell responses, children with ≥2 fold increase in T-cell counts or IFN-γ levels compared to the responses before vaccination were considered as responders. CF-specific ASC responses of ≥10 ASC/10⁶ PBMC on day 7 (post-infection) was considered positive. Responses were also compared where necessary to healthy controls. Cumulative responder frequency was defined as the responses after intake of the first and/or second dose of vaccine in vaccination studies, and at early and/or late convalescent responses compared to acute stage responses in patient studies. Results are expressed as geometric mean titer (GMT) and standard error of mean (SEM). Paired samples were assessed by the Wilcoxon signed rank test, non-paired samples by the Mann–Whitney U-test and proportion of responses using the χ² or the Fisher exact test. In addition, the Chi-Square or Fisher Exact Tests were also used for determining the Lewis phenotypes with CFA/I group ETEC diarrhea (Paper III). P values ≤0.05 were considered to be statistically significant.
RESULTS AND COMMENTS

Safety and immunogenicity of reduced doses of ETEC vaccine in Bangladeshi infants (Paper I)

In previous phase I studies in Bangladesh, the CF-CTB-ETEC vaccine was found to be safe and immunogenic in adults as well as in children 3–9 years of age (129). It was also well tolerated in children, 18–36 months of age and gave rise to robust systemic and mucosal IgA antibody responses (117). Since ETEC diarrhea is most common in younger children (127), the vaccine was also evaluated in a younger age group 6-17 months of age. A randomized, double blind placebo-controlled study carried out in this age group showed that a full dose of the ETEC vaccine gave rise to adverse events in the form of vomiting and hence the study was terminated before being completed (159).

Therefore, studies were undertaken to evaluate whether a lower dose of the ETEC vaccine would be safe and immunogenic in Bangladeshi infants. For this purpose, a dose finding study was first carried out in 2-12 year old children, to determine the immunogenicity of a full, half and a quarter dose of the ETEC vaccine. These analyses showed comparable plasma antibody responses to vaccine specific antigens against the different doses of vaccine in these children. Thereafter, half and quarter doses were tested in children 6-17 months of age showing that the quarter dose was safe and gave almost comparable immune responses as the higher doses. Based on these results, a randomized double-blind placebo controlled trial of the reduced quarter dose of the vaccine was carried out in infants. The latter study showed no differences in symptoms between the vaccinees and the placebo recipients, confirming that a quarter dose of the vaccine was safe in children aged 6-17 months. The studies also showed that post-vaccination immune responses in the 6-17 month old children were comparable after a half and quarter doses of vaccine.

We also found that response rates and magnitude of responses to CFA/I were somewhat lower in the infants than in the older children, whereas responses to CTB were comparable or even slightly higher in the youngest age group given a quarter dose (Figure 7).
These findings suggest that optimal doses of vaccine for children in developing countries may not be the same as for adults in developed or developing countries. Thus, our results suggest that vaccines need to be evaluated independently in different populations and in different doses to identify optimal dosage for the respective target groups.
Mucosal and systemic immune responses to CS6-expressing ETEC in hospitalized diarrheal patients (Paper II)

Based on the high prevalence of CS6 positive ETEC in different regions of the world, the antigenicity of CS6 has been evaluated as a candidate vaccine antigen expressed by live attenuated strains (172, 173) or recombinantly produced, and administered by different routes (19, 42, 172, 189). In all these studies, immune responses against CS6 have been weak. Hence, we studied the immunogenicity of CS6 after natural infection to determine the levels of anti-CS6 responses in patients with CS6 positive ETEC.

Identification of CS6-ETEC patients

To determine the natural immune responses to CS6, both adult and children patients infected with CS6 expressing ETEC were studied for systemic and mucosal immune responses to CS6 using different immunological techniques, and for analyzing different clinical specimens. Patients with history of diarrhea ranging between 2 and 72 h prior to arrival at the hospital and with confirmed CS6 positive ETEC (CS6 alone or CS6 co-expressed with CS5) as the only pathogen in stool were enrolled for the study; the adult patients suffered more from severe dehydration compared to the children (46% vs. 11%). The CS6 only ETEC strains were mostly of the ST only phenotype (85%) and less of the LT/ST phenotype (15%), whereas the CS5+CS6 strains were equally distributed among ST only (48%) and LT/ST (52%) toxin types.

Immune responses to CS6

Studies of CS6-specific ASC responses at the acute (day 2) and early convalescent stage (day 7) of diarrhea showed ~30-fold higher ASC responses of the IgA isotype \( (P=0.005) \) (Figure 8 A) and also weak responses of IgG and IgM isotypes by day 7 compared to at the acute stage. Response rates of 89-100% were seen in the IgA isotype in children and adults, and all the patients showed IgG responses to CS6 by day 7. Similar trends of IgA responses to CS6 were also seen using the ALS assay. ASC or ALS responses in healthy controls were negligible in both the IgA and IgG isotypes compared to those seen at day 7 post-onset in the patients \( (P<0.001) \). CS6-IgA antibody responses in serum were also seen in patients infected with ETEC expressing CS6 (Figure 8B). The IgA antibody
responses had declined by day 21 as compared to the responses seen on day 7 \((P<0.001)\). However, IgG antibodies to CS6, which peaked at the early convalescent stage, remained elevated up to late convalescence. Comparable antibody responses to CS6 were seen in children and adults with ETEC diarrhea. In addition, both children and adults infected with CS6 expressing ETEC responded with CS6 specific IgA antibodies in stool by day 7 of infection (Figure 8C); the day 7 antibody levels in the patients were significantly higher than the antibody levels seen in stool specimens from healthy controls \((P<0.001)\).

**Figure 8.** CS6-specific systemic and mucosal IgA responses at the acute (d2) and at early (d7) convalescent stage (GMT+SEM). Both children and adults including individuals infected with CS6 alone or CS6 together with CS5 are included in these analyses.

In summary, the results show that CS6 is immunogenic and give comparable immune responses in children and adults infected with ETEC irrespective of whether CS6 is
expressed alone or together with CS5, and that those responses can be measured in mucosal and systemic clinical specimens. Thus, substantial antibody responses of both the IgA and IgG isotypes were induced in plasma as well as in ASCs and ALS specimens. Highest immune responses in serum were found at the early convalescent stage and these levels had decreased already within a couple of weeks in most instances. Thus, our study gives evidence that natural ETEC infection gives rise to robust anti-CS6 responses and that it may be possible to induce such responses by a vaccine expressing sufficient amounts of CS6 in immunogenic form.

**Children with Lewis (a+b-) blood group are more susceptible to diarrhea caused by ETEC expressing CFA/I group fimbriae (Paper III)**

CFA/I has been shown to bind to blood group antigens, particularly Lewis a and related glycolipids, that may be expressed on intestinal epithelial cells in humans (67). To evaluate if individuals with certain Lewis blood groups, e.g. Lewis (a+b-), are more susceptible to infection with ETEC expressing certain CFs, a study was carried out in Bangladeshi children to determine the possible association between Lewis phenotype and susceptibility to ETEC expressing the CFA/I group fimbriae. These studies were undertaken as a follow up of a previous birth cohort study (127). As many as 179 children of the 254 children participating in the original BC study were eligible for follow up.

**Determination of ETEC infection in BC children**

ETEC had been the cause of at least one symptomatic diarrheal episode in 56%, and one or more asymptomatic ETEC infection in 37% of the children in the original birth cohort. Among the 13 colonization factors searched for in the ETEC strains isolated from the BC children, CFA/I, CS3 and CS6 were the major ones identified in isolates from symptomatic as well as asymptomatic stool samples. The frequencies of ETEC strains that had been isolated from diarrheal stool samples and that expressed CFA/I, CS3 (alone or together with CS1 or CS2) and CS6 (alone or together with CS5) were 9.8%, 7.5% and 23.0%; for asymptomatic stool samples the frequencies were 7.7%, 4.0% and 11.4%.
Lewis blood group phenotypic distributions
Lewis blood group phenotypes were determined by a dot blot immunoassay using saliva samples and by a tube agglutination test using fresh red blood cells (RBC) from the 179 BC children aged ≥4 years of age, and also from a group of 112 younger children (aged <2 years) from the same area to evaluate if Lewis phenotypes had been comparable at the time for ETEC infection, i.e. before 2 years of age. When using the saliva test, similar frequencies of Le (a-b+) and Le (a+b-) were found in the older and younger children as well as in adults (Figure 9). However, when analyzing Lewis phenotypes with the RBC agglutination test, 17 of the younger control children were found to have the Le (a+b+) phenotype. Since it has been shown that the Le (a+b+) RBC phenotype converts to the Le (a-b+) phenotype by the age of two years (33), these children were included in the Le (a-b+) group. Our findings support that saliva samples can be used for determination of Lewis phenotypes even in infants as well as in large population based studies.

![Figure 9: Distribution of Lewis blood groups in Bangladeshi children of different age groups and adults as determined by salivary test.](image-url)
Lewis blood group phenotypes and association with ETEC expressing major CFs and different toxin profiles

When analyzing the distribution of symptomatic and asymptomatic ETEC infections in relation to Lewis antigen phenotypes of the BC children, we observed that the Le (a+b-) children were significantly more prone to have symptomatic (71%) than asymptomatic ETEC infections (29%) \((P<0.001)\). In contrast, the prevalence of symptomatic (56%) and asymptomatic (44%) ETEC infections did not differ significantly in the Le (a-b+) children. In addition, when analyzing for a possible association between Lewis phenotype of the BC children and infections with ETEC expressing CFA/I group fimbriae (CFA/I, CS14 and CS17), we observed a significantly higher incidence of symptomatic ETEC infections in the Le (a+b-) than in the Le (a-b+) group \((P=0.032)\); this relationship was even higher for children infected with ETEC expressing CFA/I-group fimbriae in combination with CS3, i.e. CS1+CS3 and CS2+CS3 \((P<0.001)\) (Figure 10). When analyzing the association between Le (a+b-) phenotype and ETEC expressing CFA/I only, no significant relationship was found, probably due to too low number of such infections in the study group.

![Graph](image)

**Figure 10.** Association between Lewis blood group phenotypes and symptomatic ETEC expressing CFA/I alone or CFA/I group fimbriae alone and in combination.
When studying the relationship between infections with ETEC expressing CS5+CS6 or CS6 only and Lewis blood group phenotypes, we did not find any significant relationship, either for children with symptomatic or asymptomatic infections. Similarly, no association between the Lewis blood group phenotype and the toxin profile of the ETEC strains isolated from the BC children was found.

**Combined association of ABO and Lewis blood group with ETEC infection**

We have previously shown that ETEC diarrheal episodes were more prevalent in children of blood group A and AB than of other blood groups (127). When analyzing for the occurrence of ETEC diarrhea in BC children with different combinations of Lewis blood group phenotypes and ABO blood groups, we found that among the blood group A children, Le (a+b-) children were more prone than those with Le (a-b+) to ETEC diarrhea (82% vs. 43%), i.e. children with Lewis secreting blood group antigens were less susceptible to symptomatic infection, although this difference did not reach statistical significance ($P=0.061$).

In summary, our results in children support previous experimental studies of specific binding of CFA/I group fimbriae to certain glycosphingolipids (67). This study is also the first one to suggest that a relationship exists between Lewis blood group (a+b-) phenotype, i.e. non-secretor status, and susceptibility to a bacterial enteric infection.

**Studies of immune responses to cholera vaccine in young Bangladeshi children and the effect of different interventions (Paper IV & V)**

First, the safety and immunogenicity, as well as possible interventions to increase the immunogenicity of the licensed oral cholera vaccine Dukoral was evaluated in 6-18 month old Bangladeshi children. In a second smaller study, we also investigated if immunization with the oral cholera vaccine may induce specific T-cell responses in children 10-18 month old and whether zinc supplementation may enhance such responses.
Cholera vaccination and evaluation of reactogenicity

In these studies, a total of 400 Bangladeshi infants from Mirpur were enrolled for studies of safety and immunogenicity of differently administered oral cholera vaccine (Dukoral) (Table 2). Two different age groups of children, 6-9 month and 10-18 month, were given two doses of vaccine either in different vaccine formulations or after breast milk withdrawal or zinc supplementation (Figure 4). All children were breast fed and healthy according to study requirements and the average baseline zinc levels did not differ significantly among the different intervention groups. Surveillance for possible reactogenicity during 3 days after each vaccine administration did not reveal any adverse events either in children of the different age groups or after vaccination in combination with any of the interventions tested.

Systemic and mucosal antibody responses

In the initial large cholera vaccination study (Paper IV), significantly increased vibriocidal antibody responses were observed both in the older and in the younger age groups of children after intake of two oral doses of Dukoral administered with standard buffer; the overall response rates were comparable in the two age groups (56% vs. 57%). However, whereas the vibriocidal responses were significantly higher after intake of the second dose in the younger children, they were not significantly increased after intake of the second as compared to after the first vaccine dose in the older age group. Both age groups of children responded with higher rates and magnitudes of antitoxin responses after intake of the second than after the first dose of vaccine, both with CTB-IgA as well as CTB-IgG responses (P<0.001). Similar vibriocidal as well as antitoxin antibody responses were also obtained in the second, smaller Dukoral study in 10-18 month old children compared to the first study (Paper IV and V). The post vaccination IgA (25-34%) and IgG (13-34%) responses to LPS were lower than to CTB in all study groups in both studies.

We also compared the immune responses with different vaccine formulations (Paper IV). Except for the significantly lower antitoxin responses when using water or not adding any fluids as compared to standard buffer formulation in the older, 10–18-month old children,
comparable antibody responses were observed in children receiving Dukoral with these three formulations.

Vaccine specific mucosal antibody responses were also determined in ALS specimens using ELISA (Paper IV). Increased CTB specific IgA ALS antibody responses, that were comparable in both age groups, were observed after intake of the first (P<0.05) and second vaccine dose (P<0.05) in all study groups (cumulative responder frequency, 72-88%). No differences in ALS antitoxin IgA titers were observed between the different intervention groups. Responses to LPS in IgA ALS assay were low (~30% responder frequency). These results show that a full dose of Dukoral is safe and immunogenic also in very young children.

**Cellular immune responses**

Since T-cell responses are difficult to measure in children due to the limited volume of blood that is available for testing, we adopted the FASCIA method that only requires small volumes of blood for our studies (Paper V). Initial establishment of the FASCIA technique for analysis of T-cell responses to the oral cholera vaccine was performed using blood samples collected from the adult Swedish volunteers before and after vaccination with Dukoral. In the initial validation assay in adult Swedish volunteers, we found robust T cell blast responses to a variant CTB molecule (mCTB) with reduced GM1 binding capacity in comparison to rCTB. Therefore, this mCTB molecule was used in forthcoming experiments. We also compared the FASCIA method with traditional thymidine incorporation assay and found good agreement between the two methods when assessing T-cell responses to the cholera vaccine. Concentrations of different cytokines were measured in culture supernatants from the FASCIA cultures using ELISA as well as by the cytometric bead array technique.

After two doses of Dukoral, mCTB gave rise to significantly increased numbers of CD4+ T-cell blasts compared to the pre-vaccination levels (P=0.011) whereas the proliferative T-cell response to a membrane protein preparation of *V. cholerae* O1 bacteria did not differ significantly from the baseline responses in any of the vaccination groups (P>0.05) (Figure 11).
Figure 11. Vaccine specific T-cell responses to mCTB and cholera MP (chMP) in Bangladeshi children before (pre) and after (post) intake of two doses of Dukoral. Asterisk indicates significant rise of the T-cell response ($P<0.05$).

Vaccination also induced increased production of IFN-$\gamma$ in response to mCTB stimulation compared to in secretions collected before vaccination ($P<0.05$). No detectable levels of other cytokines (IL-4, IL-5, IL-2, IL-10, IL-13 and TNF-$\alpha$) were found in the culture supernatants.

The results show that the oral cholera vaccine can induce a vaccine specific Th1 T-cell response to mCTB in young children. However, lack of responses to chMP may be due to the presence of a large number of cross reacting antigens in these primed children, who were previously infected with other enteric pathogens.

Interventions to improve vaccine specific antibody responses

The younger, 6-9 month old infants, who were temporarily not breast fed i.e. for 3 hours before vaccination, showed comparable vibriocidal responses as the infants, who received vaccine in the standard buffer formulation (Paper IV). In the older, 10-18 months old children, however, withholding of breast milk resulted in significantly higher vibriocidal antibody responses, both with regard to magnitude and responder frequency than in children given vaccine by the standard protocol ($P<0.001$). This difference in the two age
groups may be due to delayed emptying of breast milk from the intestine of the younger infants compared to the older children. Withholding of breast milk had no influence on antitoxin immune responses in either of the two age groups (Paper IV).

Supplementation with zinc resulted in amplification of the vibriocidal responses in the older children after intake of two doses of vaccine. This result was first obtained in the large Dukoral study (Paper IV) and was later confirmed in the second cholera vaccine study (Paper V, Figure 12). About 3-4-fold higher vibriocidal responses were observed in the zinc supplemented vaccine group compared to in the groups given only the standard vaccination in both studies (P<0.001). However, zinc had no influence on vibriocidal antibody responses in the younger age group (Paper IV). The reason for the differences observed in the different age groups could be because the younger children were less zinc deficient than the older ones. Another explanation may be that the immunomodulating effect of zinc can only be seen in children whose immune system is more developed and receptive to the effect of zinc supplementation. Zinc supplementation did not affect the antitoxin immune responses in either age group (Paper IV and V).

Figure 12. Effect of zinc supplementation on vibriocidal antibody responses to cholera vaccine in 10-18 month old Bangladeshi children before (pre) and after (post) intake of two doses of Dukoral (Paper V). ‘Vacc’ stands for vaccine and ‘ZnVacc’ stands for zinc plus vaccine groups. Asterisks indicate significant rise of the vibriocidal antibody response in pre- versus post-vaccination samples (P<0.05).
We also studied if zinc supplementation influences antibody responses differently in zinc deficient and zinc sufficient children (Figure 13). The effect of zinc on the vibriocidal responses was primarily seen in zinc deficient children, since zinc supplementation significantly enhanced the responses in this subgroup, whereas the responses among zinc sufficient children were comparable with and without zinc supplementation. Among the zinc deficient children, the responder frequencies increased from 54% to 89% as a result of the supplementation (P<0.05), whereas the responder frequencies were comparable in the zinc sufficient children with and without zinc supplementation (71% vs 78%). However, no significant influences of baseline zinc status or zinc supplementation were observed on the CTB- or LPS-specific antibody responses.

**Figure 13:** Zinc deficient children show higher vibriocidal antibody responses (fold increases) to oral cholera vaccine after supplementation with zinc. ‘ZnDef’ stands for zinc deficient and ‘ZnSuf’ for zinc sufficient children. ‘Vacc’ stands for vaccine and ‘ZnVacc’ for zinc plus vaccine groups. Asterisk indicates significantly higher vibriocidal antibody response (P<0.05) in the ZnDef than ZnSuf group after zinc supplementation and ‘ns’ indicates no significant difference between groups.
Our studies have thus documented that brief temporary breast milk withdrawal as well as zinc supplementation for a couple of weeks in zinc deficient children may enhance the antibacterial immunogenicity of the oral cholera vaccine, but that these effects were restricted to children above 9 months of age.

**Influence of zinc on vaccine specific cellular responses**

We also evaluated the influence of zinc supplementation on T-cell activation in 10-18 month old children. Supplementation with 20 mg of zinc before and during the vaccination period did not affect the level of T-cell proliferation induced by mCTB (P>0.05), but enhanced the production of IFN-γ more than 4-fold (P=0.008) (Figure 14). However, we could not detect any clear differences in T-cell responses between children who were zinc sufficient or zinc deficient at the start of the study. This may be due to the small sample size of each subgroup of children and further larger studies might reveal a difference between zinc deficient and sufficient children.

![Figure 14](image)

*Figure 14.* Effect of zinc supplementation on IFN-γ T-cell responses to mCTB in Bangladeshi children before (pre) and after (post) intake of two doses of Dukoral. ‘Vacc’ stands for vaccine and ‘ZnVacc’ stands for zinc plus vaccine groups. Asterisks indicate significant rises of the IFN-γ response to vaccination (P<0.05).
In summary, these are the first studies to show that Dukoral is safe and immunogenic in children as young as six months of age. The vaccine induced both antibody and T-cell responses in these young children. Simple interventions such as changing the breast feeding pattern in relation to vaccination or increased intake of zinc induced significant increases in the vibriocidal antibody responses, which are indirect markers of immunity. The effect of zinc was not restricted to the B-cell responses but also influenced the T-cell responses and induced increased IFN-γ production in response to vaccination.
GENERAL DISCUSSION

The main goals of these studies were to evaluate safety, immunogenicity, optimum immunization regimens and efforts to improve vaccine responses to oral cholera and ETEC vaccines in young children in a developing country. For these purposes, two oral inactivated whole cell vaccines, containing the same CTB component against ETEC diarrhea and cholera, respectively, were evaluated in young children less than 18 months of age in Bangladesh.

Due to adverse reaction in the form of vomiting after taking the ETEC vaccine in 6-17 month old children, we lowered the dose of vaccine given to this age group. We have shown that a lowered, quarter dose of ETEC vaccine was immunogenic in children 6 months to 12 years of age. Both antitoxic and antibacterial antibody response rates to a quarter dose of the vaccine were also almost comparable to those seen to the full dose of the ETEC vaccine in this and earlier studies carried out in Bangladesh and Egypt (57, 117, 145). However, the magnitudes of the antibacterial (anti-CF) antibody responses to the quarter dose were somewhat lower than to the full dose in the 6-17 months old children. Furthermore, the magnitudes of the anti-CF responses were generally lower in the infants than in children >2 years of age to all doses of vaccine in this study as well as when compared to responses in previous studies in Bangladesh (117, 129). Similar findings were found in Egypt where lower post-immunization titers to the ETEC vaccine were seen in young infants (145) compared to in older children (57, 144).

The full dose of Dukoral was not only safe but also immunogenic in young children less than 18 months. We observed similar response rates for vibriocidal antibodies as in a previous study in older children (4). However, like the ETEC vaccine studied in different age groups, lower magnitudes of responses were seen in young (6-18 month) than in older children in spite of that a higher vaccine dose per weight of the child was given to the youngest (4, 118). An age descending, lower magnitude of immune response has also been seen to the live oral attenuated vaccine, Peru-15, in Bangladesh (120). This is probably due to previous priming of older children, resulting in higher responses, as well as to a more mature immune system in these children.
Both the ETEC and cholera vaccines gave rise to comparable mucosal and systemic vaccine specific immune responses to certain common component as well as to homologous LPS. Thus, both vaccines induced strong, comparable levels of systemic IgA and IgG antibody responses to CTB and poor antibody responses to LPS. The ETEC vaccine also induced enhanced, significant antibody responses against the bacterial CFs and Dukoral induced significant antibacterial, i.e. vibriocidal antibody responses in serum.

Although B-cell responses to both cholera and ETEC vaccines have been extensively investigated, the involvement of T cells in protection against cholera has to date not been thoroughly investigated. In this study, we also measured cholera vaccine specific T-cell responses in 10-18 month old children and our results confirm that Dukoral can induce CD4+ T-cell responses against CTB. The stimulated T cells primarily secreted the Th1 type cytokine IFN-$$\gamma$$, but not measurable levels of the Th2 cytokines IL-13, IL-4 or IL-5. The responses were clearly detectable one week after administration of the second vaccine dose, a time point known to be optimal for analysis of ASC response in peripheral blood (32). These findings are also consistent with a previous study demonstrating the presence of increased numbers of IFN-$$\gamma$$ producing cells in the small intestine of adult volunteers one week after administration of the oral cholera vaccine (130). However, the present results reveal for the first time the capacity of Dukoral to induce a vaccine specific Th1 T-cell response in young children, which are likely to influence the B-cell responses induced by infection and vaccination.

Vomiting was observed when the ETEC vaccine was tested in a full dose in children 6-17 month old, but was not seen in those given a quarter doses. The reason for observing more side effects to the full dose of ETEC vaccine in infants than in older children (116, 169, 180) may be directly related to the differences of nutritional status as well as the size of the children. Generally speaking, children in Bangladesh are smaller in size and lower in weight than children of the same age group in developed countries (160). Even children in Egypt, in whom some symptoms of vomiting were observed, had better nutritional status than the Bangladeshi children (145). In contrast, the same number of *V. cholerae* bacteria as used as in the ETEC vaccine could be given without causing any
adverse events even to 6 months old Bangladeshi infants. An interesting observation was that there was also reactogenicity in recipients given a full dose of the *E. coli* K-12 placebo and that a quarter dose of the placebo resulted in decreased frequency of vomiting. This suggests that the number of *E. coli* bacteria needs to be decreased when used for infants, probably due to the lipid A bound 3-deoxy-D-manno-octulosonic acid content of LPS in *E. coli* which is believed to be higher than *V. cholerae* O1 and that might cause this differing toxicity (142, 161).

One limitation to the use of mucosal vaccines is that these vaccines are less immunogenic in children than adults, which is thought to partly be a result of immature lymphocytes and antigen presenting cells (47, 174, 190). In addition, there are a number of factors that may be accountable for inducing lowered immunological and/or protective efficacy in young children in developing countries. These include the nutritional, in particular the micronutrient status of the children, environmental factors as well as differences in genetic makeup of populations which can be contributing elements for modulating responses and can result in less than optimal efficacy of vaccines. In addition, the levels of trans-placental transferred maternal antibodies and frequent breast-feeding practices may also be responsible for this hyporesponsiveness (37, 132, 178). However, factors which may contribute to the lowered immunogenicity of vaccines have not been well studied. In this thesis, the effect on the immunogenicity of the oral cholera vaccine by different interventions, e.g. by altering the formulating buffer, by modification of the breast feeding pattern and by supplementation with zinc, is documented.

Oral cholera vaccines have generally been formulated in buffers to counteract the gastric acidity of the stomach and to protect the acid perishable CTB component from denaturation. However, sometimes buffers are constraints to the use of vaccines as a cheap public health tool since they result in more bulk, leading to costs of shipping and transportation to hard-to-reach areas in developing country settings. We compared the safety and immunogenic profile of different formulations of Dukoral. When Dukoral was reconstituted in water or given without any additional fluid, the safety profile remained unchanged and the vibriocidal responses were comparable with those obtained with standard buffer formulation. However, in the 10-18 months old children, two doses of the
vaccine gave significantly higher antitoxin responses when using standard buffer formulation as compared to when using water or not adding any fluids, whereas there was no difference in the antitoxin responses in the youngest, 6-9 month old children when using buffer or water. These findings suggest that Dukoral may be given with water to young children <10 months to avoid bulk supply and to reduce shipment costs, whereas an acid neutralizing buffer is required to retain the immunogenicity of the CTB component in children >10 months.

We also evaluated different strategies to further improve the immunogenicity of Dukoral. One of the factors that has been suggested to be related to hyporesponsiveness of vaccines in developing countries is frequent intake of breast milk, which may inhibit vaccine take rates (24, 132, 176). Our studies show that the vibriocidal antibody responses were increased when breast-feeding was temporarily withheld for 3 hours prior to immunization. This effect was only seen in children 10-18 month of age and not in younger infants. This may be due to that breast milk remains for a longer time in the intestine of the younger children due to slower gastric emptying (23) and hence withholding breast feeding for 3 hours may not be sufficient in very young children.

We found that about 50% of the children participating in our studies were zinc deficient. Previous studies have shown that zinc is required for normal T-cell function and zinc deficiency has been shown to be associated with decreased vaccine specific T-cell and antibody responses as well as memory responses (36, 43, 44, 113). However, the mechanisms responsible for the positive effects of zinc treatment observed after vaccination as well as in different infections have not been elucidated. Furthermore, it is still unclear if zinc only promotes immune responses in zinc deficient individuals. Our studies of the influence of zinc supplementation on B- and T-cell responses to the cholera vaccine in young children in Bangladesh showed that zinc supplementation can enhance the vibriocidal antibody responses to the oral cholera vaccine, but only in older children 10-18 month of age. We also revealed that the IFN-γ responses after stimulation with a modified CTB molecule were significantly stronger in vaccinated children receiving zinc supplementation compared to children who received only vaccine. In contrast, no influence of zinc was observed on the T-cell proliferative responses to CTB. Previous
studies indicate that zinc supplementation can promote both proliferative and cytokine responses in T cells (1, 38, 43, 112, 113), and it is unclear at present why zinc supplementation only influenced the IFN-γ responses in our study.

We also analyzed if zinc sufficient and deficient children responded differently to the vaccine after having been supplemented with the micronutrient. We observed that zinc supplementation enhanced the vibriocidal antibody responses in the zinc deficient children more than in those children who were not deficient at onset of vaccination. However, this is in contrast to observations in zinc sufficient adults in Norway, who responded with significantly higher vibriocidal antibody responses after zinc supplementation than those given vaccine without zinc (77).

In contrast to the B-cell responses, we did not see any difference in the T-cell responses between children with different baseline zinc status. This may suggest that T cells and B cells have different requirements for zinc for optimal function but need to be confirmed in a larger study. It has been speculated that supplementation with zinc during vaccination may hinder efficient vaccination responses in children who are zinc sufficient already before zinc supplementation (101, 108) and suppressive effects on antitoxic antibody responses by zinc have been observed in studies in older children and adults (77, 118). However, we did not observe any tendency for zinc to suppress either antibody or T-cell responses in any of the study groups. Our data thus support that zinc may be given to children older than 9 months of age, since this is the age group, who are the most at risk of micronutrient deficiency and who may need supplementation to improve vaccine take rates.

Changing patterns of ETEC CFs are now apparent worldwide. CS6 has during recent years emerged as one of the major CFs, e.g. in Mexico, Guatemala, Egypt and Bangladesh (138, 139, 146, 187), making it a key component to be included in an effective ETEC vaccine. Based on disappointing immune responses to CS6 in different candidate vaccines during recent years (19, 34, 42, 56, 79, 180, 189), we wanted to elucidate the immunogenicity of this CF after clinical infections with CS6 expressing ETEC bacteria. This was done by determining the systemic and mucosal antibody
responses to CS6 in patients hospitalized due to diarrhea caused by CS6 positive ETEC. The results of these analyses showed that by early convalescence, most patients responded with significantly increased levels of IgA and IgG antibodies in serum. Similarly, ASC, ALS and fecal IgA responses to CS6 were found in a majority of the patients, supporting the mucosal immunogenicity of CS6.

Thus, since natural infection with CS6 positive ETEC induces comparatively strong mucosal as well as systemic immune responses, inducing of protective immune response to CS6 using suitable candidate vaccines and immunization regimens may be feasible. Studies are in progress to construct *E. coli* bacteria overexpressing CS6 as candidate vaccine strains (170), since clinical ETEC isolates express comparatively low levels of this non-fimbrial CF.

To determine genetic influences on the susceptibility to ETEC infections with major CFs and toxins, we have investigated the relationship between blood group phenotypes and incidence of symptomatic and asymptomatic ETEC infections in a cohort of children in Bangladesh, who were followed from the day of birth (127). In initial studies, we found that blood group A children in this cohort were more susceptible to ETEC diarrhea than children with other blood groups (127). We have now also analyzed if there is a relation between Lewis blood groups and ETEC diseases. The distribution of Lewis blood group phenotypes of Bangladeshi participants was found to be similar to the Lewis antigen distribution in Indian and African populations (11, 22, 33, 109). However, an interesting observation was that the distribution of Lewis (a+b-) phenotype in Bangladeshi children and adults is higher than that seen in developed countries and this could certainly have implications for susceptibility to infections and vaccine interventions as well. Thus, in this study we show that children with Le (a+b-) phenotype had significantly higher incidence of diarrhea caused by ETEC expressing CFA/I group than children in the Le (a-b+) group. This may be related to the recently reported capacity of *E. coli* expressing CFA/I-, CS1- or CS4-fimbriae to bind to Le\(^a\)-terminated glycosphingolipids, while Le\(^b\)-terminated glycosphingolipids are not recognized by these CFs (67). Thus, the present findings support that ETEC bacteria expressing the CFA/I group fimbriae, i.e. CFA/I and related CFs, may bind to Le\(^a\) determinants expressed by intestinal epithelial cells. ETEC
strains expressing CS6 were not associated with the Lewis blood group phenotype. This is also supported by a recent study showing that CS6 antigen does not bind to any type of Lewis blood group determinants, but instead to sulfatide (SO$_3$-$\beta$1 galactosylceramide) (66) confirming the present clinical data. Thus, the blood group phenotype of the host might have important influences on the susceptibility to different types of ETEC in different populations, and the high prevalence of the Le (a+b-) phenotype in Bangladesh suggests that a vaccine, which provides protection against CFA/I group expressing ETEC, is particularly important in this population.

Overall, our results reveal that both a reduced dose of the ETEC vaccine and a full dose of Dukoral is safe and immunogenic in infants and young children in developing countries. Interventions, including use of zinc and brief intermission of breast-feeding, were shown to further enhance the immunogenicity of Dukoral and will be tested alone and in combination for the capacity to enhance immune responses to other oral vaccines, including ETEC vaccines, in the near future. Our data also suggest that incorporation of the CS6 antigen should be beneficial for a new generation of ETEC vaccines, since this antigen can induce robust immune response in both children and adults. Finally, a genetic preponderance of Lewis (a+b-) positive individuals for ETEC diarrhea caused by bacteria expressing CFA/I group fimbriae has been identified. In conclusion, the results of this study are encouraging for the potential use of enteric vaccines in young children in endemic areas, who need such vaccines urgently.

Based on the results of these studies, we recommend that different interventions should be utilized to provide maximal efficacy of different oral vaccines in young children in developing countries. Before going to large scale trials, dosing studies should be performed to avoid adverse reactions but still induce strong immune responses. Different simple interventions that may improve vaccine efficacy, such as temporary withholding of breast milk, should also be evaluated. Identification of proper antigens as well as possibilities to lower the number of bacteria with higher expression of protective antigens should also be considered. Timing of vaccination should also be chosen to avoid vaccination during seasonal epidemic periods. In addition, environmental factors such as arsenic toxicity, which is a common problem in Bangladesh due to poor drinking water
quality, malnutrition and helminthic and parasitic load of the host (138), interaction with vaccines used in the expanded program for immunization, booster dosing, and putative adjuvants, etc that may influence vaccine immunogenicity, should be considered to identify optimal vaccination strategies in developing country settings.
ACKNOWLEDGEMENTS

This thesis arose as a result of years of research that I have done within collaborative studies between the ICDDR,B and the University of Gothenburg. During these years, I have worked with a great number of people whose contribution in assorted ways to the research and the making of this thesis deserve to be specially mentioned. It is a pleasure to convey my gratitude to them all in my humble acknowledgment.

In the first place I would like to record my gratitude to my main supervisors, Ann-Mari Svennerholm and Anna Lundgren at the University of Gothenburg, for their supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences throughout the work. Above all and the most needed, they provided me persistent encouragement and support in various ways. Throughout my thesis-writing period, I have particularly benefited from Anna, who provided constant encouragement, sound advice, good teaching, good company, and lots of good ideas. Their truly scientist intuition has exceptionally inspired and enriched my growth as a student, a researcher and a scientist want to be. I am indebted to them more than they know.

It is difficult to overstate my gratitude to my co-supervisor, Firdausi Qadri (Apa) at the ICDDR,B in Dhaka. With her enthusiasm, her inspiration, and her great efforts to explain things clearly and simply, she helped to make research fun for me. Her involvement with her originality has triggered and nourished my intellectual maturity that I will benefit from, for a long time to come. I gratefully acknowledge Apa for her advice, supervision, and crucial contribution throughout my research and thesis writing periods. I would have been lost without her. Apa, I am grateful in every possible way and hope to keep up our teamwork in the future.

I convey special acknowledgement to Alejandro Cravioto, Executive Director of ICDDR,B, for his indispensable help for approving my PhD funds, so I could optimally carry out my studies at the University of Gothenburg.

I was extraordinarily fortunate in having Susann Teneberg as a collaborator at the University of Gothenburg. I could never have embarked on the Lewis blood group work without her vast knowledge of biochemical binding of Lewis blood group antigens and her help thus opened up unknown areas to me. Thank you.

It is a pleasure to express my gratitude wholeheartedly to Jan Holmgren for his scientific inputs and kind hospitality while I was staying with my family in Gothenburg.

I gratefully thank Amit Saha and Mohiul Chowdhury for their vital support in recruiting and overseeing the studies at the field site in Mirpur. Without their active support, all the research activities had been impossible to accomplish. I would also like to thank all the field staff and study participants who were involved in this research.

My special thanks go to Mohmmad Arifuzzaman, without his tremendous work on the FASCIA and Lewis blood group detection methods would have been unthinkable. Furthermore, I would particularly like to thank my colleagues Taufiqrur Bhuviyan, Abdullah Tarique, Atiqr Rahman and Sajib Chakraborty, who were helping me at the Lab, whenever I needed them. My thanks also go to all the other lab colleagues at the
ICDDR,B, who has done a great job for helping to do all the ELISA and vibriocidal assays. I would also like to thank Joanna Kaim, who helped to set up the FASCIA method in Gothenburg.

Collective and individual acknowledgments are also owed to my colleagues and co-authors of my papers at the ICDDR,B, University of Gothenburg and elsewhere in the world. Many thanks go in particular to Yasmin Begum, Firoz Ahmed, David Sack, Endtz Hubert, Michael Lebens, Nils Lycke, Samuel Lundin, Sanna Cardell, Simin Meydani, Dayong Wu, Daniel Novak, Hanna Stenstad, Sara Tengvall, Sukanya Raghavan, Åsa Sjöling, Ali Harandi, Joshua Tobias, Marianne Quiding, Paul Bland, Ingrid Bölin and Nadir Kadir for giving me such a pleasant time when working together.

Many thanks go to my many student colleagues in Gothenburg for providing a stimulating and fun environment in which to learn and grow. I am especially grateful to Patrik Sundström, Reza, Mamun, Bert Kindlund, Erik Nygren, Claudia Rodas, Åsa Lothigius, Veronica Olofsson, Susannah Leach and Anders Janzon.

I gratefully thank Faozul Kabir, Susanne Uhlan, Andrea Frateschi, Annika Djärf, Tinna Carlsson and Gudrun Wiklund for their indispensable help dealing with travel arrangement, office supplies, administration, shipments and bureaucratic matters during my stay and my commuting between Dhaka and Gothenburg.

I wish to thank my entire extended family for providing a loving environment for me. My parents deserve special mention for their inseparable support and prayers. My Late Father, Amjad Hossain and my Mother, Fahmida Hossain, are those who sincerely raised me with their caring and gently love. Dulabhai (Bulbul), Apa (Ivy), Taposh Bhai, Apee (Iris), Zaman, Farhana, Monty, Mumu, Babu Bhai, Bakul Bhabi, Babul Bhai, Shishir Apu, Dipto, Mohua, Parash, Barna, Niloy, Onindo, Naveel, Naveed and Rahul, thanks for being as supportive and caring family members. Words fail me to express my appreciation to my wife Chuty whose dedication, love and persistent confidence in me, has taken the load off my shoulder. I owe her for being unselfishly let her intelligence, passions, and ambitions collide with mine. Therefore, I would also thank Bazlur and Lutfi Rahman’s family for letting me take her hand in marriage, and accepting me as a member of the family, warmly. And most importantly, my thanks to my daughters, Ariana and Tanisha for giving me happiness and joy.

Finally, I would like to thank everybody who was important to the successful realization of this thesis, as well as expressing my apology that I could not mention all of you personally one by one.

This work was supported by grants from the Swedish Agency for International Development and Corporation (Sida-SAREC), the Marianne and Markus Wallenberg Foundation through the support to GUVAX, the Swedish Medical Research Council, the Sahlgrenska Academy of the University of Gothenburg, and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B).
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