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tachment 1. Date 1/9/96

FACE SHEET) ETHICAL REVIEW COMMITTEE, ICDDR,B.

rincipal Investigator Firdausi Qadri Trainee Investigator (if any) UU

pplication No. 96-014 Supporting Agency (if Non-ICDDR,B) SAREC

itle of Study Further evaluation of the oral ETEC vaccine and studies on the immune responses in acute watery diarrhoea.

Project status:
(✓) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

ircle the appropriate answer to each of the following (If Not Applicable write NA).

. Source of Population:
(a) Ill subjects (Yes) No
(b) Non-ill subjects (Yes) No
(c) Minors or persons under guardianship (Yes) No

. Does the study involve:
(a) Physical risks to the subjects (Yes) No
(b) Social Risks Yes No
(c) Psychological risks to subjects Yes No
(d) Discomfort to subjects (Yes) No
(e) Invasion of privacy Yes No
(f) Disclosure of information damaging to subject or others Yes No

. Does the study involve:
(a) Use of records, (hospital, medical, death, birth or other) (Yes) No
(b) Use of fetal tissue or abortus Yes No
(c) Use of organs or body fluids (Yes) No

Are subjects clearly informed about:
(a) Nature and purposes of study (Yes) No
(b) Procedures to be followed including alternatives used (Yes) No
(c) Physical risks (Yes) No
(d) Sensitive questions Yes No
(e) Benefits to be derived Yes No
(f) Right to refuse to participate or to withdraw from study (Yes) No
(g) Confidential handling of data (Yes) No
(h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure (Yes) No

5. Will signed consent form be required:
(a) From subjects (Yes) No
(b) From parent or guardian (if subjects are minors) (Yes) No

6. Will precautions be taken to protect anonymity of subjects (Yes) No

7. Check documents being submitted herewith to Committee:
___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
✓ Protocol (Required)
✓ Abstract Summary (Required)
✓ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
✓ Informed consent form for subjects
✓ Informed consent form for parent or guardian
___ Procedure for maintaining confidentiality
✓ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

Firdausi Qadri
Principal Investigator

Trainee

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CHECK-LIST FOR SUBMISSION OF PROPOSALS TO THE RESEARCH REVIEW COMMITTEE (RRC)

[Please tick (✓) the appropriate box]

1. Has the proposal been reviewed, discussed and cleared at the Division level ?

   Yes ☑

   No ☐

   If 'No', please clarify the reasons: _____

2. Has the proposal been peer-reviewed externally ?

   Yes ☑

   No ☐

   If the answer is 'NO', please explain the reasons: _____

3. Has the proposal scope to address gender issues ?

   Yes ☐

   No ☑

   If the answer is 'YES', have these been adequately incorporated in the proposal. Please indicate: _____

4. Has a funding source been identified ?

   Yes ☑

   No ☐

   If the answer is 'YES', please indicate the name of the donor: SAREC/SIDA

5. Whether the proposal is a collaborative one ?

Yes [✓]

No [ ]

If the answer is 'YES', the type of collaboration, name and address of the institution and name of the collaborating investigator be indicated:

Dept. of Medical Microbiology and Immunology, University of Goteborg, Goteborg, Sweden
Prof. Ann Mari Svennerholm and Prof. Jan Holmgren

6. Has the budget been cleared by Finance Division ?

Yes [✓]

No [ ]

If the answer is 'NO', reasons thereof be indicated: _____

_____

_____

7. Does the study involve any procedure employing hazardous materials, or equipments ?

Yes [ ]

No [✓]

If 'YES', fill the necessary form.

1/9/96
Date

Firdausi Qadri
Signature of the
Principal Investigator

APPLICATION FOR PROJECT GRANT

1. TITLE PAGE

a) TITLE OF PROJECT : Further evaluation of the oral inactivated ETEC vaccine and studies on the immune responses in acute watery diarrhoea

b) INVESTIGATORS AT ICDDR,B :

PRINCIPAL INVESTIGATOR : Firdausi Qadri

COINVESTIGATORS : Drs. Rubhana Raqib/Dilara Islam
Dr. M.A. Salam
Dr. N.H. Alam
Dr. P.K. Bardhan
Dr. A.S.G. Faruque

CONSULTANT : Dr. George Fuchs

INVESTIGATORS IN SWEDEN* : Prof. Ann-Mari Svennerholm
Prof. Jan Holmgren
Dr. Christine Wenneras
Dr. Anna Helander

*Department of Medical Microbiology and Immunology, University of Goteborg, Goteborg, Sweden.

c) TOTAL AMOUNT OF FUNDING : US$ 137,950 FOR ICDDR,B AND 134,800 FOR UNIVERSITY OF GOTEBORG, SWEDEN

d) PROPOSED STARTING DATE : July 1996

e) DURATION OF PROJECT : Two and half years

f) FUNDING SOURCE : SAREC

g) HEAD OF PROGRAMME : M. John Albert
Acting Division Director
Laboratory Sciences Division

A) SCOPE

GENERAL AIM

To further evaluate the oral inactivated vaccine against ETEC diarrhoea in Bangladesh with regard to B- and T-cell immune responses and to compare these responses with those seen after acute watery diarrhoea.

SPECIFIC AIMS

1) To study systemic and mucosal immune responses in children after immunization with the oral B subunit (BS),colonization factor antigen (CFA) ETEC vaccine (BS-CFA ETEC vaccine) developed in Sweden. Safety and capacity to induce antibody secreting cell (ASC) responses in peripheral blood as well as antibody responses in serum and stool specimens against the key protective antigens will be assessed.

2) To determine cytokine production at the single cell level in blood and in small intestinal biopsies taken from adult patients (18-45 years) with watery diarrhoea due to infection with ETEC or *Vibrio cholerae* O1 or O139, as well as from age-matched volunteers receiving the oral BS-CFA ETEC vaccine. This includes studies of the involvement of Th1 (IL2, IFN-gamma) and Th2 (IL4, IL5, IL10) and proinflammatory (TNF-alpha, IFN-gamma and IL6) cytokines as well as receptors for these cytokines in patients and vaccines. It also includes studies of the localization in intestinal biopsies of cells containing mRNA for selected cytokines. Immunohistochemical analyses of intestinal section from patients with watery diarrhoea and vaccinated volunteers will also be conducted to evaluate the relative contribution of different cell types in the induction of immunity.

3) To compare immune responses against homologous and heterologous ETEC colonization factors (CFs) in different age-groups of patients with watery diarrhoea due to CF-positive ETEC. The responses in patients will be compared with those induced by the oral BS-CFA ETEC vaccine in nonprimed Swedes and in Bangladeshi children and adults, respectively. These studies are undertaken to evaluate whether ETEC CFs are capable of inducing immune responses against heterologous CFs in immunologically primed subjects.

4) To determine prospectively the prevalence of 12 different CFs (i.e. CFA/I and CS1-CS6) that are contained in the BS-CFA ETEC vaccine as well as newly identified putative colonization factors (PCF 0159, PCF 0166) and coli surface (CS) antigens (CS7,CS17) (that are not present in the ETEC vaccine) on ETEC isolated from Bangladeshi patients with ETEC diarrhoea.

SIGNIFICANCE

Acute diarrhoeal diseases are a major health problem in developing countries as well as in travellers venturing to these regions. Infection with ETEC is

the most common cause of diarrhoea among travellers and residents of developing countries. Since ETEC organisms also are very common pathogens encountered in children in the developing countries of the world, children will benefit most from a vaccine. Recently a prototype ETEC vaccine composed of a mixture of formalin-killed ETEC strains expressing CFA/I and CFA/II and containing the B-subunit of cholera toxin has been found to be safe and immunogenic in adult Swedish volunteers. A recent study with a similar vaccine containing ETEC strains expressing CFA/I, CFA/II and CFA/IV antigens and recombinantly produced cholera B-subunit (rBS) has been found to be safe and immunogenic in adult Bangladeshi volunteers. Studies have also been carried out in the United States, Egypt and Israel. Since children in the 1-5 years range will benefit most from an effective vaccine in the future the safety and immunogenicity of the vaccine, needs to be tested in the paediatric age group particularly in older children and if found to be safe and immunogenic, subsequently in younger children. A phase III study has been initiated in Cairo where children (age 6-12 years) have been immunized with 2 doses of the vaccine (Savarino and Svennerholm, unpublished data). The dosing appears to be well tolerated by the children tested so far.

Although studies have shown that in Bangladesh, CFA/I, CFA/II and CFA/IV are the most common colonization factors expressed on ETEC strains isolated from diarrhoeal patients, presence of other CF antigens and putative colonization factors (PCFs) have not been investigated previously. A systematic study is, therefore, necessary to understand the relative contribution to ETEC diarrhoea from other adhesion antigens. Since immune response to the CF antigens are important for protection, this information is very important for the design of a vaccine that will be effective in developing countries such as Bangladesh and elsewhere.

The investigation of the immune response in patients, both adults and children, to CF antigens other than those present on the infecting strain is also important and will help in understanding whether a vaccine will be able to provide protection to ETEC expressing cross-reactive or heterologous CF. Such information will also help in understanding whether natural ETEC infection may protect against subsequent infection with ETEC with heterologous CFs.

Acute watery diarrhoea (caused by *V. cholerae* and ETEC) results in B cell responses to specific antigens present on the surface of the bacterial pathogen (LPS, CFs, other adhesion antigens, as well as enterotoxin). These antibody responses are regulated by cytokines as well as T lymphocytes and other cells of the lymphoid system. Although information is available on the B cell responses in peripheral blood and on the local antibody response in the gut, not much is known about the cytokine profile and group of cytokines (Th1/Th2) involved in the pathogenesis and immunity in patients with noninvasive acute watery diarrhoea. Since the gut-associated immune response is of importance in diarrhoea, a study of the local cytokine production in the gut will help in understanding the mechanism of the immune response in disease. A comparison of the local cytokine profile with the cytokine profile in blood will also be carried out.

RATIONALE

Enterotoxigenic *Escherichia coli* are a common cause of acute watery diarrhoea in infants in countries of the developing world leading to growth retardation and death in the paediatric age group. A vaccine is, therefore, needed. Since recent studies have shown that in a phase I study, an oral ETEC vaccine is safe and immunogenic in adult volunteers in Sweden and Bangladesh, it is important that the vaccine also is tested in children in Bangladesh. In addition, studies need to be carried out in children with ETEC diarrhoea which will help in comparing the immune response generated after natural disease with that stimulated by oral immunization. More information is also needed on the immune response in natural disease to CF antigens, on the CF antigens which are expressed on ETEC which cause dehydrating diarrhoea, and on the B and T cell responses, including cytokine responses, in ETEC vaccinees as well as in patients. Such information will be helpful in a better understanding of the immune response ETEC diarrhoea and whether the response generated by immunization is adequate and appropriate. Also comparison of the responses in patients with acute watery diarrhoea caused by ETEC and *V. cholerae* will generate useful information on the immune mechanisms.

BACKGROUND

Cholera toxin (CT) and heat-labile toxin (LT)/heat stable toxin (ST) mediated diarrhoeas result in fluid secretion without any apparent gross histopathology of the gut by light microscopy. However, ultrastructural studies (Asakura *et al.*, 1974) have shown significant abnormalities in the villus and crypt enterocytes and the lamina propria. These include infiltration of surface epithelium and lamina propria by neutrophils and degranulation of mast cells and eosinophil (Mathan *et al.*, 1995). Both *V. cholerae* and ETEC are noninvasive enteropathogens which colonize the small intestine by means of fimbrial structures (CFAs for ETEC and TCP, as well as possibly the putative adhesin, MSHA for *V. cholerae* O1/O139). In these diseases, antibodies present on the mucosal surfaces of the gut can protect against subsequent infection (Svennerholm *et al.*, 1984; Holmgren and Lycke, 1986). Protective immunity in these diseases is dependent on the stimulation of the mucosal immune system and generation of secretory IgA antibodies in the gut associated lymphoid tissue (GALT). Considerable information is available on the antibody responses to specific antigens in natural diseases and after administration of oral vaccines in adults.

B-cell responses

Recently, information on the lymphocytes involved in the immune response has also emerged. Cholera and ETEC diarrhoeas result in specific antibody-secreting cell (ASC) responses which are detected in peripheral blood as well as in small intestinal biopsies. A rise in the levels of lipopolysaccharide- (LPS) and CT-specific ASCs isolated from blood can be seen around 7 days after onset of cholera (Qadri *et al.*, 1994, 1995, manuscript in preparation). A similar increase in CFA- and toxin-specific ASC responses are seen in patients with ETEC diarrhoea (Qadri *et al.*, manuscript in preparation). Similarly, antigen CT-specific antibody secreting cell responses are observed in peripheral blood after oral immunization with inactivated ETEC (Wenneras

et al., 1992, 1994) or cholera vaccines (Czerkinsky *et al.*, 1991; Quiding *et al.*, 1991; Jertborn *et al.*, 1996). The peripheral blood B cell responses are believed to be derived from the GALT, which after circulating in the blood, home-back to the mucosa for antibody secretion (Czerkinsky *et al.*, 1987). Therefore, these cells appear to be good markers of the immune response of the GALT to locally presented antigens.. Determination of specific B cell responses in peripheral blood has been found to be a reliable proxy measure of the mucosal immunogenicity in natural disease and to enteric vaccines. Very recently a strong correlation between IgA ASC responses in peripheral blood and specific IgA antibody response in intestinal lavage after oral immunization with ETEC vaccine was observed (Åhren *et al.*, manuscript in preparation). The migratory behaviour of the mucosal lymphocytes appears to be under regulation by homing receptors and adhesion molecules present on lymphocytes and ligands present on high endothelial venules which direct the lymphocytes back to the gut (Yednock and Rosen, 1989; Butcher, 1994; Czerkinsky and Holmgren, 1994). B cell function is dependent on T cells which control its proliferation and differentiation and is, therefore, dependent on signalling by cytokines. Intestinal T cells also display migratory properties in humans and oral ETEC vaccination induces appearance of T cells in blood (Wenneras *et al.*, 1994). Thus, both B cells and T cells induced by oral immunization has been shown to migrate to the peripheral blood before homing back to mucosal surfaces including the lamina propria of the gut. Direct evidence of cellular immune responses locally in the intestinal mucosa is now also emerging using intestinal lymphocytes isolated from duodenal biopsy specimens. Immunization with oral cholera vaccine resulted in a strong specific antibody-secreting cell response in the gut in the intestinal mucosa which was of a higher magnitude than that seen in blood (Quiding *et al.*, 1991). Similarly, after oral immunization with ETEC vaccine (Wenneras *et al.*, manuscript in preparation) or after natural disease (Wenneras *et al.*, 1995, manuscript in preparation) large numbers of CFA- and CT-specific antibody-secreting cells were seen in the gut. Recently, it was found that patients with *V. cholerae* O1 and O139 infections (Qadri *et al.*, manuscript in preparation) mounted high increases of LPS- and CT-specific ASC response in gut.

T-cell responses

T-cell response has also been observed to the oral inactivated vaccines (Quiding *et al.*, 1991; Wenneras *et al.*, 1994). Thus, immunization with the rCTB-CFA ETEC vaccine was shown to result in IFN-gamma responses in peripheral blood (Wenneras *et al.*, 1994) although increases in levels of IL2 were not observed. Quiding *et al.* (1991) have found higher increases in numbers of IFN-gamma secreting cells in the intestine than in peripheral blood after oral cholera vaccination. Increases of IFN-gamma have also been seen in saponin extracts of duodenal biopsies in patients with ETEC diarrhoea or after oral immunization with the ETEC vaccine (Wenneras *et al.*, 1994, manuscript in preparation).

Immune responses to bacterial antigens are generally dependent on T cell (CD4 T helper cell) recognition of antigen in association with antigen presenting cells of the body. These T cells function by producing a variety of cytokines which cause activation and differentiation of other immune cells. Antibody responses are greatly dependent on T helper cells for generation of memory B

cells and the induction of different isotypes and subclasses of antibodies. The T helper cells can be divided into two populations of CD4 T cells, Th1 and Th2, distinguished by the type of cytokines produced (Cherwinski *et al.*, 1987; Mosmann and Coffman, 1989). Th1 cells produce IFN-gamma and IL2 which favour either cell-mediated and inflammatory immunity and Th2 lymphocytes which produce IL4, IL5, IL6, IL9, IL10 and IL13, favour antibody-mediated humoral immunity (Mosmann, 1994). The Th1 cytokines can also have some antibody promoting effects. CD4 T lymphocytes play a role in host defence against CT-induced diarrhoea due to *V. cholerae* O1 (HÖrnquist *et al.*, 1991). The involvement of IL4-dependent Th2 type of response in oral immunization with CT as mucosal adjuvant has also been suggested in studies involving IL4-deficient mice (Vajdy *et al.*, 1995). These mice failed to show antigen-specific T cell responses demonstrating that IL4 and the Th2 cell type are required for induction of the gut mucosal immune response. The induction by CT of a Th2 type of response in Peyer's patches is also suggested by other studies (Dertzbaugh *et al.*, 1991; Ramarathinam *et al.*, 1991). However, studies in mice have suggested that CT induces both a Th1 and Th2 type of cytokine secretion (Horniquist, 1993). There is also evidence to suggest that the B subunits of LT induce IL12 mRNA expression at mucosal sites (Bost and Clements, 1995). IL12, a newly discovered cytokine has been shown to increase IFN-gamma production by Th1 lymphocytes and diminish development of Th2 lymphocytes. Only limited information is available on the Th1 or Th2 pattern of cytokines or of other cytokines that are induced in acute watery diarrhoea caused by *V. cholerae* or ETEC in patients. Since both ETEC and *V. cholerae* are noninvasive diseases of the gastrointestinal tract and result in antigen-specific local and systemic antibody responses, it is expected that a Th2 type of response will be induced. Although available data in experimental animals suggest this may be true, there are also reports that a Th1 type of response may be involved by the increases seen in vaccinees (Quiding *et al.*, 1991; Wenneras *et al.*, 1994) of IFN-gamma (a Th1 cytokine). It is, therefore, of interest to study the cytokine profiles of both the Th1 and Th2 types of T helper cells. An overall investigation of these cytokine profiles both locally in the duodenal mucosa and in the peripheral blood of patients in an endemic area will help in understanding the regulation and control of the immune responses. Using different techniques for analyzing the cytokine profile will complement and help in better unraveling the findings in different immune compartments (mucosal and systemic) of the body. Knowledge of the cytokine profile in natural disease and a comparison with cytokine responses after oral vaccination will also increase the knowledge of the oral inactivated cholera and ETEC vaccine. In this respect it will be useful to compare the responses in patients and vaccinees in this study with corresponding responses in adult volunteers orally immunized with two doses of the bivalent (O1/O139) or monovalent (O1) BS- whole cell cholera vaccines (Protocol No 94-019). Blood, feces and duodenal biopsies are being collected in this ongoing study. The stored PBMC, duodenal biopsies, fecal extracts, and plasma will be also used for comparisons.

Immune response in children

So far, most studies of mucosal immune responses to watery diarrhoea and to oral vaccines have been conducted in adults. This is partly due to the fact that methods available to assess intestinal immune responses have been less suitable for use in children. Thus, intestinal lavages are time-consuming and

require consumption of large volumes of fluid although conduction of intestinal lavages in children has been reported (Hodges *et al.*, 1995). Studies of antibody secreting cell (ASC) responses in children require large volumes of blood and invasive methods like collection of intestinal biopsies are less suitable for children. Recently, however, the methodology for determination of ASCs in peripheral blood has been optimized, requiring less volumes of blood (down to 5 ml) and methods to assess intestinal immune responses to watery diarrhoea pathogens and vaccines in faecal extracts have been developed (Åhren *et al.*, manuscript in preparation). Since ETEC disease is predominantly a problem in children in endemic countries , studies of new ETEC vaccines should be focused on their usefulness in young children. Against this background, studies of the safety and mucosal immunogenicity of the oral BS-CFA ETEC vaccines should include a comparison of immune responses in different age groups in an endemic area, e.g. in Bangladesh.

SUMMARY OF RESULTS OBTAINED SO FAR (1993-1995)

Immune responses in adult cholera and ETEC patients and vaccinees

Immune responses to oral inactivated ETEC vaccine and after ETEC diarrhoea in Bangladesh

An oral ETEC vaccine containing rBS (rCTB) and a mixture of formalin-killed ETEC strains expressing CFA/I, CFA/II and CFA/IV has been tested for safety and immunogenicity in 27 adult volunteers (Qadri *et al.*, manuscript in preparation). None of the vaccinees suffered any major side-effects and reported only mild symptoms. The majority of the vaccinees responded with CFA- and CTB-specific antibody secreting cells in peripheral blood and gut (Wenneras *et al.*, 1996). The magnitude of the CTB-specific response was higher than the CFA-specific response. The response in the IgA isotype was higher for both CFAs and CTB closely followed by the response in the IgG isotype.

The vaccinees showed a significant CFA/I-specific response in plasma which was of the IgA isotype and showed a poor response in the IgG isotype. Significant responses both in IgA isotype and IgG to CTB were seen after vaccination.

Twenty adult patients with acute watery diarrhoea due to CFA/I-, CFA/II- or CFA/IV-positive ETEC strains have been studied, samples were collected early in the infection (day 3) and about 6 days later (day 9). Patients showed an ASC response to homologous CFA and CTB which was mainly of the IgA isotype, although responses of the IgG and IgM isotypes were also observed. Compared to healthy controls, the patients showed increased CFA- and CTB-specific ASC responses as early as 3 days after onset of diarrhoea. The patients showed ASC responses of the IgA isotype to homologous CFA antigens and also responded with CFA-specific plasma antibody responses of both the IgA and IgG isotypes. Although both vaccinees and patients responded with CFA-specific responses, the magnitude of the response in peripheral blood was higher in patients than in the vaccinees.

A majority of the patients and vaccinees responded significantly with CFA- and CTB-specific antibody secreting cells isolated from duodenal biopsies as well

as in saponin extracts of intestinal tissue. The magnitude of the ASC response in the gut were comparable in the two study groups. IgA was the most prevalent isotype although IgM and IgG responses were also seen. ETEC-specific antibodies could also be detected in intestinal lavage and faeces of both ETEC vaccinees and patients.

Saponin extracts of duodenal punch biopsies (2 pieces) were used in a novel approach to test for the local IFN-gamma and IL10 production. A modest increase in the levels of IFN-gamma could be seen in the intestinal biopsies of the vaccinees but not of IL10. These results suggest that using very small amount of biopsies (~5 mg), cytokine production and antibody responses can be monitored in the gut (Wenneras *et al.*, 1994, manuscript in preparation).

Immune response in patients with cholera due to V. cholerae O1 and O139

Vibrio cholerae O139 is now considered to be a second organism (WHO, 1993) together with *V. cholerae* O1, capable of causing epidemics of severe dehydrating cholera. Since *V. cholerae* O139 is a newly recognized pathogen which is capable of causing severe disease, a investigation comparing the immune responses in patients infected with this pathogen and in patients with *V. cholerae* O1 infection was undertaken. *V. cholerae* O139 and O1 both produce CT and share many virulence characteristics but differ in the LPS antigen (Hisatsune, 1993). In addition, *V. cholerae* O139 possesses a loose polysaccharide capsule (Waldor *et al.*, 1994; Weintraub *et al.*, 1994) that makes bacteria resistant to host defence immune mechanisms. Since the epidemics of cholera due to *V. cholerae* O139 (Albert *et al.*, 1993; Ramamurthy *et al.*, 1993) mainly affect adults, it was suggested that people in endemic areas of cholera due to *V. cholerae* O1 were not protected and raised the question whether *V. cholerae* O1 infection or the vaccines currently available may not protect against *V. cholerae* O139. The aim was, therefore, to compare the immune response in the two groups of patients to obtain information which has been useful in designing a new cholera vaccine that may protect against both *V. cholerae* O1 and O139. Below is a summary of results obtained so far:

Vibriocidal antibody response: The standard vibriocidal assay for *V. cholerae* O1 was modified when *V. cholerae* O139 was used as target organism (Qadri *et al.*, 1995) and tested with sera from patients. Patients (n=33) with cholera caused by *V. cholerae* O139 showed highly significant differences in vibriocidal antibody activity from the acute stage to the convalescent stage of infection and showed an 18-fold increase in response by day 7 of onset of disease. However, the response was only to the homologous O139 serogroup with no response to *V. cholerae* O1. Patients with *V. cholerae* O1 infection (n=18) similarly showed vibriocidal activity to *V. cholerae* O1 organisms only and no heterologous response. The serogroup-specific response in the patient supported that an oral vaccine containing both *V. cholerae* O1/O139 components is needed to protect against both types of cholera.

B cell responses to cholera antigens: The kinetics of the immune response to different cholera antigens was studied in *V. cholerae* O1 (n=31) and *V. cholerae* O139 (n=23) patients (Qadri *et al.*, 1995, manuscript submitted). *V. cholerae* O139 patients showed a peak ASC response to homologous LPS around 7 days after onset of cholera which was of the IgA and IgM isotypes, and a much lower IgG response. *V. cholerae* O1 patients similarly responded with O1

LPS-specific IgA, and IgM-ASCs, and much lower IgG-ASCs. No response was seen to O139 LPS. Both groups of patients responded with CT-specific IgG and IgA-ASCs of comparable magnitudes in the two patient groups.

V. cholerae O1 patients showed a CT-specific IgA response in plasma which showed a peak increase by day 7 of onset of illness. A higher increase in CT-specific IgG response was seen which peaked by day 22 of onset of cholera. O139 patients showed a similar response to CT but with peak increases by day 11 in the IgA, and IgG isotype.

The role of a putative adhesin, MSHA, in cholera was also studied by analyzing local and systemic immune response to this antigen in natural disease. Experiments in animal models have suggested that MSHA is immunogenic and protective (Jonson *et al.*, 1991; Osek *et al.*, 1992). Most *V. cholerae* O1 patients (n=21) responded with MSHA-specific ASCs, mainly in IgA but also in IgM, with little contribution in the IgG isotype. MSHA-ASC response could be studied in only few patients with *V. cholerae* O139 infection since the incidence of cholera due to *V. cholerae* O139 had decreased greatly over time when the assay had been optimized.

Patients with *V. cholerae* O1 infection (n=28) showed significantly increased MSHA-specific IgA and IgG responses in most instances. Analysis of plasma samples collected from *V. cholerae* O139 patients (n=23) for MSHA-specific response revealed statistically significant increases in IgA as well as IgG isotypes. These results were comparable to those seen in *V. cholerae* O1 patients. The gut antibody response is also being studied using faecal extracts and both *V. cholerae* O1 and O139 patients responded with MSHA-specific IgA antibodies. These results thus suggest that MSHA is an immunogenic component which elicits both mucosal and systemic immune responses in patients.

A study of the CT-specific IgG subclass specific response in plasma showed that in both *V. cholerae* O1 (n=13) and *V. cholerae* O139 (n=13) patients, the pattern of distribution at the acute stage was IgG1>IgG2>IgG3=IgG4 which changed to IgG1>IgG2>IgG4>IgG3 at convalescence confirming results obtained earlier in *V. cholerae* O1 patients (Jertborn *et al.*, 1988). The CT-specific subclass distribution of the IgA isotype in plasma differed from that seen in faecal extracts. The plasma IgA response was restricted to the IgA1 and the faecal antibodies to both IgA1 and IgA2 antibodies.

Proliferation and stimulation of MNC from peripheral blood

In order to understand whether cholera induces antigen-specific T cell responses in blood, lymphocyte proliferation assays and stimulation of MNC from peripheral blood have been carried out. Preliminary results show that LPS and MSHA induced very poor proliferative responses in PBMC obtained from patients at the acute (day 2) or convalescent stages (day 7, day 11) of infection. However, the proliferative response to phytohaemagglutinin was high and showed significant increase by day 11 of onset of illness as compared to the acute stage.

The cytokines IL6, IL10 and IL4 were assayed in culture supernatants of MNC stimulated with LPS (both O1 and O139) and MSHA. Only MSHA induced increased

levels of IL6 and IL10 which were from 2- to 10-fold higher than that seen in cells cultured in the absence of the antigen. Increases in levels of IL4 were also seen. These preliminary data suggest an involvement of a Th2 type of cytokine pattern in *V. cholerae* O1/O139-induced acute watery diarrhoea. The contribution of the Th1 type of cytokines also needs to be studied.

STUDIES PLANNED

Determination of the safety and immunogenicity of the ETEC vaccine in Bangladeshi children

Hitherto, numerous studies have been undertaken on the safety and mucosal immunogenicity of BS-WC ETEC vaccine in adults in different countries (Sweden, the United States, Bangladesh, Egypt and Israel). All these studies have shown that the vaccine is safe and gives rise to significant mucosal immune responses in a majority of the volunteers. Recently, studies have been initiated in Cairo, Egypt (Clemens *et al.*, personal communication) to determine the safety and the immunogenicity of the vaccine also in young children. Within the frame of this proposal, we plan to conduct similar types of studies of the ETEC vaccine also in Bangladeshi children who probably have been differently primed with ETEC. Initially, children in the age groups 5-10 years will be given two doses of the vaccine two weeks apart and safety and mucosal immunity, assessed as ASC responses in peripheral blood as well as specific antibody responses in faecal extracts will be determined on specimens collected immediately before and 7 to 9 days after the first and the second immunization. Provided that these studies confirm that the vaccine is safe and give rise to significant ASC responses, the vaccine will be further studied in younger age groups (children below 5 years). Groups of 20 children in each will be immunized. Twenty children will be included as a placebo group for studying the safety aspect. The older children (5-10 years) will receive full doses (the same as given to adults) of the vaccine, whereas young children below 5 years will receive half doses (provided that ongoing studies in adults in Sweden and in children in Egypt confirm that decreased vaccine doses provide significant immune responses in a majority of the vaccinees). However this will be carried out in a separate study.

Cytokine assays

Oral immunization or natural infection with *V. cholerae* O1 or ETEC organisms are known to result in B cell responses. However, antibody responses are regulated by T lymphocytes, other cells of the lymphoid system and by cytokines and only limited information is available on the pattern of cytokines released in patients with acute watery diarrhoea or in vaccinees. The cytokine profiles of both the Th1 and Th2 types of T helper cells, as well as the contribution of the proinflammatory cytokines IL6 and TNFα will be studied. An overall investigation of these cytokines both locally in the duodenal mucosa and in the peripheral blood of patients and vaccinees will help in understanding the regulation and control of the immune responses. For this purposes, adults with ETEC diarrhoea and with cholera will be studied to look at the cytokine responses in the gut using immunohistochemical procedures. In addition, 20 ETEC vaccinees will be studied for comparison of responses between vaccinees and patients. The cytokine-specific mRNA in

peripheral blood mononuclear cells will be studied by using reverse transcriptase-PCR technique. Cytokines and receptors will be studied in plasma and stool using ELISA. In children (both patients and vaccinees), plasma and stool will be analyzed for cytokine responses using similar procedures as those used for adults. In addition, cytokine responses will be studied in culture supernatants obtained from PBMC stimulated with specific antigens.

Specificity of immune responses against ETEC CFAs

In studies in experimental animals and human volunteers. it has been found that ETEC CFAs only give rise to immune responses against the homologous but not heterologous CFAs (Åhren *et al.*, 1985; Svennerholm *et al.*, 1988, 1990; Levine *et al.*, 1990). However, it has recently been shown that symptomatic infection with CFA/I-positive ETEC in adult Bangladeshi patients induced significant immune responses not only against CFA/I but also against several other ETEC CFs, e.g. CS4, PCFO166, CS17, CS1 and CS2, whereas North American volunteers challenged with CFA/I-positive ETEC only developed significant immune responses against the homologous fimbriae (Rudin *et al.*, 1996, manuscript in preparation). Based on results from animal studies that ETEC CFs with related amino acid sequences (e.g. CFA/I and CS4) can prime and boost immune responses against each other (Rudin, 1994), it is likely that subjects living in ETEC endemic areas who are probably subjected to repeated priming with ETEC expressing different CFs may develop immune responses not only against the homologous CFs of the infecting strain or against the CFs expressed by an oral ETEC vaccine, but also against other, related CFs.

To evaluate this hypothesis, we plan to compare immune responses against different CFs in Bangladeshi infants and adults who develop diarrhoea due to infection with CF-positive ETEC strains. Immune responses against different CFs in Swedes and Bangladeshi volunteers of different age-group receiving the oral BS-CFA-ETEC vaccine will also be assessed. This includes a comparison of immune responses against the homologous CFs, i.e. the one expressed by the infecting strain or by those expressed by the ETEC vaccine, i.e. CFA/I and CS1-CS5, as well as to a number of heterologous CFs. By these studies we hope to evaluate whether vaccination with strains expressing a relatively limited number of CFs in endemic areas will provide immunity also against a number of different CFs not included in the vaccine. For this purpose, 20 children and 20 adults with ETEC diarrhoea will be enrolled. Blood and faecal specimens will be collected after diagnosis of infection (day 3) as well as 7 and 21 days of onset of illness.

Determination of CFs on ETEC isolated from Bangladeshi patients

In previous studies at ICDDR,B, the relative distribution of CFA/I and CFA/II on ETEC isolated from patients with watery diarrhoea has been determined (Gothefors *et al.*, 1984). In subsequent studies the relative distribution of CFA/I and the different subcomponents of CFA/II and CFA/IV was evaluated on LT-producing ETEC strains. These studies have shown that CFA/I, CFA/II and CFA/IV are present in relatively high frequencies on ETEC isolated from patients with moderate to severe ETEC disease in Bangladesh. However, no systematic evaluation of the prevalence of the different CFs and a number of

recently identified "new" *E. coli* CFs (e.g. PCFO159, PCFO166, CS7, CS17) and CFA/III on ETEC isolated from cases with different clinical severity has been conducted. Within the frame of these studies we plan to conduct such a study prospectively on ETEC strains isolated from a routine 2% surveillance sampling (Stoll et al. 1982) of all diarrhoeal patients seen at Clinical Research and Service Centre collected at ICDDR,B. As part of the surveillance relevant clinical information is routinely collected. These samples represent specimens collected from patients and will allow prospective analyses of enterotoxin and CF profiles during, e.g. a full year. For this purpose, about 2000 faecal specimens collected from the surveillance system will be cultured on CFA agar medium for isolation of ETEC strains (Evans et al. 1977). Enterotoxin production by ETEC will be determined by ELISAs (Svennerholm et al.,1986, Svennerholm and Holmgren 1978) or by specific DNA probes (Albert et al., 1995). *E. coli* colonies will be analyzed for 12 different *E. coli* CFs by means of quick dot-blot tests, using specific monoclonal antibodies developed at the Department of Medical Microbiology, and Immunology, Göteborg University as described previously (Lopez-Vidal *et al.*, 1988, 1990; Viboud *et al.*, 1993).

METHODOLOGY

Patients: Both children and adults will be enrolled in these studies. Adult patients with CFA-positive ETEC or *V. cholerae* O1 and O139 infective cases will be enrolled. The microbiological confirmation of V.cholerae in stools obtained from patients is possible around day 2 of onset of disease whereas for ETEC it is around day 3 of onset. Blood and faecal samples from patients will be collected at the acute stage (day 2 or 3 days of onset) and at convalescence (about 7 days and 21 days of onset). Duodenal biopsies will be collected only from adults at onset and either at 7 days (10 patients) or 21 days (10 patients) of onset. This will help in comparing the immune response early in convalescence (day 7) and about 3 weeks later (late convalescence) when the pathological changes may have reverted to those seen in the preimmune stage. Comparison of these changes with those seen in healthy controls (Day 0, pre-immune samples from vaccinees) will help better understand the alterations that take place in the disease and recovery.

Vaccinees: Before entering into the study, all subjects will be carefully examined by a physician. Where necessary, laboratory tests will be carried out. Presence of any one of the following will cause exclusion from the study: (a) hypertension, (b) heart disease, (c) epilepsy, (d) glaucoma, (e) chronic renal disease, (f) pregnancy (in case of women), (g) acute diarrhoea requiring hospitalization during the last 2-3 months, and (h) any other complications.

Age-matched adult Bangladeshi volunteers (both adults [n=20] and children [n=20] will be given two doses of the oral ETEC vaccine 2 weeks apart and specimens collected before immunization on day 0 and 7 days after the first dose and/or on 7 days after the second dose. Blood and fecal samples from vaccinees will be collected before immunization (day 0) and 7 days after the first dose and 7 days after the second dose. Duodenal biopsies will be collected at day 0 and either at 7 days after the first dose (10 vaccinees) or 7 days after the second dose (10 vaccinees), (Flow chart A). From children who will be vaccinated, blood will be collected prior to immunization (day 0)

and 7 days after the first dose (day 7) as well as 7 days after the second dose (day 21) (Flow chart B).

The following investigations will be carried out:

Bacteriology: Stools from patients will be cultured to isolate *V. cholerae* O1, *V. cholerae* O139 and CFA ETEC strains, as well as other enteric co-pathogens according to previously described methods. In addition, the 2% systemic surveillance will be used for detecting CFs on ETEC strain that will be isolated.

Peripheral blood: Blood (10 ml from adults and 5 ml from children) will be obtained by venipuncture and centrifuged on Ficoll-Paque for separation of plasma and mononuclear cells. Serum specimens will be collected as well and stored at -20°C for antibody assays. One ml of blood will be collected in EDTA-containing tubes for cytokine assays and kept at -70°C until used.

For detection of IFN-gamma in plasma and stool, a sandwich ELISA assay will be used (Andersson *et al.*, 1989). For the other cytokines and soluble receptors, ELISA kits or reagents available commercially will be used (from Endogen, benzyme, Pharmingen, R and D systems) where appropriate.

Duodenal biopsies (collection of sample): Punch biopsies (4-5) will be obtained from patients (day 2 or 3 after onset [acute] and 7 days or 21 days after onset [early or late convalescence] and from adult vaccinees on day 0 and 7 days after the first dose or second dose of vaccine). Two pieces will be collected in histocon and immediately frozen in liquid nitrogen to be used for preparing cryostat sections for immunohistochemical and cytokine studies. In addition, one piece will be collected in 10% neutral buffered formalin for embedding in paraffin. The rest of the biopsies will be used for extraction of lymphocytes for studying cytokine-specific responses at the single-cell level using reverse ELISPOT technique similar to those that will be used for blood.

Histopathology of duodenal biopsies: Histopathology of formalin fixed paraffin biopsies processed at ICDDR,B will be carried out, and all slides will be examined by two pathologists, unaware of the clinical findings (Raqib *et al.*, 1994). The paraffin sections will be used for morphological as well as immunohistological analyses (phenotyping of cells, activation markers, homing receptors, etc.).

Immunohistochemistry for detection of cytokines and phenotypic characterization of biopsies: Duodenal biopsies will be processed for detection of cytokines and receptors (IL2, IL4, IL5, IL6, IL10, TNFa and IFN-gamma etc.) and visualized using the avidin-biotin horseradish peroxidase procedure (Raqib *et al.*, 1995a,d). To test for staining specificity, natural or recombinant cytokines will be used to block specific cytokines.

Immunohistochemistry of cryostat sections will be used to analyze phenotypes of cells and activation markers (Raqib *et al.*, 1994). Monoclonal antibodies will be used for detection of antigens specific for CD4, CD8, CD16, CD19, CD20, CD23, CD56 and MHC class II antigens as well as others (Becton-Dickenson, Dakopatts). Distinct surface markers on B cell both early maturation markers (HLA-DR) and/or more mature B cells and plasma cells

(proposed cell marker, CD28) will be studied. The peroxidase-antiperoxidase (PAP) procedure will be used for visualization (Raqib *et al.*, 1994).

Cytokine detection by in situ hybridization: The in situ hybridization technique will be used to study cytokine mRNA expression and localization using synthetic oligonucleotide radio-labelled probes (Scandinavian Gene Synthesis, Koping, Sweden) using procedures optimized recently by Raqib *et al.* (1995c) for similar studies in patients with shigellosis.

Reverse transcriptase-PCR (RT-PCR): Peripheral blood mononuclear cells will be used for RT-PCR (1.5×10^6/ml and 2.0×10^5/ml) using procedures described recently (Klappworth *et al.*, 1995). Total RNA will be isolated from MNCs using TRIZOl reagent (Gibco). RT-PCR for analysis of cytokines will be performed following methods of James (1992). PCR primers used will be those used previously (Klappworth *et al.*, 1995). The effect of different bacterial antigens (CFAs, MSHA) on the expression of different Th1 and Th2 types of cytokines will also be studied using stimulated MNCs.

Vaccine: Vaccine is being produced at the Swedish Bacteriological Laboratories Vaccin (SBL), Stockholm, Sweden, under conditions allowing clinical trials in humans according to Swedish and European Pharmacopeia regulations. The vaccine will be a liquid formulation and distributed in individual ampoules, i.e. one vaccine per ampoule, sufficient for one vaccine dose. Each dose of vaccine consists of a mixture of 10^{11} formalin-killed ETEC bacteria expressing CFA/I and CFA/II (CS1, CS2, CS3) and CFA/IV (CS4, CS5 and CS6), together with one mg of recombinant CTB (rCTB). The vaccine will be mixed with bicarbonate buffer (Samarin, Cederroth AB; Sweden) to counteract the effect of low pH in the stomach.

Data and analysis

Statistical analyses will be carried out using appropriate tests, such as the Wilcoxon's rank sum, the Mann-Whitney, the Kruskall-Wallis or other tests, where appropriate, using the statistical package SigmaStat (Jandel Scientific Software, San Rafael, CA, USA)

SIGNIFICANCE OF EXPECTED FINDINGS

It is expected that the study will increase our knowledge on the immune responses generated in acute watery diarrhoea in different age groups. The studies in children, both patients and vaccinees, will also help to evaluate the effectiveness of the vaccine in the pediatric age group. The knowledge of the immune response to cross-reactive CF antigens in patients is necessary for the formulation of an effective vaccine. Identification of the various CFs that are expressed by ETEC that cause acute watery diarrhoea in Bangladesh is important in the design of a vaccine with broad protective coverage in different parts of the world.

COLLABORATIVE ARRANGEMENT AND INTERACTION WITH RELATED PROJECTS AT ICDDR,B FUNDED BY SAREC

This study is a collaborative one between the International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B) and The Department of Medical Microbiology and Immunology, University of Goteborg, Sweden. The investigators from Goteborg, Sweden will interact closely in the study by transfering recently adapted techniques of cytokine assays, antigen specific antibody assays and by supplying immunodiagnostic reagents which will be needed for the study. Continuous link will be maintained by visits of scientists to each other's laboratories. In addition, there will be collaboration with scientists from the Karoliska Institute, Stockholm Sweden. Recently, Drs. Rubhana Raqib and Dilara Islam have returned to ICDDR,B after completion of their post-graduate studies at the Karolinska Institute, Huddinge Hospital. They have been trained and have gained expertise in sensitive immunological techniques involving B and T cell responses in patients with shigellosis. New techniques will be introduced at ICDDR,B which will involve looking at the immune response using tissue sections of gut biopsies. It is hoped that with this interaction an in-depth study of the immune response in acute watery diarrhoea can be carried out. By sharing equipment and immunoreagents in parallel studies on the immune response in patients with shigellosis, we anticipate optimal use of the funds provided by SAREC for the different studies of immune responses in diarrhoeal diseases at ICDDR,B. For example initially we hope to procure a microtome as well as a cryostat with these funds. This will enable us to carry out sensitive immunological studies in ICDDR,B using tissue sections. In the past samples had to be shipped in liquid nitrogen to laboratories abroad for such studies.

DISSEMINATION OF THE RESEARCH FINDINGS

It is hoped that the information generated from this study will result in a better understanding of the B and T cell responses in patients and vaccinees in acute watery diarrhoea. It is hoped that the results obtained will help in the formulation of an optimal ETEC vaccine for use in children in developing countries.

PROTECTION OF HUMAN SUBJECTS

The following groups will be studied:

Subject criteria	No. of* subjects	Age of patients (yrs)	Samples	Source of subjects
Watery diarrhoea due to *V. cholerae* O139	20	18-45	Peripheral blood (10 ml) faeces and duodenal biopsies from 20 patients	ICDDR,B
Diarrhoea due to *V. cholerae* O1 (Inaba/Ogawa)	20	18-45	Peripheral blood (10 ml) faeces and duodenal biopsies from 20 patients	ICDDR,B
Diarrhoea due to ETEC	20	18-45	Peripheral blood (10 ml) faeces and duodenal biopsies from 20 patients	ICDDR,B
Diarrhoea due to ETEC	20	5-10	Peripheral blood (5 ml) faeces from 20 patients	ICDDR,B
**Volunteers (of similar socioeconomic status as patients, non-diarrhoeal) receiving two doses of ETEC vaccines	20 20	18-45 5-10	Peripheral blood, faeces (duodenal biopsies only from adults)	Volunteers (Bangladeshi)
Volunteers (from nonendemic area) receiving 2 doses of ETEC vaccines	20 20	18-45 5-10	Peripheral blood (10 ml) faeces and duodenal biopsies (only from adults)	Volunteers (Swedish)

*From patients, samples will be collected at the acute stage (day 2 or 3 of onset) and at the convalescent stage (day 7 and 21). Intestinal biopsies will be collected twice, once at the acute stage and for half the patients at 7 days and half, 21 days after onset of diarrhoea.

**Volunteers must be without any history of diarrhoea for at least 2 - 3 months. They should also have no history of illness (fever, cold, cough) in the last one month. Adult volunteers should preferably be people who are not working in the ICDDR,B staff. They will be recruited by investigators and technicians involved in the study after having signed a consent form or in

case of children after consent of parent. The Bangladeshi volunteers will be chosen so that they are of the same socioeconomic status as the patients. The preimmunization specimens from volunteers will be used as healthy control specimens.

The following samples will be obtained each time:

Peripheral blood (for differential leucocyte, Hb, ASC, serum and plasma antibody, cytokine assays (Adults = 10 ml, Children = 5 ml)	:	On day of diagnosis (i.e. d2/d3) 7 days after onset of disease 21 days of onset of disease
Faeces (5 g)	:	As above
Duodenal biopsy (only from adults)	:	On day 2/day 3 and within 7 days or 21 days after onset of disease

Patient Selection

Adults both males and females coming to the ICDDR,B Clinical Research Centre (CRC) with acute watery diarrhoea of not more than 24-h duration will be initially enrolled in the study. Samples will be taken from the patients culture positive for *V. cholerae* O1 (Inaba/Ogawa) or *V. cholerae* O139 (which is usually 2 days of onset) and ETEC positive for different CFs (usually 3 days of onset). Specimens will be collected from the volunteers, as in patients but prior to immunization (day 0, control samples) and 7 days after the first and second immunization. In half of the adult vaccinees, duodenal biopsies will be collected 7 days after the first immunization (day 7) and in the rest 7 days after the second immunization (day 21). The volumes of blood and of biopsies to be obtained will not be harmful for the patients. From children only 5 ml of blood will be collected. The patients will be clinically evaluated by a daily physical examination and will have a standard six-hourly monitoring of oral temperature, pulse rate and respiration. The haemoglobin percentage, total and differential leucocyte counts will be measuredon admission. The study will not interfere with the management and treatment of the patients and none of the procedures will be harmful. Written consent will be obtained from the patients and volunteers. In case of children, consent will be obtained from their parents/guardians. Separate written consent forms for (a) blood and stool, and (b) endoscopy and duodenal biopsies will be obtained. Patients will be released from the hospital when diarrhoea is controlled and requested to return twice for follow-ups.

The clinical aspects of the study, such as patient and volunteer enrollment and management, will be carried out by the clinicians as per the schedule followed in the Clinical Research and Service Centre, (CRSC), ICDDR,B.

TIME-FRAME

1st six months

a) Enrollment of children, both patients and volunteers. Collection of samples and carry out some assays immediately (ASC response to antigens.

b) Initiation of detection of CFs on ETEC strains using the ICDDR,B, 2% surveillance stool specimens.

c) Optimization of procedures for immunohistochemical analysis of biopsies using samples collected from cholera vaccinees (Protocol 94-019).

d) Optimization of techniques for study of cytokine m-RNA using RT-PCR. The method has been set up in the Department of Microbiology and Immunology at the University of Göteborg and will be established in the Immunology Laboratory, ICDDR,B.

Next 12 months

a) Complete enrollment of patients and volunteers in the study as well as carrying out ELISPOT assays for antibody secreting cells. Start ELISA (IgA, IgG and IgM isotype) with stored plasma and fecal extracts.

b) Continue and complete isolation of CF positive ETEC strains from surveillance samples.

c) Carry out immunohistochemical analysis of biopsies.

d) Complete RT- PCR assays.

Last 12 months

a) Continue with completion of studies mentioned above.

b) Carry out antigen and cytokine specific ELISAs with plasma and fecal samples.

c) Analyse data and write manuscript for dissemination of result.

TASK OF EACH INVESTIGATOR

Dr. Firdausi Qadri

- Setting up of procedures for cytokines, RT-PCR, ELISAs etc.
- Supervise work in the laboratory and coordinate specimen collection from patients and volunteers.
- Analyze data.

Drs. Christine Wenneras and Anna Helander

- Collaborative activities in laboratory work by helping set up techniques, etc. Carry out volunteers study in Sweden.

Profs. Ann-Mari Svennerholm and Jan Holmgren

- Coordinate study in Bangladesh with that carried out in Göteborg, Sweden in terms of supply of specimens, vaccines and reagents.
- Scientific and academic feedback.

Dr. M. J. Albert

- Scientific and academic feedback.

Dr. A.S.G. Faruque

- Coordinate collection of stool specimens from the 2% systemic surveillance system.

Dr. M.A. Salam

- Clinical management of patients.

Dr. P.K. Bardhan/N.H. Alam

- Volunteer enrollment, clinical management, follow up of toxicity surveillance of vaccine, endoscopy and collection of duodenal biopsies from patients and volunteers.

Medical Officer

- Assist in patient and volunteer enrollment and management.

Drs. Rubhana Raqib and Dilara Islam

- Help set up techniques for processing and sectioning duodenal biopsies and immunohistochemical analysis.
- Collaborate in exchange of ideas and techniques.

Research Officers (2)

Technical assistance in the laboratory.

Microbiology - Isolation and detection of CFs on ETEC strains using slide agglutination with specific Mabs as well as dot blot assays.

Blood: Fractionation for separation of PBMC. Separation of plasma and storage at $-20^{o}C/-70^{o}C$ as required. Storage of PBMC or use in assays immediately. Freshly isolated PBMC to be used in a modified two-colour ELISPOT assay for antigen specific antibody secreting cell assays.

Faeces: Collection and extraction of faecal antibodies and filtration. Fractionation and storage in presence of protease inhibitors at $-70^{o}C$.

Dudoenal biopsies: Extraction of intestinal lymphocytes from biopsies. Sections for histological and immunohistochemical analysis.

FACILITIES REQUIRED

Existing Hospital, laboratory and office facilities are adequate. New equipment needed will be ordered.

COLLABORATIVE ARRANGEMENTS

This is a collaborative study between the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) and Department of Medical Microbiology and Immunology, University of Göteborg, Sweden..PA

BUDGET FOR ICDDR,B FOR TWO AND HALF YEARS (US$)

		Jul 1 to Dec 31, 1996	1997	1998	Total
Salary Laboratory Technician Laboratory Attendant Clinician Hospital staff		0	30,000	33,000	63,000
Travel		200	2,500	2,500	5,700
Laboratory reagents Hospital supplies etc.		9,400	6,000	5,850	21,250
Interdepartment services (including patients and hospital charges)		2,200	2,700	2,500	7,400
Other services at ICDDR,B		3,000	4,800	2,300	10,100
Capital expenditure (cryostat)*		30,000	1,000	0	31,000
Total	US$	44,800	47,000	46,150	137,950

	BUDGET PROPOSAL			
PROJECT TITLE- FURTHER EVALUATION O		INES IN THE IMMUNE RESPON		PONSE IN
ACUTE WATERY DIARRHOEA				
PROJECT DURATION- 2.5 YEARS STARTING FROM July 1996				
PRINCIPAL INVESTIGATORS-FIRDAUSI QADRI (ICDDR,B)				
	PROF. A-M SVENNERHOLM		(GOTEBORG UN	UNIVERSITY,
	PROF. JAN HOLMGREN		SWEDEN)	
LINE ITEM	**Ist six months**	**2nd year**	**3rd year**	**TOTAL US$**
LOCAL SALARIES	**0**	**30000**	**33000**	**63,000**
	A	B	C	D=A+B+C
PERSONNEL				
TRAVEL				
LOCAL TRAVEL	200	500	500	1200
INTERNATIONAL TRAVEL	0	2000	2000	4000
Sub-total	**200**	**2500**	**2500**	**5,200**
SUPPLIES AND MATERIALS				
Drugs		500	500	1000
Hospital supplies	200	200	250	650
Office supplies	200	200	200	600
Laboratory Supplies				
* immuno-reagents etc.	8,000	2,000	3,000	13,000
culture media. RT-PCR reag.				
cytokine reag.				
Misc. chemicals		600	600	1,200
Tissue culture plastics	500	2000	1000	3500
Microbiology media	500	500	300	1300
Sub-total	**9,400**	**6,000**	**5,850**	**21,250**
OTHER CONTRACTUAL				
SERVICES				
Repair.renovation.	100	200	500	800
Fax.telex.phone.electricity				
bill, etc.	100	500	500	1100
Training, workshop.volunteers	1000	1000	1000	3000
stipend for trainees and				
fellows				
Patient food and diet	1000	1000	500	2500
Sub-Total	**2200**	**2700**	**2500**	**7400**

Budget continued				
	1st year	**2nd year**	**3rd year**	**TOTAL US$**
INTERDEPARTMENTAL				
SERVICES				
Computer,email.	50	200	200	450
Audio-visual unit		500	500	1000
Xerox,Library service	50	100	100	250
Clinical laboratory charges	1000	2000	1000	4000
Hospital charges for patients				
and staff clinic charges	2000	2000	500	4500
Sub-Total	3000	4800	2300	10100
CAPITAL EXPENDITURE	**			
Cryostat	30,000	1000	0	31000
TOTAL PROJECT COST	44,800	47,000	46,150	137,950
* Budgeted here but additional reagents will be obtained from Goteborg University for study.				
**Budgetted here but to be used				
for studies carried out by Drs. Radib and Islam also.				

There is no budget provision for o/h. 70% of P.I's salary is budgeted here rest of the (30%) is covered by other grant. This budget has provision for Lab. Equipment ($30,000) which will be shared by others.

S Li
25/4/96

BUDGET FOR LABORATORY SUPPLIES TO ICDDR,B TO BE PURCHASED BY GÖTEBORG UNIVERSITY (account in Göteborg)

	1/7–31/12 1996 US$	1997 US$	1998 US$
Laboratory reagents	5,000	10,000	10,000
TOTAL	5,000	10,000	10,000

GRAND TOTAL: $25,000

BUDGET GÖTEBORG UNIVERSITY

	1/7–31/12 1996 US$*	1997 US$*	1998 US$*
Salary			
1 laboratory technician 75% including LKP 40%	15,000	30,000	30,000
1 researcher, 1 month/year including LKP 40%	4,600	4,600	4,600
Travel			
1 visit (~14 days) for one of the researchers to ICDDR,B - 97 and 98	--	3,000	3,000
Laboratory reagents			
For analyses of clinical specimens, production of MAbs etc.	5,000	15,000	15,000
Shipment of reagents, courir mail etc.	1,000	2,000	2,000
TOTAL	**25,600**	**54,600**	**54,600**

GRAND TOTAL: $134,800

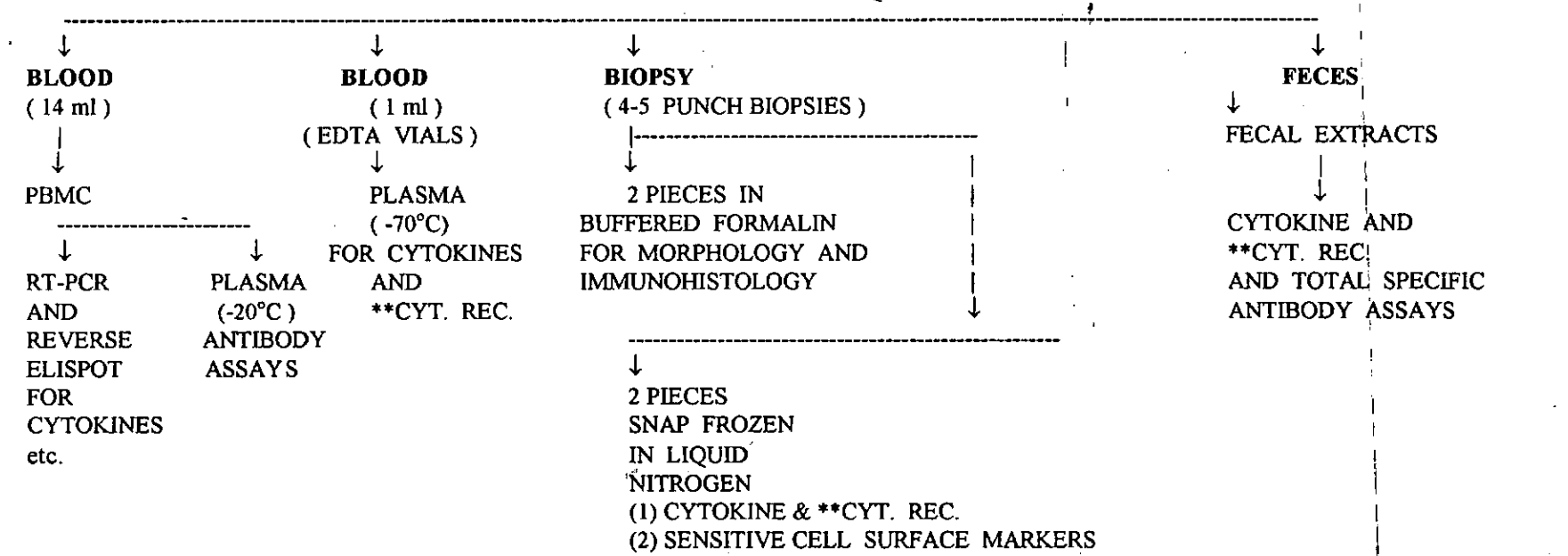
*Estimated 1 dollar = 6,60 SEK

FLOW CHART (A)

***ADULTS**

(age-18-45y)

SAMPLE COLLECTION AND SEQUENCE OF WORK



* SAMPLES FROM ADULT PATIENTS WILL BE COLLECTED AFTER BACTERIOLOGICAL CONFIRMATION OF ILLNESS (ACUTE STAGE, Day 2/3) AND 7 AND 21 DAYS OF ONSET OF ILLNESS. BLOOD AND FECES WILL BE COLLECTED AT ACUTE STAGE AND AT 7 AND 21 DAYS OF ILLNESS. BIOPSIES WILL BE COLLECTED TWICE , AT ACUTE STAGE AND EITHER AT 7 DAYS OR 21 DAYS OF ONSET. FOR VOLUNTEERS BLOOD AND FECES WILL BE COLLECTED PRIOR TO IMMUNIZATION (Day 0) AND 7 DAYS AFTER EACH DOSE OF THE VACCINE. BIOPSIES WILL BE COLLECTED TWICE , AT DAY 0 AND EITHER 7 DAYS AFTER INTAKE OF THE FIRST DOSE OR 7 DAYS AFTER THE SECOND DOSE.

** CYTOKINE RECEPTORS RELEVANT TO THE CYTOKINE THAT WILL BE STUDIED. THE DAY 0 SAMPLES FROM VOLUNTEERS WILL ALSO BE USED AS CONTROL SAMPLES.

FLOW CHART (B)

*SAMPLE COLLECTION AND SEQUENCE OF WORK

(CHILDREN)

(age-5-10y)

PATIENTS AND VACCINEES

↓		↓	↓
BLOOD		**BLOOD**	**FECES**
4.0 ml		**1.0 ml**	↓
↓	↓	(EDTA VIALS)	↓
PBMC	**PLASMA**	↓	**EXTRACT**
↓	(FROZEN UNTIL USED)	**PLASMA**	↓
ELISPOT	ELISA -20°C FOR	(-70°C)	FROZEN (-70°C)
FOR	ANTIBODY ASSAYS	FOR CYTOKINE ASSAY	ELISA FOR ANTIBODIES
ASC		AND **CYT. REC.	(SPECIFIC AND TOTAL)
RESPONSES		ASSAYS.	AND CYTOKINE / CYT. REC.

*SAMPLES FROM PATIENTS TO BE COLLECTED AFTER BACTERIOLOGICAL CONFIRMATION AT ONSET (DAY 2/3) AND 7 AND 21 DAYS OF ONSET OF ILLNESS AND AT CONVALESCENCE. SAMPLES FROM VOLUNTEERS WILL BE COLLECTED PRIOR TO IMMUNIZATION (DAY 0) AND 7 DAYS AFER THE FIRST DOSE AND SECOND DOSE OF INTAKE OF THE VACCINE.

** CYT.REC. INDICATES CYTOKINE RECEPTORS RELEVANT TO THE CYTOKINES THAT WILL BE STUDIED.

THE DAY 0 SAMPLES WILL ALSO BE USED AS CONTROL SAMPLES.

ITEMIZED BUDGET SUMMARY FOR ICDDR,B

		US Dollar Total for 2.5 years
a)	Local salary ...	63,000
b)	Travel (local and international)	5,200
c)	Supplies and materials* ... (drugs, hospital supplies, office supplies, labwares, lab chemicals, media, reagents, etc.)	21,250
d)	Contractual services ... (electricity, communication, training, workshop, patient food and diet)	7,400
e)	Interdepartment services ... (clinical lab charges, hospital charges for volunteers and patients, etc.)	10,100
f)	Capital expenditure ... (Cryostat**)	31,000
	TOTAL US$	137,950

*Additional reagents (US$ 25,000) to be supplied by the Department of Medical Microbiology and Immunology, University of Göteborg, Sweden. Fund in Sweden to be used for studies that have to be conducted there.

**To be shared by other SAREC-funded protocols.

Papers and manuscripts from ongoing cholera and ETEC studies funded by SAREC

Qadri F, Wenneras C, Bardhan PK, Hossain J, Albert MJ, Sack RB, Svennerholm A-M. B cell response to enterotoxigenic *Escherichia coli* (ETEC) in vaccinees and patients after oral immunization and infection (submitted).

Qadri F, Mohi G, Hossain J, Azim T, Khan AM, Salam MA, Sack RB, Albert MJ, Svennerholm A-M. Comparison of the vibriocidal antibody response in cholera due to *V. cholerae* O139 Bengal with the response in cholera due to *V. cholerae* O1. 1995. Clin. Diag. Lab. Imm. Vol(2):685-688.

Rudin A, Wiklund G, Qadri F, Wenneras C, Svennerholm A-M. Infection with colonization factor antigen I (CFA/I) expressing enterotoxigenic *Escherichia coli* may boost immune responses against heterologous CFAs in primed subjects. (submitted).

Wenneras C, Qadri F, Bardhan PK, Sack RB, Svennerholm A-M. Intestinal antibody responses to enterotoxigenic *Escherichia coli* in vaccinees and patients after oral immunization and infection (in manuscript).

Qadri F, Jonson G, Wenneras C, Hossain J, Begum YA, Salam MA, Albert MJ, Svennerholm A-M. Immune response to the mannose sensitive hemagglutinin in patients with cholera due to *Vibrio cholerae* O1 and O139. (in manuscript).

Qadri F, Wenneras C, Hossain J, Begum YA, Moni G, Salam MA, Sack RB, Albert MJ, Svennerholm A-M. Immune response in cholera due to *Vibrio cholerae* O139. A coparison with response in cholera due to *V. cholerae* O1 (submitted).

Papers presented in International Meetings.

Qadri F, Wenneras C, Hossain J, Begum YA, Chowdhury A, Azim T, Sack RB, Salam MA, Khan MA, Albert MJ, Svennerholm A-M. Immune response in patients with cholera due to *Vibrio cholerae* serogroups O1 and O139. 31st US-Japan cholera and related diarrhoeal diseases conference, December 1-3, 1995, Kiawah Island, USA, p.192.

Wenneras C, Qadri F, Czerkinsky C, Bardhan P, Sack RB, Svennerholm A-M. Intestinal immune responses in patients infected with enterotoxigenic *E. coli*. 8th International congress of mucosal immunology, 17-20 July, 1995, San Diego, USA. Clin Immunol Immunopath 1995; 76:S103, 601.

Qadri F, Hossain J, Begum YA, Jonson G, Azim T, Sack RB, Albert MJ, Svennerholm A-M. Immune response to the mannose sensitive hemagglutinin (MSHA) in patients with cholera caused by *Vibrio cholerae* serogroups O1 and O139. 8th International Congress of Mucosal Immunology. Clin Immunol Immpath 1994; 76:S105, P615.

Qadri F, Hossain J, Chowdhury A, Azim T, Salam MA, Khan AM, Sack RB, Albert MJ. Antigen-specific antibody secreting cells in peripheral blood due to *Vibrio cholerae* O139 (W20/34). 12th European Immunology Meetings. Barcelona, June, 1994.

Wenneras C, Qadri F, Bardhan P, Sack RB, Czerkinsky C, Svennerholm A-M. Antibody and cytokine response determined in duodenal biopsy extracts after oral immunization of human volunteers with a prototype vaccine to enterotoxigenic *Escherichia coli*. 12th European Immunology Meeting, 14-17 June, 1994, Barcelona, Spain. p.500.

REFERENCES

Åhren C, Wenneras C, Holmgren J, Svennerholm A-M. Intestinal antibody response after oral immunization with a prototype cholera B subunit colonization factor antigen enterotoxigenic *Escherichia coli* vaccine. Vaccine 1993; 11:929-934.

Åhren M, Svennerholm A-M. Experimental enterotoxin-induced *Escherichia coli* diarrhea and protection induced by previous infection with bacteria of the same adhesin or enterotoxin type. Infect Immun 1985; 50:253-261.

Albert MJ, Siddique AK, Islam MS, Faruque ASG, Ansaruzzaman M, Faruque SM, Sack RB. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh [Letter]. Lancet 1993; 341:704.

Andersson G, Ekre H-PT, Alm G, Perlmann P. Monoclonal antibody two site ELISA for human IFN-gamma. Adaptation for determination for human serum or plasma. J Immunol Method 1989; 125:59-69.

Asakura H, Yoshioka M. Cholera toxin and diarrhoea. J Gastroenterol Hepatol 1994; 9:196-193.

Bost KL, Clements JD. In vivo induction of interleukin-12 mRNA expression after oral immunization with *Salmonella* dublicn or B subunit of *Escherichia coli* heat-labile enterotoxin. Infect Immun 1995; 63:1076-1083.

Butcher EC. Role of α4 integrins in lymphocyte homing to mucosal tissues *in vivo*. J Immunol 1994; 152:3282-3293.

Callard R, Gearing A, editors. The cytokine network. In: The Cytokine Facts Book, chap. 3. San Diego: Academic Press, 1994: 12-17.

Cherwinski HM, Schumacher JH, Brown KD, Mosmann TR. Two types of mouse helper T cell clones. J Expl Med 1987; 166:1229-1244.

Clemens J, Sack DA, Harris JR, Chakraborty J, Neogy PKB, Stanton B, Huda N, Khan MU, Kahn MR, Ansaruzzaman M, Yunus M, Rao MR, Svennerholm A-M, Holmgren J. Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile toxin-producing enterotoxigenic *Escherichia coli* results of alarge-scale field trial. J Infect Dis 1988; 158:372-377.

Czerkinsky CZ,, Holmgren J. Exploration of mucosal immunity in humans:relevance to vaccine development. Cell Mol Biol 1994; 40:37-44

Czerkinsky CZ, Moldoveanu Z, Mestecky J, Nilsson L-A, Ouchterlony O. A novel two colour ELISPOT assay. I. Simultaneous detection of distinct types of antibody secreting cells. J Immunol Method 1988; 115:31-37.

Czerkinsky CZ, Svennerholm A-M, Quiding M, Ronsson R, Holmgren J. Antibody-producing cells in peripheral blood and salivary glands after oral cholera vaccination of humans. Infect Immun 1991; 59:996-1001.

Czerkinsky CZ, Prince SJ, Michalesky SM, Jackson,s, Rusell,MV, Moldoveanu Z, IgA antibody producing cells in peripheral blood after antigen ingestion:a evidence for a common mucosal immune system in humans. Proc Natl Acad Sci USA, 84,2449-2453

Dertzbaugh MT, Elson C.O. Cholera toxin as a mucosal adjuvant. In: Spriggs DR, Koff WC, editors. Topics in Vaccine Adjuvant Research. Boca Raton: CRC Press Inc., 1991: 119-131.

Evans DG, Evans Jr DJ, Tjoa W. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhoea: correlation with colonization factor. 1977,18, 330-337

Gothefors L, Åhren C, Stoll B, Barner DK, Orskov F, Salek MA, Svennerholm A-M. Presence of colonization factor antigens on fresh isolates of fecal *Escherichia coli*: A prospective study. J Infect Dis 1985; 152:1128-1133.

Hisatsune K, Kondo RS, Isshiki Y, Iguchi T, Kawamata Y, Shimada T. O-antigenic lipopolysaccharide of *Vibrio cholerae* O139 Bengal, a new epidemic strain for recent cholera in the Indian subcontinent. Biochem Biophys Res Commun 1993; 196:1309-1315.

Hodges M, Kingstone K, Brydon WG, Sallam J, Ferguson A. Use of gut lavage fluid to measure intestinal humoral immunity, gastrointestinal bleeding and protein losses in Sierra Leonean children. Adv Expl Med Biol 1995; 371B:813-835.

Holmgren J, Lycke N. Immune mechanisms in enteric infections. In: Holmgren J, Lindberg A, Mollby R, editors. Development of Vaccines and Drugs Against Diarrhea. Lund: Studentlitteratus, 1986: 9-22.

Hornquist E, Goldschmidt TJ, Holmdahl R, Lycke N. Host defense against cholera toxin is strongly CD4+ T cell dependent. Infect Immun 1991: 59:3630-3638.

Hornquist E, Lycke N. Cholera toxin adjuvant greatly promotes antigen priming of T cells. Eur J Immunol 1993; 23:2136-2143.

James SP. Detection of cytokine mRNA expression by PCR. In: Coligan JE, Kruisbeek AM, Margulias DH, Shevah EM, Strobe W, editors. Current

Protocols in Immunology. New York: Current Protocols, 1993: 10.23.1-10.23.10.

Jonson G, Holmgren J, Svennerholm A-M. Identification of a mannose binding pilus on *Vibrio cholerae* El Tor. Microb Pathog 1991; 11:433-441.

Klapproth J-M, Donnenberg MS, Abraham JM, Mobley HL-T, James SP. Products of enteropathogenic *Escherichia coli* inhibit lymphocyte activation and lymphokine production. Infect Immun 1995; 63:2248-2254.

Klimpel GR, Asuncsion M, Hathwat J, Niesel DW. Cholera toxin and *Salmonella typhimurium* induce different cytokine profiles in the gastrointestinal tract. Infect Immun 1995; 63:1134-1137.

Levine MM. Vaccines against enterotoxigenic *Escherichia coli* infections. In: Woodrow G, Levine MM, editors. New Generation Vaccine. New York: Mercel Dekka, 1990: 649-660.

Lopez-Vidal Y, Klemm P, Svennerholm A-M. Monoclonal antibodies against different epitopes on colonization factor antigen I of enterotoxin producing *Escherichia coli*. J Clin Microbiol 1988; 26:1967-1972.

Lopez-Vidal Y, Svennerholm A-M. Monoclonal antibodies against the different subcomponents of colonization factor antigen II of enterotoxigenic *Escherichia coli*. J Clin Microbiol 1990; 28:1906-1912.

Lycke N, Severinson E, Strober W. Cholera toxin acts synergistically with IL4 to promote IgG1 switch differentiation. J Immunol 1990; 145:3316.

Mathan MM, Chandy G, Mathan VI. Ultrastructural changes in the upper small intestine mucosa in patients with cholera. Gastroenterology 1995; 109:000-000.

Mosley WH. Vaccines and somatic antigens. The role of immunity in cholera. A review of epidemiological and serological studies. Tex Rep Biol Med 1969; 27:227-241.

Mossman TR. Properties and function of interleukin-IL-10. Adv Immunol 1994; 56:1-26.

Mossmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Ann Rev Immunol 1989; 7:145-173.

Osek JJ, Svennerholm A-M, Holmgren J. Protection against *Vibrio cholerae* El Tor infection by specific antibodies against mannose binding hemagglutininin pili. Infect Immunol 1992; 60:4961-4964.

Paul-Eugene N, Pene J, Bousquet J, Dugas B. Role of cyclic nucleotides and nitric oxide in blood mononuclear cell, IgE production structured by IL4. Cytokine 1995; 7:64-69.

Qadri F, Azim T, Chowdhury A, Hossain J, Sack RB, Albert MJ. Production, characterization, and application of monoclonal antibodies to *Vibrio cholerae* O139 synonym Bengal. Clin Diagn Lab Immunol 1994; 1:51-54.

Qadri F, Hossain J, Begum YA, Jonson G, Azim T, Sack RB, Albert MJ, Svennerholm A-M. Immune response to the mannose sensitive hemagglutinin (MSHA) in patients with cholera caused by *Vibrio cholerae* serogroups O1 and O139. 8th International Congress of Mucosal Immunology. Clin Immunol Immpath 1994; 76:S105, P615.

Qadri F, Hossain J, Chowdhury A, Azim T, Salam MA, Khan AM, Sack RB, Albert MJ. Antigen-specific antibody secreting cells in peripheral blood due to *Vibrio cholerae* O139 (W20/34). 12th European Immunology Meetings, Barcelona, June, 1994.

Qadri F, Wenneras C, Bardhan PK, Hossain J, Albert MJ, Sack RB, Svennerholm A-M. B cell response to enterotoxigenic *Escherichia coli* (ETEC) in vaccinees and patients after oral immunization and infection. (submitted).

Qadri F, Wenneras C, Hossain J, Begum YA, Chowdhury A, Azim T, Sack RB, Salam MA, Khan MA, Albert MJ, Svennerholm A-M. Immune response in patients with cholera due to *Vibrio cholerae* serogroups O1 and O139. 31st US-Japan cholera and related diarrhoeal diseases conference, December 1-3, 1995, Kiawah Island, USA, p.192.

Qadri F, Wenneras C, Hossain J, Begum YA, Mohi MG, Salam MA, Sack RB, Albert MJ, Svennerholm A-M. Immune response in cholera due to *Vibrio cholerae* O139. A comparison with response in cholera due to *V. cholerae* O1. (submitted).

Quiding M, Norstrom I, Kilander A, Andersson G, Hansson LA, Holmgren J, Czerkinsky C. Intestinal immune response in human. J Clin Invest 1991; 88:143-148.

Ramamurty T, Garg S, Sharma R, Bhattacharya SK, Nair GB, Shimada T, Takeda T, Kanasawa T, Kurazano H, Pal A, Takeda Y. Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India [Letter]. Lancet 11993; 341:703-704.

Ramarathinam L, Shaban RA, Niesel DW, Klimpel GR. Interferon gamma (IFN-gamma) production by gut-associated lymphoid tissue and spleen following oral *Salmonella typhimurium* challenge. Microb Pathog 1991; 11:347-356.

Raqib R, Lindberg AA, Wretlind B, Bardhan PK, Andersson U, Andersson J. Persistence of local cytokine production in shigellosis in acute and convalescent stages. Infect Immun 1995a; 63:289-296.

Raqib R, Ljungahl Å, Lindberg AA, Wretlind B, Andersson J. Dissociation between cytokine mRNA expression and protein production in *Shigella dysenteriae* type 1 infection. 1995d (submitted).

Raqib R, Ljungdahl Å, Wretlind AA, Andersson U, Andersson J. Local entrapmen of IFN-gamma in recovery from *Shigella dysenteriae* type 1 infection. 1995c (submitted).

Raqib R, Reinholt F, Bardhan PK, Karnell AA, Lindberg AA. Immunopathological patterns in the rectal mucosa of patients with shigellosis: expression of HLA-DR antigens and T lymphocytes subsets. APMIS 1994. 103:371-380.

Raqib R, Wretlind B, Andersson J, Lindberg AA. Cytokine secretion in acute shigellosis is correlated to disease activity and directed more to stool than to plasma. J Infect Dis 1995b; 171:376-384.

Rudin A, Svennerholm A-M. Colonization factor antigens (CFAs) of enterotoxigenic *Escherichia coli* can prime and boost immune responses against heterologous CFAs. Microb Pathog 1994; 16:131-139.

Rudin A, Wiklund G, Qadri F, Wenneras C, Svennerholm A-M. Infection with colonization factor antigen I (CFA/I) expressing enterotoxigenic *Escherichia coli* may boost immune responses against heterologous CFAs in primed subjects. (submitted).

Stoll BJ, Glass RI, Huq MI, Khan MU, Holt JE, Banu H. Surveillance of patients attending a diarrhoeal disease hospital in Bangladesh. Br Med J 1982; 285:1185-1188.

Svennerholm A-M, Gothefors L, Sack DA, Bardhan PK, Holmgren J. Local and systemic antibody responses and immunological memory in humans after immunization with cholera B subunit by different routes. Bull WHO 1984; 62:909-918.

Svennerholm A-M, Holmgren J. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA) procedure. Curr Microbiol 1978; 1:9-23.

Svennerholm A-M, Lopez-Vidal Y, Holmgren J, McConnell MM, Rowe B. Role of PCF8775 antigen and its coli surface subcomponents for colonization, disease, and prospective immunogenicity of enterotoxigenic *Escherichia coli* in rabbits. Infect Immun 1988; 56:523-528.

Svennerholm A-M, Wenneras C, Holmgren J, McConnell MM, Rowe B. Roles of different coli surface antigens of colonization factor antigen II in colonization by and protective immunogenicity of enterotoxigenic *Escherichia coli* in rabbits. Infect Immun 1990; 58:341-346.

Svennerholm A-M, Wikstrom M, Lindblad M, Holmgren J. Monoclonal antibodies against *Escherichia coli* heat-stable toxin (ST) and theri use in a diagnostic ST ganglioside GM1-enzyme-linked immunosorbent assay. J. Clin Microbiol 1986; 24:585-590.

Vajdy M, Kosco-Vilbois MH, Kopf M, Kohler G, Lycke N. Impaired mucosal immune responses in interleukin 4-targeted mice. J Expl Med 1995: 181:41-53

Vibrud GI, Binsztein N, Svennerholm A-M. Characterization of monoclonal antibodies against putative colonization factors of enterotoxigenic

Escherichia coli and their use in an epidemiological study. J Clin Microbiol 1993; 31:558-564.

Waldor MK, Colwell R, Mekalanos JJ. The *Vibrio cholerae* O139 serogroup antigen includes capsular and oipopolysaccharide virulence determinants. Proc Natl Acad Sci USA 1994; 91:11388-11392.

Weintraub A, Widmall G, Jansson P-E, Hansson M, Hultenby K, Albert MJ. *Vibrio cholerae* O139 Bengal possesses a capsular polysaccharide which may confer increased virulence. Microb Pathog 1994; 16:235-241.

Wenneras C, Qadri F, Bardhan P, Sack B, Czerkinsky C, Svennerholm A-M. Antibody and cytokine responses determined in duodenal biopsy extracts after oral immunization of human volunteers with a prototype vaccine to enterotoxigenic *Escherichia coli* [Abstract]. 12th European Immunology Meeting 14-17 June, 1994 (W54/24).

Wenneras C, Qadri F, Bardhan PK, Sack RB, Svennerholm A-M. Intestinal antibody responses to enterotoxigenic *Escherichia coli* in vaccinees and patients after oral immunization and infection (manuscript in preparation).

Wenneras C, Qadri F, Czerkinsky C, Bardhan P, Sack RB, Svennerholm A-M. Intestinal immune responses in patients infected with enterotixigenic *E. coli*. 8th International congress of mucosal immunology, 17-20 July, 1995, San Diego, USA. Clin Immunol Immunopath 1995; 76:S103, 601.

Wenneras C, Svennerholm A-M, Åhren C, Czerkinsky C. Antibody-secreting cells in human peripheral blood after oral immunization with an inactivated enterotoxigenic *Escherichia coli* vaccine. Infect Immun 1992; 60:2606-2611.

Wenneras C, Svennerholm A-M, Czerkinsky C. Vaccine-specific T cells in human peripheral blood after oral immunization with an inactivated enterotoxigenic *Escherichia coli* vaccine. Infect Immun 1994; 62:874-879.

Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol-water and further application of the procedure. Methods Carbohydr Chem 1965; 5:83-91.

World Health Organization. Epidemic diarrhoea due to *Vibrio cholerae* non-01. Weekly Epidemiol 1993; 68:141-142.

Xu-Amano J, Jackson RJ, Fujihashi K, Kiyono H, Staats HT, McGhee JR. Helper Th1 and Th2 cell responses following mucosal or systemic immunization with cholera toxin. Vaccine 1994; 12:903-911.

Yednock TA, Rosen SD. Lymphocyte homing. Adv Immunol 1989; 44:313-378.

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INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM FOR VOLUNTEERS (ADULTS) - BLOOD DRAWING AND STOOL COLLECTION

IMMUNE RESPONSE STUDIES TO ORAL ETEC VACCINE

We are carrying out research on an ETEC vaccine which may help protect the body against diarrhoea... In the greater interest of the country, people and humanity, we hope you will agree to take part in this research.

For this purpose, you will be asked to drink this vaccine twice, fourteen days apart, and we will collect blood samples and stools from you. We will take ten ml of blood (about two teaspoonful) and stool from you, three times, once in the beginning of the study and then again 7 and 21 days later.

You are to decide if you want to participate in the study, and you can withdraw from the stuy at any time that you wish.

We will compensate for any wage loss and travel costs that you may incur while participating in this study.

If you agree to participate in this study, please put your signature or your left thumb imprint at the specificed space below.

Thank you for your cooperation.

Signature or left-thumb impression of the volunteer	Date
Signature of witness	Date
Signature of investigator	Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM FOR VOLUNTEERS (ADULTS) - ENDOSCOPY

IMMUNE RESPONSE STUDIES TO ORAL ETEC VACCINE

We are carrying out research on an ETEC vaccine which may help protect the body against diarrhoea. In the greater interest of the country, people and humanity, we hope you will agree to take part in this research.

For this purpose, you will be asked to drink this vaccine twice, fourteen days apart. You will be examined by an instrument called the endoscope. This instrument has a tube which will be passed through your mouth to examine the stomach and intestine. Some medicines will be given to you so that you will not feel any discomfort or pain in your throat during the examination. During this examination, some tiny pieces of biopsies (about the size of mustard seeds) (4-5) will be taken from your gut. Although the procedure should not be harmful, you will be kept under observation for 3-4 hours after the endoscopic examination to ensure it. You will, however, not feel any pain or suffer any harm as a result of this. You will be examined twice by this instruemnt, once in the beginning of the study and either 7 days or 21 days later.

You are to decide if you want to participate in the study, and you can withdraw from the study at any time that you wish.

We will compensate for any wage loss and travel costs that you may incur while participating in this study.

If you agree to participate in this study, please put your signature or your left-thumb impression at the specified space below.

Thank you for your cooperation.

_____ _____

Signature or left-thumb impression of the volunteer — Date

_____ _____

Signature of witness — Date

_____ _____

Signature of investigator — Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM FOR PATIENTS (ADULTS) WITH ACUTE WATERY DIARRHOEA
- BLOOD AND STOOL

IMMUNE RESPONSE STUDIES TO ORAL ETEC VACCINE

You are suffering from a diarrhoeal disease which may be cholera or very much like cholera. In order to understand more about this illness we will need to study such patients. For this purpose we seek your assistance in collecting blood and stool samples from you. About 10 ml of blood (2 spoonful) and stool (5-10 g) will be collected from you three times during the study. These will be one day after admission, 5 days and 14 days after that. The collection of these samples will not be harmful to you in any way. During the study you will have to stay in the hospital until you have recovered and will be discharged. We will request you to come back to this hospital twice after discharge, when we will collect blood and stool samples from again.

The tests that we will carry out will be very helpful in understanding the reason why such a severe diarrhoea occurs in Bangladesh. Also, this study may help development of vaccine to prevent infection from this germ.

You are to decide if you want to participate in the study. Even if you do not agree to participate, you will receive the standard treatment of this hospital. Even after initial participation in the study you have the right to withdraw yourself at any time at your will. If you decide to withdraw from the study you will not miss the opportunity of getting standard treatment from this hospital.

If you agree to participate in the study, please put your signature or your left thumb impression at the specified space below.

Thank you for your cooperation.

Signature or left-thumb impression of the patient	Date
Signature of witness	Date
Signature of investigator	Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM FOR VOLUNTEERS (CHILDREN) - BLOOD DRAWING AND STOOL COLLECTION

IMMUNE RESPONSE STUDIES TO ORAL ETEC VACCINE

We are carrying out research on an ETEC vaccine which may help protect the body against diarrhoea. In the greater interest of the country, people and humanity, we hope you will allow your child to take part in this research.

For this purpose, your child will be asked to drink this vaccine twice, fourteen days apart, and we will collect blood and stools from your child. We will take five ml of blood (about one teaspoonful) and stool from your child, three times, once in the beginning of the study and then again 7 and 21 days later.

You are to decide if you would allow your child to participate in the study, and you can withdraw your child from the study at any time that you wish.

We will compensate for any wage loss and travel costs that you may incur while allowing your child to participate in this study.

If you agree that your child can participate in this study, please put your signature or your left thumb imprint at the specified space below.

Thank you for your cooperation.

_____ _____

Signature or left-thumb impression of the guardian — Date

_____ _____

Signature of witness — Date

_____ _____

Signature of investigator — Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM FOR CHILDREN WITH ETEC DIARRHOEA
- BLOOD DRAWING AND STOOL COLLECTION

IMMUNE RESPONSE STUDIES TO ORAL ETEC VACCINE

Your child is suffering from watery diarrhoea caused by a germ called Enterotoxigenic *Escherichia coli* (ETEC). In order to understand more about this illness, we are conducting a study which, in future, may help us prevent or treat it. We would like your permission to enroll your child in this study.

During the study, some specimens will be collected from your child. Stool and 5 ml of venous blood (approximately 1 teaspoon) will be collected on the day of admission and again 5 days and 14 days later. We will also collect stool from your child on these days. All these procedures are safe. However, we will provide treatment for any untoward effect if it were to occur.

We will ask you to bring your child back twice, on day 7 and 21, after discharge when we will again collect samples of blood and stool.

Your child will receive the same care and treatment that is normally provided whether he/she is enrolled in the study or not. If at any time you wish to withdraw your child from the study, you are free to do so without causing any effect on his/her treatment at the CRSC. All information obtained during the study will be confidential and if you wish to know the results, we will provide those to you on request, as they become available.

If you agree to let your child participate in the study, please sign or put your left-thumb impression below.

Signature or left-thumb impression of the guardian	Date
Signature of witness	Date
Signature of investigator	Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM FOR PATIENTS (ADULTS) WITH ACUTE WATERY DIARRHOEA
– ENDOSCOPY AND COLLECTION OF DUODENAL BIOPSY

IMMUNE RESPONSE STUDIES TO ORAL ETEC VACCINE

You are suffering from a diarrhoeal disease which may be cholera or very much like cholera. In order to understand more about this illness we will need to study such patients. For this purpose we seek your assistance in carrying out endoscopy and collecting duodenal biopsy samples from you. About 4-5 very small pieces of biopsies (1-2 mmm in size,about the size of mustard seeds) will be collected from you two days. This will be on day afer admission and 5 days or 14 days after that. The collection of these samples will not be harmful to you in any way but you may feel a bit uncomfortable. However, you will not feel any pain during this procedure.

The tests that we will carry out will be very helpful in understanding the reason why such a severe acute watery diarrhoea occurrs in Bangladesh. Also, this study may help development of vaccine to prevent infection from this germ.

You are to decide if you want to participate in the study. Even if you do not agree to participate, you will receive the standard treatment of this hospital. Even after initial participation in the study you have the right to withdraw yourself at any time at your will. If you decide to withdraw from the study you will not miss the opportunity of getting standard treatment from this hospital.

If you agree to participate in the study, please put your signature or your left thumb impression at the specified space below.

Thank you for your cooperation.

Signature or left-thumb impression of the patient	Date
Signature of witness	Date
Signature of investigator	Date

Reaction surveillance after oral administration of oral vaccine.

Name : _____

Date of vaccination : _____

Primary (P) or secondary immunization : _____

Examiner : _____

LOCAL REACTION

	1	2	3	4	5
Abdominal pain	| |	| |	| |	| |	| |
cramps	| |	| |	| |	| |	| |
distension	| |	| |	| |	| |	| |
Vomiting	| |	| |	| |	| |	| |
If yes, number/day	( )	( )	( )	( )	( )
Diarrhoea	| |	| |	| |	| |	| |
If yes, specify number/day	( )	( )	( )	( )	( )
Other	| |	| |	| |	| |	| |

If yes (1-3)*, specify nature of symptoms _____

SYSTEMIC REACTION

Fever	| |	| |	| |	| |	| |
If yes, temperature (^{o}C)	( )	( )	( )	( )	( )
Nausea	| |	| |	| |	| |	| |
Dizziness	| |	| |	| |	| |	| |
Exanthema	| |	| |	| |	| |	| |
Oedema	| |	| |	| |	| |	| |
Other	| |	| |	| |	| |	| |

If yes (1-3)*, specify nature of symptoms _____

0 = No
1 = Mild (Reaction does not affect your wellbeing)
2 = Moderate (Reaction affects your wellbeing, but does not interfere with daily activities)
3 = Severe (Reaction affects wellbeing and impedes daily activities)

030804

History form for volunteers of the ETEC vaccination study

Name of volunteer : _____

Sex : MALE / FEMALE

Age : _____

Current medication : _____

Diarrhoeal episodes within previous 3 months : _____

HISTORY OF -

Abdominal surgery : _____

Digestive problems : _____

Intestinal ulcers : _____

Cardiac/Vascular disease : _____

Metabolic disease (such as diabetes) : _____

Allergies : _____

Dr. Frederick Sachs

Title: "Further evaluation of the oral inactivated ETEC vaccine and studies on the immune responses in acute watery diarrhoea."

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project		✓ *	
Adequacy of Project Design		✓ *	
Suitability of Methodology		✓ *	
Feasibility within time period	✓		
Appropriateness of budget	✓		
Potential value of field of knowledge	✓		

Most high but some low

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification ☐

- on technical grounds ✓
- on level of financial support ☐

I do not support the application ☐

Name of Referee:

Signa
Posit

Insti

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

Title:

PI:

Reviewer: .....

TITLE - FURTHER EVALUATION OF THE ORAL INACTIVATED ETEC VACCINE AND STUDIES ON THE IMMUNE RESPONSES IN ACUTE WATERY DIARRHOEA

GENERAL COMMENTS

This work will continue and extend an important programme of research on host-pathogen interactions in ETEC diarrhoeas by bacteriological studies of colonisation factors; producing further information on mucosal and systemic antibody responses, in the Bangladeshi population, to homologous and heterologous ETEC antigens after natural infection and vaccination. Rather separately, studies are proposed on cellular mechanisms of immunity by immunohistochemistry and cytokine studies particularly using duodenal biopsies.

I am broadly supportive of the application. This is important work. The bacteriology and the humoral immune studies are virtually guaranteed to be successful, on the basis of the track records of the Centre's staff and proposed participants, the powerful collaborative links with Swedish groups and the integration with other ongoing studies. I have some concerns and make constructive criticisms on the proposed work on cellular mechanisms, but this also is an important line to be pursued and the project, slightly simplified and improved, would certainly be worthy of support.

The application has generally been prepared to a high standard. The arguments and previous literature have been well laid out, the work which has led up to the present project is clearly described and thoroughly referenced. Some of the application is rather repetitious and unfortunately a number of key pieces of work which are critical to the present project have not yet been published and this makes it difficult to be completely certain that the techniques are indeed completely sound.

SPECIFIC COMMENTS

Aim 4 - Microbiological studies

I have no criticisms of this essential and vital work. This project will only be possible because of the continuation of the 2% sampling protocol: since fortunately I have visited ICDDRB, I can be completely satisfied as to the excellent clinical information, sampling techniques and bacteriological skills which will allow this important work to be undertaken. Clearly, the collaboration with Goteborg in relation to monoclonal antibody production, is a further corner-stone which will contribute to success.

Aims 1 and 3 - Investigations of antibody responses, including antibody producing cells in blood, after vaccination of children and after natural infection

The proposed studies are in my view completely ethical and again, important.

In setting out the rationale and their proposed interpretation of the results, the applicants link the presence of antibody producing cells in blood with intestinal antibody secretion. They should be aware that all of the positive studies in this regard have related to periods of change, i.e. in the early inductive phase of an immune response to a mucosal agent, such as vaccination. Indeed the positive results in humans have generally related to powerful inductive agents such as oral cholera vaccines. Other work which the applicants have not cited, using food antigens in children, has failed to show any relationship between antibody producing cells in the blood and intestinal antibodies in a more steady state (this referee has unpublished experience, again of the steady state, which indicates that antibody producing cells are not a good index of mucosal antibodies in this situation). A key paper supporting the applicants proposed study is still in preparation.

As the index of intestinal antibody production the investigators plan to use antibodies prepared from faeces and once again the key reference, Ahrens, is still in preparation. I know and have met with Dr

Ahrens. She has found that antibody is much more frequently detected if soft or diarrheal stools are used, than if a formed stool is used even in recently vaccinated volunteers. Data suggesting that antibody is spuriously apparently absent in formed stools, is also present in a recent paper by Ferguson et al in Clinical and Experimental Immunology about 2 years ago. The applicants must appreciate that although positive results obtained in diarrheal stools are likely to be a correct index of mucosal antibody status, negative results in formed stools may, in fact, be false negatives.

In order to address this issue might I suggest that in addition to measuring antibodies to the antigens of interest such as colonising factors, in faeces, the applicants also measure total IgA production and per cent water in the stools, and particularly when serial samples are to be collected, they also measure IgA antibody to reference antigens unlikely to change in the course of a few weeks such as bacteroides outer membrane, cholera toxin or a food antigen. This would be particularly relevant for the studies in the naturally acquired ETEC diarrhoeas where the first samples on day 3 of infection will have totally different characteristics from the faecal samples collected in convalescence.

Have the applicants considered studying IgM responses? In view of the large amount of material which is to be collected, addition of this isotype of antibody would involve little extra work and might give key and important new data particularly on the kinetics of intestinal antibody responses. I do not suggest that IgM antibody producing cells should be studied as this would increase greatly the volume of blood required to be collected.

Aim 2 - Studies of cytokine production by single cells in blood and small bowel biopsies

One of the reasons for planning this work is that the applicants have experience in the same techniques using rectal biopsies collected from patients with shigellosis. I would raise the following questions:

a) Is there evidence that the duodenum is representative of the mucosa affected by ETEC and cholera? There is abundant evidence

with other intestinal pathogens such as helminths, giardia, various viruses, that the mid intestine or the ileum may be preferentially infected and damaged, and that proximal small bowel biopsies taken from the second part of duodenum may show less pathological changes than more distal gut. (This, of course, is not a problem with shigella and studies of the rectum.)

b) In the information given to the volunteers, it is stated that the endoscopic biopsies are 1-2mm across, the size of a millet seed. Such biopsies, presumably taken with a paediatric endoscope, are not suitable for immunohistochemistry and are completely unsuitable for any form of quantitative microscopy (see below). Again this is an entirely different situation to proctoscopy and rectal biopsies taken with large forceps under direct vision. There are now available excellent wide channel endoscopes and dedicated intestinal biopsy forceps. I suspect that the upper GI endoscope currently available at the Centre is of small calibre producing small biopsies. I would strongly recommend the inclusion of appropriate endoscopy and biopsy facilities, unfortunately $30,000 - $40,000, as being absolutely vital if the proper benefit from the ambitious research on duodenal cells and cytokines is to be properly carried out.

c) Is there, in fact, any evidence of TH1 responses in the infections under consideration? The applicants indicate that intestinal pathology is absolutely normal. Perhaps this, of course, is based on duodenal biopsies? Or are postmortem studies available showing that intestinal pathology is entirely normal in the more distal gut? There will, of course, be data on animal models with these agents. I know that in animal models of cholera the whole gut is histologically normal. This would imply that there is not a delayed type hypersensitivity response of any sort since the most sensitive index of this in the gut is histopathological changes, along with increased expression of HLADR in the crypts.

Thus one would predict complete absence of any TH1 expression in the mucosa. This is apparently at variance with the reported interferon gamma production.

The applicants must be aware of the work from MacDonald in London who, in studies of children with inflammatory bowel disease, reported positive results in his control specimens. This is a controversial matter, and it has been suggested to Professor MacDonald that because his control children had non-specific diarrheal illnesses, in fact he was simply proving that induction of interferon gamma in the gut is an extremely frequent occurrence and is present in acute and chronic form in apparently trivial illnesses.

d) The references cited by the applicants suggest non-specific immune activation in the mucosa and peripheral blood T-cells. I am uncertain as to whether there is any evidence of antigen specific T-cell activity in animal models or in any clinical settings with the ETEC range of antigens. The authors should be quite clear as to how they will interpret their findings, in other words non-specific immune modulating effect, in a type of adjuvent effect, either as a result of the infection itself or the associated gut mucosal injury and permeability, or a true antigen specific T-cell response. The talents you have available at ICDDRB, your collaborators, and the clinical material which is available to you, would allow this very important point to be answered.

e) The applicants propose to use a whole range of different techniques, many of them extremely expensive involving purchased reagents and kits, to study a large number of cytokines in tissues, in molecular biological techniques, as protein, in whole blood samples and in faeces and by ELISPOT methods.

I think this section of the work is badly thought through. In particular, once again the analogy with shigella is a dangerous one. Faecal cytokines may be detectable in shigella stools which are after all full of inflammatory cells pouring into the gut lumen from the rectal mucosa and reaching the stool perhaps only minutes before it is evacuated. On the other hand cytokines which may be relevant in the small intestine may or may not survive transit through the colon and before conducting large numbers of studies on faecal cytokines in particular, the authors should think critically and carry out recovery experiments etc. to establish whether what they aim to do is scientifically valid.

A final point relates to pathological studies of the mucosal biopsies which will be taken in this study.

It is totally unacceptable to simply mention in passing that the biopsies will be studied by a diagnostic Pathologist. Enormous information can be obtained from properly collected, processed and stained mucosal biopsies, by using simply morphometry and/or computerised image analysis. These are highly relevant to mechanisms of immunological injury in the gut. The proposed project is somewhat invasive, submitting volunteers to 2 upper endoscopies with biopsy. I believe that, ethically, the most broad range of information should be collected from the biopsies thus obtained, by tight and accurate pathological techniques. I would be happy to advise the applicants further on this point if the project is funded and if it would be helpful.

D. F. Barker

Proposal: Further evaluation of the oral inactivated ETEC vaccine and studies on the immune response in acute watery diarrhea

PI: Firdausi Qadri

This is a very ambitious proposal with the following objectives:

1. Determination of the safety and immunogenicity of the ETEC vaccine in Bangladeshi children.

For this study, a total of forty children (20: 5-10 year old and 20: below 5 years) will receive two doses two weeks apart of the BS-WC ETEC vaccine. Seven to nine days after each immunization, the investigators will assess ASC responses in peripheral blood and antibody in fecal extracts.

2. Determination of cytokine production in response to natural infection or disease in adults and children.

In adults, cytokine specific mRNAs will be determined in PBMC by RT-PCR. Reverse ELISPOT will be employed for cytokine production from PBMC and duodenal biopsies. Cytokines and receptors in plasma and stool will be determined by ELISA. In children, both patients and vaccine recipients, plasma and stool will be examined for cytokine response.

3. Specificity of the immune response against ETEC CFAs.

For this study, the investigators propose to compare the immune response against different CFs in infants and adults who develop diarrhea due to infection with CF-positive ETEC. Twenty children and 20 adults with ETEC diarrhea will be enrolled for this study. A comparison will also be made between the response in Swedes and Bangladeshi volunteers in different age groups.

4 Determination of CFs on ETEC isolated from Bangladeshi patients.

This is a prospective study for enterotoxin production and CF profile on approximately 2,000 fecal specimens from natural infections occurring in Bangladesh. ELISA and DNA probes will be utilized for toxin production; slide agglutination or dot-blot will be used for CF determination.

The rationale for this proposal is sound, the experimental design appropriate, and the methodology suitable. It is, however, unlikely that the project will be completed within the specified time period. It is also not clear which of these analyses will be performed in Bangladesh and which will be performed in Sweden. If the ELISA for cytokines and RT-PCR for mRNA are to be performed in Bangladesh, then that budget is grossly underestimated. The ELISA for cytokines and RT-PCR for mRNA are very expensive and could easily double or triple the second and third year estimates. Moreover, the duodenal biopsy analysis adds little to the overall project and should be dropped, perhaps with the funds for the cryostat being distributed over the remainder of the project for reagents associated with the cytokine and mRNA assays.

I support the application, with qualification on level of financial support.

gy

ABSTRACT

ANTIGEN-SPECIFIC ANTIBODY SECRETING CELLS IN PERIPHERAL BLOOD IN CHOLERA DUE TO *VIBRIO CHOLERAE* 0139

F. Qadri, J. Hossain, A. Chowdhury, T. Azim, M.A. Salam, A.M. Khan, R.B. Sack, M.J. Albert, A-M Svennerholm. Laboratory Sciences Division, ICDDR,B, Dhaka, Bangladesh

Epidemics and pandemics of cholera are known to be caused by strains of *Vibrio cholerae* 01. However, *V. cholerae* 0139, a new serogroup has recently caused severe cholera-like disease in Bangladesh and elsewhere. Antigen-specific antibody secreting cell (ASC) response was studied in peripheral blood of adult patients with *V. cholerae* 0139 infection. The ASC response was studied in the acute stage (day 1), as well as on days 7 and 11 after the onset of the disease. The ASC response to whole cell antigen prepared from *V. cholerae* 0139 peaked at seven days after infection with the predominant isotypes being immunoglobulin M (IgM) and IgA with negligible IgG ASCs. The ASC response to purified cholera toxin (B subunit) also showed the highest response seven days after infection although the predominant isotype was IgG and IgA with negligible IgM-ASC response. These results indicate that cholera due to *V. cholerae* 0139 induces a strong mucosal response as suggested by the presence of antigen-specific ASCs in peripheral blood very soon after infection.

12th European Immunollgy Meeting, 14-17 June, 1994, Barcelona, Spain. p.165.

ABSTRACT

IMMUNE RESPONSE IN PATIENTS WITH CHOLERA DUE TO *VIBRIO CHOLERAE* SEROGROUPS O1 AND O139

Firdausi Qadri[1], C. Wenneras[2], J. Hossain[1], Y.A. Begum[1], A. Chowdhury, T. Azim[1], R.B. Sack[1], M.A. Salam[1], A.M. Khan[1], M.J. Albert[1], A-M Svennerholm[2].

Laboratory Sciences Division, International Centre for Diarrhoeal Disease Research, Bangladesh, GPO Box 128, Dhaka-1000, Bangladesh[1]. Department of Medical Microbiology and Immunology, Goteborg University, Sweden[2].

Vibrio cholerae of serogroups O1 and O139 are now both considered to be the causative agents of severe cholera. The kinetics of the immune response was studied in these patients at the acute stage of the disease (day 2) and at convalescence. It was observed that the antibody secreting cell (ASC) responses in peripheral blood peaked to homologous lipopolysaccharides (LPSs) and cholera toxin (B subunit) around 7 days after infection. The responses to LPS was serogroup-specific and predominantly in the IgA and IgM isotypes. The response to cholera toxin was mainly seen in the IgA and IgG isotypes. A comparison of the antigen-specific ASC responses with the antibody responses in plasma and in the gut was carried out. Results showed that as expected, patients with cholera due to *Vibrio cholerae* O1 and O139 both respond to cholera toxin but the response to LPS is serogroup-specific.

31st US-JAPAN cholera and related diarrheal diseases conference, December 1-3, 1995, Kiawan Island, SC, USA, p.192.

ABSTRACT

IMMUNE RESPONSE TO THE MANNOSE-SENSITIVE HEMAGGLUTININ (MSHA) IN PATIENTS WITH CHOLERA CAUSED BY *VIBRIO CHOLERAE* SEROGROUPS O1 AND O139

F. Qadri[1], J. Hossain[1], Y.A. Begum[1], G. Jonson[2], T. Azim[1], R.B. Sack[1], M.J. Albert[1] and A-M. Svennerholm[2], [1]International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh, [2]University of Göteborg, Göteborg, Sweden.

The MSHA pilus is a putative adhesion in *Vibrio cholerae* and specific antibodies against it have been shown to protect against experimental El Tor cholera. To understand more about the role of MSHA in natural disease, the immune response to this antigen was studied in adult patients with cholera caused by *V. cholerae* O1 or the new pathogen *V. cholerae* O139. In *V. cholerae* O1 patients, antibody secreting cell (ASC) responses to MSHA were seen in peripheral blood which increased significantly in most of the cases within 7 days of onset of cholera and responses were mainly in IgA isotype. In patients with *V. cholerae* O139 disease, both the magnitude and the frequency of responses were lower. The MSHA responses in sera from patients correlated with the ASC responses. A comparison of these results with the MSHA-specific mucosal immune response in the gut is being carried out.

8th International Congress of Mucosal Immunology, 17-20 July, 1995, San Diego, California, USA. Clin Immunol Immunopath 1995; 76:S105.

ABSTRACT

INTESTINAL IMMUNE RESPONSES IN PATIENTS INFECTED WITH ENTEROTOXIGENIC *ESCHERICHIA COLI*.

C. Wennerås, F. Qadri, C. Czerkinsky, P. Bardhan, B. Sack, A.-M. Svennerholm.
Dept. Medical Microbiology and Immunology, University of Göteborg, Sweden and International Center for Diarrheal Disease Research, Dhaka, Bangladesh.

Enterotoxigenic *E. coli* (ETEC) give rise to diarrhea among children in developing countries by colonizing the small intestinal mucosa by means of colonization factor antigens (CFAs) and producing one or two enterotoxins. The objective of this study was to monitor the intestinal immune responses elicited by this non-invasive enteropathogen. Lymphocytes were isolated from peripheral blood and duodenal punch biopsies and numbers of antibody-secreting cells specific for CFAs and enterotoxin were determined. ETEC-specific antibody levels were monitored in fecal extracts and extracts of duodenal biopsies. Duodenal biopsy extracts were also tested for cytokine contents. All patients responded with large numbers of antibody-secreting cells both in the blood and intestine. IgA was the most prevalent isotype, although IgM and IgG responses were also seen. Interestingly, ETEC infection induced high numbers of antibody-secreting cells not only to the CFA of the infecting strain, but also to heterologous CFAs. ETEC-specific antibodies could also be detected in the feces of most patients. High levels of interferon-γ were seen in the duodenal mucosa. To conclude, ETEC infection elicited a vigorous B cell response both in the blood and intestine against CFAs as well as enterotoxin.

8th International Congress of Mucosal Immunology, 17-20 July, 1995, San Diego, California, USA. Clin Immunol Immunopath 1995; 76:S103.

ABSTRACT

ANTIBODY AND CYTOKINE RESPONSES DETERMINED IN DUODENAL BIOPSY EXTRACTS AFTER ORAL IMMUNIZATION OF HUMAN VOLUNTEERS WITH A PROTOTYPE VACCINE TO ENTEROTOXIGENIC *ESCHERICHIA COLI*

C. Wenneràs[1], F. Quadri[2], P. Bardhan[2], B. Sack[2], C. Czerkinsky[1], and A.-M. Svennerholm[1].

[1]Dept. Medical Microbiology and Immunology, University of Göteborg, Göteborg, Sweden and [2]International Center for Diarrheal Disease Research Bangladesh, Dhaka, Bangladesh.

Enterotoxigenic *E. coli* (ETEC) are one of the main causes of childhood morbidity and mortality among children in developing countries. These noninvasive enteropathogens give rise to diarrhea by colonizing the small intestinal mucosa and elaborating one or two enterotoxins which induce secretion of electrolytes and water in the gut. An ETEC vaccine consisting of formalin-killed ETEC strains expressing the major colonization factor antigens in addition to the B-subunit of cholera toxin (CTB) was given perorally to 9 adult Bangladeshi volunteers. Duodenal punch biopsies were taken prior to and 1 week after two oral ETEC immunizations. The purpose of this study was twofold: to determine immune responses in the intestinal mucosa to vaccine components and to evaluate a new method for monitoring local immune responses in the gut, i.e. saponin extraction of duodenal punch biopsies. Two punch biopsies, each weighing about 5 mg, were incubated overnight in a 2% solution of the detergent saponin in saline, after which the biopsies were discarded. Contents of vaccine-specific antibodies and cytokines in the saponin extracts were determined by ELISA. It was found that a majority of the volunteers, 6-7/9 responded with significant IgA titer increases to both bacterial whole cell components and CTB. Furthermore, an increase in the levels of interferon-gamma could be seen in the intestinal biopsies of most volunteers. These results indicate that a mucosal immune response was induced in the duodenum of most vaccinees, which could be detected in saponin extracts from minute amounts of intestinal tissue.

12th European Immunology Meeting, 14-17 June, 1994, Barcelona, Spain. p.500.

REPLY TO REVIEWERS' COMMENTS

(First reviewer)

Response to relevant comments have only been made.

General comments

The total IgA content and specific response to cholera toxin will be measured in stool extracts to exclude chances of incorrect assessment of the immune response.

IgM responses will be also be measured for antibody secreting cells. This will not increase the volume of blood since a 2-color ELISPOT technique will be used in which two isotypes can be detected at the same time. In ELISA assays too, the IgM responses will be included as has been suggested

Specific comments

a and b

The biopsies will be collected from between the 3rd and 4th part of the duodenum from different sites. We have previously used this part for extracting lymphocytes for ASC and cytokine responses and found it to be adequate. Duodenal biopsies have been used earlier in volunteer studies as well as in studies on the ultrastructural changes in patients with cholera (Mathan et al. 1995). The 1-2 mm sized biopsies have been used for morphological studies and histopathologic grading and found to be satisfactory. Since biopsies will be collected from one location of the duodenum from 4-5 different sites, this will allow us to get a complete picture when immunohistochemistry of the cryostat sections are carried out.

c

The background information has been changed to insert information that ultrastructural studies show changes in the upper small intestinal mucosa in patients with cholera. A Th1 type of response has been shown earlier in cholera and ETEC vaccinees from Sweden.

e

We will not carry out reverse ELISPOT assays for cytokines and will concentrate on ELISAs, RT-PCR and the immunohistochemical analysis of duodenal biopsies. An exploratory study will be conducted with faecal specimens and if cytokines are not detected, this part will be left-out.

We now have two histopathologists, all from Bangladesh (Dr. Mukarram Ali) and one from India (Dr. Minnie Mathan), who have agreed to be a part of the study for histology. In addition, we also plan to look at the phenotypes, activation markers, etc. so that as much information as possible can be obtained. We do not have facilities for quantitative image analysis. However, tissue sections can be analyzed in Sweden by our collaborating investigators.

REPLY TO REVIEWERS COMMENTS
(Second reviewer)

1. We plan to immunize 20 children with the ETEC vaccine. Children of the 5-10 year range will only be studied. Younger children and infants will not be immunized with the vaccine. Similarly we do not plan to study younger children with diarrhoea due to ETEC infections.

4. The study on the Swedish volunteers both adults and children will be carried out in Sweden and laboratory work carried out there. However the same reagents will be used in Bangladesh and Sweden. The reagents will be shared between University of Goteborg and ICDDR,B which will bring down the cost considerably.
We hope that by investigating the response directly at the local site using duodenal biopsies will add to the information on the immune response in acute watery diarrhoea. The funds for the cryostat may appear to be high at present but setting up of the technique will add immensely to the facilites already available. We will share reagents with the other SAREC funded study that will be initiated in ICDDR,B and with the the Department of Microbiology and Immunology at the University of Goteborg, Sweden. We hope that we can approach funding agencies in addition to SAREC to support parts of this work.