Attachment 1.

Date 7/23/80

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr Svennerholm — Trainee Investigator (if any) _____

Application No. 80-029 Gothefors — Supporting Agency (if Non-ICDDR,B) _____

Title of Study Local and Systemic immune responses to cholera B subunit and whole cell in persons with different immune preparations — Project status:

( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle 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 blood, milk — (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 Not applicable
(c) Physical risks — Yes No NA
(d) Sensitive questions — Yes No NA
(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 NA
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.

R. Stan for Dr Sach, Svennerholm, Gothefors
Principal Investigator

Trainee

Received
25.7.80

SECTION I - RESEARCH PROTOCOL

1) Title: Local and systemic immune responses to cholera B subunit and whole-cell vaccine in persons with different immune preparations

2) Principal investigator: Ann-Mari Svennerholm, MD.
University of Göteborg, Göteborg, Sweden.

3) Starting date: September 1980

4) Completion date: August 1981

5) Total direct cost: $ 16,950

6) Abstract summary:

This study, being a logical continuation of our previous study of the immune response to purified cholera B subunit, is designed to answer several important questions about gut mucosal antibody formation and immunologic memory to vaccine candidate cholera antigens: 1) Will a combination of B subunit and whole-cell vaccine (B + WCV) stimulate mucosal antitoxic as well as antibacterial antibody formation? 2) Is the response comparable to that attained by clinical cholera infection? 3) How does the immunologic memory for a mucosal response to immunization compare between naturally-exposed volunteers, the same volunteers after a single immunization, convalescents after clinical cholera, and convalescents after E. coli-LT diarrhea? We will determine local and systemic antibodies to cholera toxin and lipopolysaccharide in lactating women and other adult volunteers in response to a first and second immunization with B + WCV, and we will compare these

convalescents at comparable times after their clinical illness and after they, about one month later, have been cholera immunized with B subunit + WCV. All procedures have been practised in our previous studies.

7) Scientific Program Head:

This protocol has been approved by the Host Defense Working Group.

Signature of Scientific Program Head: W.B. [signature]

8) Review:

(a) Ethical Review Committee: _____

(b) Research Review Committee _____

(c) Director: _____

(d) BMRC: _____

(e) Controller/Administrator: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective:

The long range goal is to develop effective vaccination against cholera. The objective of this study is specifically to determine the local and systemic antibody response to a combined immunogen consisting of cholera B subunit and killed whole-cell vaccine in three categories of volunteers presumably representing different intensity of immunological preparedness: persons with no recent episode of watery diarrhea, cholera patients about one month after their disease, and E. coli LT-disease convalescents.

2. Background:

a. Evidence of protective cholera immunity. There are a few examples of infectious diseases in which the natural infection does not evoke an immune response which renders the individual resistant to reinfection, yet it has been possible to develop solid protective immunity by vaccination; the classical example is tetanus. Generally speaking, the prospects for development of effective immunoprophylactic measures, however, are much greater if the natural infection is known to stimulate a strong, protective immune response in the surviving convalescents. Knowledge about cholera in this regard mainly derives from age-incidence data, second attack rate figures and volunteer studies.

Although all age groups have been susceptible to cholera when it has spread into new areas it is predominantly a disease of childhood in endemic areas. Studies in Bangladesh have shown a close inverse relationship between attack rate of cholera and age and also with serum (vibriocidal) antibody titers.[1] This kind of epidemiologic pattern is characteristic of infections which evoke immunity following either disease or inapparent infection.

The most convincing evidence that cholera evokes protective immunity has come from volunteer studies. Recovery from disease, induced by perorally administered cholera vibrios, is associated with solid resistance for several months to rechallenge with either homologous or heterologous vibrio serotypes. [2] This protection not only prevents the clinical manifestations of disease, but also reduces multiplication of vibrios in the bowel so that organisms cannot be found in stool cultures.

These results are consistent with epidemiologic data from endemic areas which indicate that second attacks of cholera within a year or two are extremely rare.

b. Nature of protective immunity. Studies of convalescents have shown that clinical cholera evokes a significant vibriocidal as well as enterotoxin-neutralizing antibody response in serum. In endemic areas such antibodies are also formed in response to inapparent infection as judged from the age-related titer increases. [1] The vibriocidal antibodies are essentially directed against the cell wall lipopolysaccharide (LPS) of Vibrio cholerae. Studies in animals have clearly shown that both the anti-LPS and the anti-toxin antibodies can protect against experimental cholera, and, furthermore, the two types of antibodies, when present in the gut, show syngergistic protective cooperation. [3] The identification of these two protective antibodies does not exclude the possible presence of additional as yet unidentified protective antibody specificities, directed for instance against flagellar or outer membrane protein antigens.

Although the protective specificities of antibodies appear in serum after cholera infection it is unlikely that the serum antibodies have more than a marginal protective role. The cholera vibrios do not invade the intestinal tissue but remain in the lumen or are reversibly attached to the epithelium. Also the pathogenic enterotoxin is confined to the lining intestinal epithelium. Only antibodies present in the gut lumen, in the mucus layer or on the epithelial surface can thus protect against development of disease,

mainly by counteracting colonization and multiplication of the vibrios (anti-LPS antibodies) and preventing binding of enterotoxin to the intestinal epithelium (antitoxin antibodies). [4] Although very high concentrations of serum antibodies by way of diffusion into the intestine may contribute to protection, it is presently felt (and supported by results from animal studies [5, 6]) that the main protective role is played by secretory IgA antibodies produced locally in the gut. Recent studies have shown that natural cholera infection evokes local formation of secretory IgA antibodies against both V. cholerae LPS and enterotoxin. [7] The associated serum antibody levels seem to represent a combination of IgA and, possibly, IgM "spill-over" from the intestinal synthesis, and IgG and IgM antibodies formed by lymph nodes and spleen in response to immunogenic fragments of bacteria and toxin absorbed from the gut.

Recent studies in animals have yielded new knowledge about the local immune response to cholera antigens. An important finding, which was unexpected in view of some previous results from local immunity in the respiratory tract, is the clear presence of immunologic memory that may be stimulated by antigen. The results in animals futher indicate that the persistence of a potent local immunologic memory for an IgA response is usually of much longer duration than the IgA response in itself. While the intestinal IgA response to cholera antigens in, for instance, rats has been maximal within a week following immunization and then gradually subsided, the memory for a new IgA response has been equally strong for several months[8]. It is presently unknown whether the long-lasting immunity after clinical cholera infection is due to the persistence of a protective antibody synthesis or to an immunologic memory which responds so efficiently to renewed antigen contact that it turns into a protective IgA antibody response, which aborts the new infection before it has given rise to clinical symptoms.

c. Goal of vaccination. The overall goal of cholera vaccination is of course to stimulate long-lasting protection against the disease in the afflicted

populations. The extent to which this goal has been achieved can only be evaluated by a field trial, a costly, laborious "experiment" which should be regarded as a final - not early - test of a vaccine. Before this stage, as a means to develop new or improved vaccines and to determine the best way of immunization, it may be stated a goal of immunization studies to identify safe immunogens and administrations capable of evoking a local immune response that closely resembles that obtained by natural infection. This mimicry in response should ideally include similar magnitude and duration of IgA antitoxin and anti-LPS formation as well as stimulation of immunologic memory for subsequent boosting.

d. Candidate immunogens. Since animal studies indicate that both anti-toxin and anti-LPS antibodies are protective and cooperate synergistically, a cholera vaccine should contain appropriate immunogens for stimulating formation of these two kinds of antibodies. Whole cell cholera vaccines (WCV) stimulate anti-LPS antibody formation but no antitoxic immunity, while the purified toxin B subunit has been shown to stimulate effective antitoxic immunity in animals [9]. A combination of WCV and B subunit is therefore attractive for immunization against cholera provided that its administration results in antibody formation and memory development locally in the gut.

e. Methods for evaluation of local immune response. Conventional cholera serologic methods are not well suited for measurement of the local antibody response in the gut, because intestinal fluid contains anticomplementary activity interfering with vibriocidal antibodies and non-immunoglobulin toxin-neutralizing activity disturbing neutralizing antibody estimates. Furthermore, these do not reveal the immunoglobulin class of the antibody measured. The ELISA method for immunoglobulin class-specific determinations of anti-toxin and anti-LPS antibodies [10] is a primary binding method without these problems, combining very high sensitivity with excellent specificity with regard both to the antibody measured and its immunoglobulin class. Used in conjunction with other immunochemical methods the ELISA technique

thus permits sensitive quantification of locally produced secretory IgA cholera antibodies in intestinal fluid. Recent studies in cholera patients and immunized volunteers (see below) have shown that the so-called intestinal lavage method yields a useful, representative intestinal fluid specimen for the antibody analyses (7, Svennerholm et al., to be published). This method means that the study person drinks an isotonic saline buffer solution at a sufficient rate to overcome the intestinal absorptive capacity (250 ml every 10 minutes) resulting in a transient watery diarrhea - lavage fluid - which is collected, enzyme-inactivated and concentrated for antibody analyses.

There is also recent evidence that antibody determinations in milk and saliva may give proxy information about the local immune response in the gut. The mechanism for this may be 2-fold: antigen-stimulated cells from the intestine have been shown to migrate to the mammary gland and mature to IgA-secreting cells [11, 12] and/or dimeric IgA of intestinal origin which passes into the lymph and blood-stream is selectively taken up by the mammary gland epithelium and transported into the milk [13]. The ELISA method is very effective also in detecting these milk or salivary antibodies immunoglobulin class specifically [14].

When it comes to evaluation of immunologic memory this must presently be done functionally by measurement of the antibody response to boosting. At the systemic immune level an anamnestic antibody response differs from a primary response in several respects: it appears earlier because of the greater number of "committed" cells for the antigen, it reaches a higher peak level, and it can usually be elicited by less antigen because of presence of a high-affinity memory cell population. Basically these differences should apply also to the mucosal immune system, and although memory in this system was only recently discovered, there is already experimental evidence for a higher peak response and affinity associated with local memory for cholera antigens in animals [15].

f. Overall design of proposed study. Our goal is to compare the immune response

after immunization with a combination of cholera B subunit and WCV with the response, known to give long-lasting protection, obtained by clinical cholera disease.

1. We will therefore follow the local and systemic responses of anti-toxin and anti-LPS antibodies in volunteers (half of them lactating women to allow milk sampling) immunized either perorally (PO) or intramuscularly (IM) and similarly the response of these antibodies in convalescent cholera patients. This will allow evaluation of the magnitude and time course of the "acute" antibody response to the two most relevant identified, protective antigens.

2. However, equally important, we will also try to evaluate the degree of immunologic memory attained by the clinical infection in comparison with that induced via natural "endemic" antigen exposure and/or artificial immunization. This is done by immunizing the cholera convalescents about 1 month after their disease and comparing their antibody response with the responses obtained in the "normal" volunteers after the first immunization and after a reimmunization.

3. The adenylate-cyclase activating enterotoxin represents an important cross-reactive antigen of V. cholerae and LT-enterotoxigenic E. coli. In Bangladesh as in other parts of the world these E. coli rather than V. cholerae are the main cause of "natural" enterotoxin-neutralizing antibodies in the population (via clinical as well as inapparent infections). It is of considerable importance to clarify whether E. coli LT infection modulates the antitoxic immune response to subsequently administered cholera B subunit immunogen. We will therefore identify a group of E. coli LT patients (LT-only or LT/ST disease) monitor their acute antitoxin response and then determine their local and systemic antibody response to vaccination with the cholera B subunit-containing immunogen. The results are compared with the vaccine responses in the 'normal" volunteeers and in the cholera convalescents.

g. Previous experiences relating to proposed study

1. Sampling and analyses of specimens:

Our previous studies have given valuable experience about the procedures that are to be used in the present study. This includes both the collection of specimens and the methods for determinations of locally and systemically formed cholera antibodies of different immunoglobulin classes (see protocols by D Sack et al: Determination of local and systemic immune responses in cholera patients, and A-M Svennerholm et al: Local and systemic antibody response in humans after immunization with cholera B subunit antigen).

In these studies the same type of serum, milk, saliva, and intestinal lavage specimens as planned for the present study were collected on 3-7 occasions from more than 40 persons. The sampling procedures have been very well accepted by the volunteers and only few minor complaints such as abodiminal distention and slight nausea in relation to intake of the lavage fluid have been reported. In our earlier studies we also obtained a good picture of the time course of the immune responses following clinical disease or immunization. This knowledge now makes it possible to reduce the number of sampling occasions without losing important information.

For the immunological analyses the ELISA method has proved to be very useful, giving sensitive, immunoglobulin class specific measurements of antibodies against cholera toxin and LPS. When used in conjunction with immunosorbent affinity columns the ELISA has allowed specific quantitation of locally produced IgA antibodies of the secretory type.

2. Immunogens and immunizations:

In the initial B subunit study two immunizations with B subunit (in the same doses as suggested for the present study) were given either i.m. or p.o. to thirty volunteers. It was found that both the p.o. and i.m.

administration of B subunit induced significant intestinal as well as systemic antibody formation in most (>80% of the volunteers). In no instance the p.o. immunization resulted in detectable or systemic side-effects. The i.m. immunization did not induce any symptoms either, but gave rise to mild local side-effects of the same type (but milder) as observed after injection of other toxoid and bacterial vaccines.

Several field trials of killed whole cell cholera vaccines (WCV) have given extensive information about the immunogenicity and about the side-effects of these vaccines when given i.m. or s.c. Oral immunization with WVC has also been tried both in a recent study in Calcutta where as much as 10^{11} killed vibrios were given to each volunteer (16) and in an ongoing study in Sweden (M Jertborn et al) where $5x10^{10}$ killed vibrios have been given in water together with bicarbonate. In no instance, neither in Calcutta nor in Sweden, the oral administration of WCV has resulted in any noticeable side-effects. Preliminary results from the Swedish study also suggest that the p.o. administration of WCV can induce a significant antibody response against V. cholerae LPS in intestine.

3. Rationale:

There is a great need for effective immunization against cholera. This will require the development of better immunogens as well as finding appropriate immunization schedules for stimulating protective immunity locally in the gut. Experimental results indicate that purified B subunit is a safe, promising toxoid immunogen which has been found to induce marked protective immunity in animals when given alone, and also to act in synergy with somatic cholera vaccines (e.g. killed whole-cell vaccine - WCV). A goal of immunization might be to stimulate mucosal IgA antibody formation and immunologic memory comparable to that induced by clinical cholera infection, since clinical illness appears to give long--lasting protection to reinfection. Immunization with a combination of

B subunit and WCV compositionally has the potentials of mimicking the clinical infection in stimulating an antitoxic as well as antibacterial immune response.

B. SPECIFIC AIMS

The specific aims of the proposed study are:

1. To determine the local and systemic immune response (antibody formation and immunologic memory) to a combined cholera B subunit-whole cell vaccine given orally (PO) or intramuscularly (IM).

2. To compare the degree of "immunological preparedness" for mounting a local immune response to this vaccine between cholera convalescents and other volunteers; the convalescents will be vaccinated 1 month after the known epidose of disease.

3. To evaluate the "immune priming" effect of E. coli diarrhea for a subsequent antibody response to cholera B subunit immunization.

C. METHODS OF PROCEDURE

1. Subjects:

Totally 32 persons will be recruited to participate in the study: 1) Eight healthy persons, aged 15-35, without any known recent history of cholera or other severe watery diarrhea. This group of volunteers will be sought for in Nandipara and preferably about half of them should be lactating women. 2) Eight convalescents from bacteriologically verified clinical cholera who will be immunized 25 days after onset of the disease.

3) Six convalescents from bacteriologically and clinically confirmed E. coli LT diarrhea who will be studied 25 days after the onset of disease.

The cholera and E. coli patients for the study will be recruited among adults, 15-50 years, coming to the ICDDR,B treatment center with a history of acute watery diarrhea and signs of severe or moderate-to-severe dehydration.

Cholera patients should fulfill the following additional criteria:

1) To be dark-field or culture-positive for V. cholerae in stool

2) To continue to purge for at least 24 hours after admission

3) To have a watery stool output of at least 5 liters during the first 24 hours after admission.

E. coli patients are selected among dark-field and cholera culture negative patients who continue to purge watery or semifluid stool for at least 24 hours and from whose admission stool one or more of 5 picked E. coli colonies are shown to produce LT or LT+ST by laboratory methods.

Candidate patients receive standard treatment, no antibiotic is given for 48 hours to allow antibacterial cholera immunity to develop.

All subjects will be fully informed about the purpose of the study, the procedures involved and possible side-effects in relation to the immunization and sampling of specimens. Informed consent will be required from everyone participating in the study - a copy of the information sheet is enclosed. Before entering the study all volunteers will be carefully examined by a physician. Subjects with one or more of the following symptoms or signs will be excluded: hypertension, heart disease, epilepsy, glaucoma, chronic renal disease and pregnancy.

2. Immunizations:

The 32 subjects will be divided into 4 different study groups, each comprising 8 persons which are immunized as shown in Table 1:

Table 1.

Group	Recent clinical diarrhea	Vaccination: Time after onset of diarrhea	Vaccination: Route	Vaccination: No. of immunizations
I	None	-	PO	2 (25 days apart)
II	"	-	IM	2 -"-
III	Clinical cholera	25 days	PO	1
IV	E.coli LT diarrhea	25 days	PO	1

For the PO immunization a combination of 500 µg B subunit and 6 ml of WCV ($8x10^9$ Inaba and Ogawa vibrios/ml, National Bacteriological Laboratory, Stockholm, Sweden) will be given and for the IM ones 150 µg B subunit and

0.75 ml WCV. The WCV for the oral immunizations will be extensively dialyzed against water and then freeze-dried. Immediately before administration it will be redissolved to the original volume by addition of sterile water.

The antigen mixture for the PO immunization will be given 5 minutes following administration of 100 cc $NaHCO_3$ (7.5 g/l). The antigen will then also be given in 100 cc of the same $NaHCO_3$ solution.

The IM immunizations will be given with syringes and 22# needles in the deltoid muscle; the WCV will be given in the right arm followed directly by injection of B subunit toxoid in the left arm.

Following the oral administration of B subunit-WCV, toxicity surveillance will be carried out daily for 5 days. After the IM injections daily check-ups will be carried out for at least 10 days or until any symptoms that can be related to the immunization have disappeared. The surveillance forms (separate for PO and IM immunizations) that were used in the B subunit pretrial 1979 will be used (enclosed).

With regard to the IM immunizations one form for the B subunit immunogen and one for the WCV will be used for each patient.

3. Clinical specimens:

The clinical specimens listed in the table below will be obtained from the volunteers in the various test groups. These specimens are taken on the day for or one day before vaccination and then 3, 9 and 25 days after the vaccination. In addition, the same specimens are collected from the cholera and E. coli patients 8 days after admission to the hospital, i.e. 17 days before vaccination, to allow comparison of the antibody responses following infection and vaccination, respectively.

Samples	Groups I + II	Groups III + IV
Serum	X	X
Milk	X[1]	
Saliva	X	X
Lavage	X	X

The serum will be separated from a finger stick specimen of blood. A Natelson tube will be used to collect approximately 200 µl with each bleeding. Following initial separation of serum, it will be diluted 1:10 in PBS and three aliquots will be made and frozen.

The milk will be collected by manual expression into a fecal cup. The milk is then centrifuged at 10.000 x g for 10 minutes and the middle layer will be aspirated, aliquotted into 3 aliquots and frozen.

Whole stimulated saliva will be obtained by asking the subject to chew on parafilm for ~5 minutes while the saliva is put in a fecal cup. The saliva is then centrifuged at 10.000 x g for 5 minutes and the supernatant collected, aliquotted and frozen.

The lavage specimens will be collected as described [7] by letting the volunteers drink an isotonic salt solution until a watery diarrhea ensues. The drinking is then continued until ~1000 cc of watery stools has been collected. This usually means that the volunteer has to drink 2500 - 4500 cc of isotonic fluid. The passed liquid lavage stool is then heated to 56°C for 15 min to inactivate proteolytic enzymes. Of this material 300 cc are sterile-filtered and concentrated to 10 ml by means of negative pressure dialysis. The concentrated material is then aliquotted in 5x2 ml volumes and frozen at -70°C until used. This procedure is the same as that used for collection of lavage specimens in the B subunit study in 1979.

4. Laboratory assays:

The specimens collected will be tested for antitoxic and antibacterial antibody and for total immunoglobulin by the following methods:

1) antitoxic antibodies in the specimens will be determined with the ELISA IgG, IgA and in some instances IgM anti-cholera toxin assay

2) antibacterial antibodies by means of the ELISA measuring IgG, IgA and IgM antibodies against purified V. cholerae lipopolysaccharide

3) total IgG and total IgA will be assayed in all secretions (milk, lavage and saliva) by means of the immunobead ELISA method.

The relative proportion of secretory IgA in relation to total IgA will be measured on peak titer lavage and milk specimens. This will be done by testing the samples for specific IgA- and SC-containing antibodies before and after passage through an affinity column with anti-SC antibodies covalently coupled to Sepharose. The column will remove the secretory IgA antibodies while non-secretory IgA passes unbound through the column [7]. Detailed procedures of these methods have recently been described [7].

5. Data analysis:

The data obtained through the various assays described will be analyzed with regard to elucidating the following issues:

1) Frequency and magnitude of antibody response after immunization. This is analyzed a) by comparing geometric mean titers of antibodies pre- and post-immunization for the various groups of vaccinees; b) by identifying significant titer rises (> 2-fold) in individual cases. The basis for these comparisons in the various secretions will be units of specific antibody per unit amount of total immunoglobulin (especially IgA).

2) Kinetics of antibody response in serum and secretions. This is done by defining the course of the antibody responses both on an individual basis and by means of the group geometric mean titers.

3) Correlations between antibodies in lavage and other specimens. It is important to clarify to which extent the antibody response detectable in lavage specimens (which we assume is the best possible, although laborious, estimate of local immunity) is reflected in saliva, milk or serum in a way allowing proxy estimation of intestinal immunity by titrations of antibody in the latter specimens. The lavage IgA antibody responses will therefore be compared individually and group-wise with the other responses as to: a) incidence and magnitude of significant titer increases; b) time for peak response; c) course of response after the peak. The degree of correlation of these parameters will be determined.

4. Assessment of immunologic memory. The intent is to compare the degree of immunological preparedness between the "normal" volunteers, the same persons

25 days after they have received their first immunization and the cholera and E. coli patients 25 days after onset of their disease. This will be done by comparing the geometric mean titers and titer fold-increases in these groups in response to the same immunization, both very early in the response (day 3) and at the presumed peak (day 9) and thereafter.

Significance

Results of animal studies indicate that antitoxic and antibacterial cholera antibodies in the gut cooperate synergistically in the host resistance to cholera by preventing toxin binding and bacterial colonization; the protective antitoxins are mainly directed against the B subunit region of the toxin and the protective antibacterial antibodies against the cell wall lipopolysaccharide. In animals these protective antibodies have efficiently been produced by immunization with a combination of cholera B subunit and killed whole cell vaccine (WCV). The studies now proposed will answer several important questions about intestinal mucosal antibody formation and immunologic memory to those vaccine candidate antigens in persons living in a cholera-endemic region: 1) Will a combination of B subunit and whole-cell vaccine (B + WCV) stimulate mucosal antitoxic as well as antibacterial antibody formation? 2) Is the response comparable to that attained by clinical cholera infection? 3) How does the immunologic memory for a mucosal response to immunization compare between naturally-exposed volunteers, the same volunteers after a single immunization, convalescents after clinical cholera, and convalescents after E. coli-LT diarrhea? The important issue, particularly in developing countries, whether infection with LT producing E. coli can "prime" the intestine to respond more efficiently to cholera toxin antigen will be elucidated as will also the possibility that antibody responses in milk and saliva can be used as proxy measures of the intestinal immune response. The study will give important information for our long-term goal which is to develop effective immuno-prophylaxis against cholera.

E. FACILITIES REQUIRED

Patients will be hospitalized in the study ward of the treatment centre. Transport will be provided to and from Nandipara. Laboratory specimens will be handled by the Immunology Laboratory.

No new facilities will be required.

F. COLLABORATIVE ARRANGEMENTS

The study will be conducted by Dr Gothefors, a staff physician and nurse at ICDDR,B with Drs Svennerholm and Sack who will be visiting during the study and Drs Holmgren and Jertborn in Sweden. Drs Sack, Svennerholm and Holmgren have all participated in collaborative research with ICDDR,B in the past.

BIBLIOGRAPHY

1. Mosley, W.H. The role of immunity in cholera. A review of epidemiological and serological studies. Tex. Rep. Biol. Med. 27 (Suppl. 1), 227, 1969.

2. Levine, M.M. Immunity to cholera as evaluated in volunteers. In Cholera and related diarrheas, eds. Ouchterlony, Ö. & Holmgren, J. 43rd Nobel Symposium, Stockholm 1978, p. 195 (Karger, Basel 1980).

3. Svennerholm, A.-M., Holmgren, J. Synergistic protective effect in rabbits of immunization with Vibrio cholerae lipopolysaccharide and toxin/toxoid. Infect. Immun. 13, 735, 1976.

4. Svennerholm, A.-M. The nature of protective immunity in cholera. In Cholera and related diarrheas, eds. Ouchterlony, Ö. & Holmgren, J. 43rd Nobel Symposium, Stockholm 1978, p. 171 (Karger, Basel 1980).

5. Svennerholm, A.-M., Lange, S. & Holmgren, J. Correlation between intestinal synthesis of specific IgA and protection against experimental cholera in mice. Infect. Immun. 21, 1, 1978.

6. Lange, S., Hansson, H.-A., Molin, S.-O., Nygren, H. Local cholera immunity in mice: Intestinal antitoxin-containing cells and their correlation with protective immunity. Infect. Immun. 23, 743, 1979.

7. Sack, D., Islam, A., Holmgren, J., & Svennerholm, A.-M.: Development of methods for determining the intestinal immune response to V. cholerae antigens in humans. Proc. 15th U.S.-Japan Joint Cholera Conference 1979, p. .

8. Pierce, N.F. & Koster, F.T. The intestinal immune response to cholera toxoid/toxin: dependence on immunization route and antigen form. In Cholera and related diarrheas, eds. Ouchterlony, Ö. & Holmgren, J. 43rd Nobel Symposium, Stockholm 1978, p. 185 (Karger, Basel 1980).

9. Holmgren, J., Svennerholm, A.-M., Lönnroth, I., Fall-Persson, M., Markman, B. & Lundbäck, H. Development of improved cholera vaccine based on subunit toxoid. Nature 269, 602, 1977.

10. Holmgren, J. & Svennerholm, A.-M. Enzyme-linked immunosorbent assays for cholera serology. Infect. Immun. 7, 75), 1973.

11. Weisz-Carrington, P., Roux, M.E., McWilliams, M., Phillips-Quagliata, J.M. & Lamm, M.E. Hormonal induction of the secretory immune system in the mammary gland. Proc. Natl. Acad. Sci. USA 75, 2928, 1978.

12. Hanson, L.Å., Carlsson, B., Cruz, J.R., Garcia, J., Holmgren, J., Shaukat, R., Kahn, Lindblad, B.S., Svennerholm, A.-M., Svennerholm, B. & Urrutia, J. Immune response in the mammary gland. In Immunology of breast milk, eds. Ogra, P.L. & Dayton, D.H., p. 145, Raven Press, New York 1979.

13. Halsay, J.F., Johnsson, B.H. & Cebra, J.J. Transport of immunoglobulins from serum to colostrum. J. Exp. Med. 151, 767, 1980.

14. Svennerholm, A.-M., Holmgren, J., Hanson, L.Å., Lindblad, B.S., Quereshi, F. & Rahimtoola, R.J. Boosting of secretory IgA antibody responses in man by parenteral cholera vaccination. Scand. J. Immunol. 6, 1345, 1977.

15. Lange, S., Nygren, H., Svennerholm, A.-M. & Holmgren, J. Antitoxic cholera immunity in mice: influence of antigen deposition on antitoxin--containing cells and protective immunity in different parts of the intestine. Infect. Immun. 28, 17, 1980.

16. Ganguly, R., Clem, L.W., Bencic, Z., Sinha, R., Sakazaki, R. & Waldman, R.H. Antibody response in the intestinal secretions of volunteers immunized with various cholera vaccines. Bull. WHO 52, 323, 1975.

ABSTRACT SUMMARY

1. The population to be studied includes village people from Nandipara and other free living Bangladeshi adults. Bangladeshi adults are needed for this study because they presumably have had previous exposure to cholera antigens, and also because they are representative of the population who would benefit from a potential vaccine containing this antigen.
2. The risks related to this study are minimal. The antigen being used has had extensive testing in animals and Swedish volunteers with no untoward effects. The blood samples are of small volume and the lavage is currently being carried out in groups of patients at ICDDR,B.
3. Daily surveillance by a physician will be maintained after giving the antigen and any reactions would be managed immediately. The doses of antigen will actually be administered in the hospital, this will further minimize the risk.
4. The volunteers will be identified only by study number. Data forms with the names attached will be kept locked in a file in the investigators office and names will be destroyed at the end of the study.
5. Signed informed consent will be obtained.
6. No interview.
7. The individual subject may benefit if the antigen provides some protection against cholera. The society in general would benefit by the development of a successful cholera vaccine, especially one that could be administered orally.

8. The project will require specimens of blood, saliva, milk, stool and lavage fluid. It will not require hospital or other records.

SECTION III - BUDGET

A. DETAILED ICDDR,B BUDGET

1. PERSONNEL SERVICES

Name	Position	% of effort	Annual salary	Taka	Dollars
Ann-Mari Svennerholm	Investigator	30%		No cost	
Marianne Jertborn	Co-investig.	30%		No cost	
Jan Holmgren		10%		No cost	
David Sack		10%		No cost	
Leif Gothefors		15%	35,000		5,250
Study physician		3 months		10,500	
Field assistant		3 months		6,000	
Lab staff-immunology		12 pers.month		24,000	
Nurse study ward		3 months		6,000	
			Sub Total:	46,500	5,250

2. SUPPLIES AND MATERIALS

Plastics, glassware (plates, pipette tips, tubes), dialysis bags	3,000
Reagents (antisera, conjugates, enzyme, etc.)	2,000
Miscellaneous clinical supplies	500
Sub Total:	5,500

3. EQUIPMENT

0

4. HOSPITALIZATION COSTS

For collection of specimens: 16 patients x 4 days
16 patients x 5 days = 144 patient days
Extra hospitalization in relation to acute diarrhea:
16 patients x 2 days = 32 patient days
Totally 176 patient days à TK 150/day

	26,400	
Sub Total:	26,400	0

5. OUTPATIENT CARE

0

6. ICDDRB TRANSPORT

20 miles/day x 20 days = 400 miles
15 miles x 64 occasions = 960 miles

	2,720	
Sub Total:	2,720	0

7. TRAVEL

0

8. TRANSPORT OF THINGS

500

9. RENT, COMMUNICATIONS, UTILITIES

Sub Total:	0	500

0

		Taka	Dollar
10. PRINTING			
Forms-stencil, xerox		1,000	
Publication costs			300
	Sub Total:	1,000	300
11. CONTRACTUAL SERVICES			
TK 120/patient x 16 patients			
TK 150/ patient x 16 patients		4,320	
	Sub Total:	4,320	0
12. CONSTRUCTION			
0			
	Total:	80,940	11,550

or US Dollars

16,950

(15 TK = 1 US Dollar)

B. ICDDR,B BUDGET SUMMARY *

Category	Taka	Dollars
1. Personnel	46,500	5.250
2. Supplies	-	5.500
3. Equipment	-	-
4. Hospitalization	26,400	-
5. Outpatients	-	-
6. ICDDRB Transport	2,720	-
7. Travel	-	-
8. Transport of things	-	500
9. Rent, communications, utilities	-	-
10. Printing	1,000	300
11. Contractual services	4.320	-
12. Construction	-	-
Total:	80,940	11,550

or US Dollars 16,950

* The costs for the non-ICDDR,B-employed Swedish scientists (salaries, travels, per diems, etc.) which are mainly covered from the Sarec grant to the Institute of Medical Microbiology, University of Göteborg are not included in the budget.

PERMISSION FORM - IMMUNIZATION WITH A COMBINATION OF WHOLE CELL CHOLERA VACCINE AND B SUBUNIT ANTIGEN

The International Centre for Diarrhoeal Disease Research, Bangladesh is carrying out research to determine the immune response to a material which may some day be used as a cholera vaccine. This material, which is composed of common whole cell vaccine and a natural toxoid (B subunit antigen) will stimulate the body to make protective substances against cholera, but it has no harmful activity. We would like you to participate in this study. If you do decide to participate you can expect the following:

1. We will give you two doses of the vaccine - B subunit mixture either by injection or by mouth.
2. We will collect samples of saliva, milk and blood from a finger prick several times during the next 2 months. The total amount of blood taken will not exceed 2 ml.
3. We will also have you do the intestinal lavage procedure 4-5 times. This is a procedure in which you will drink a large volume (up to 5 liters) of salty water and this will cause a temporary diarrhea. The diarrhea stops shortly after you stop drinking the salty water. During the lavage you will have a full feeling in the abdomen, you will gain 1-3 kg in weight but you will not have pain or any serious side-effects.
4. A field worker will visit daily after your vaccine - B subunit mixture is administered to look for any reaction.
5. We will give you a small gift (with a value of about 25 TK) for each day you have a lavage test .
6. You do not have to join the study. If you decide not to join, you will still be elligible for care at ICDDRB. You may also decide to withdraw after entering the study and this will not affect any medical care you might require now or later on.
7. Your medical records will be kept confidential.

If you decide to join the study, please sign here

_____

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র, বাংলাদেশ।

সম্মতি পত্র।

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র বর্তমানে কলেরা "বি সাব ইউনিট এ্যান্টিজেন" নামে একটি নতুন ঔষধ নিয়ে গবেষণা করছে। ভবিষ্যতে এই ঔষধটির কলেরার টিকার সাথে সংযোজিত হওয়ার সম্ভাবনা আছে। এই ঔষধটি শরীরে কলেরা রোগের প্রতিরোধ ক্ষমতা বাড়িয়ে তুলতে সাহায্য করবে, কিন্তু কোন প্রকার অনিষ্টকর প্রতিক্রিয়া করবেনা। আমরা আশাকরি আপনি এই গবেষণায় অংশগ্রহন করবেন।

১) আপনাকে দুইবার ইনজেকশন দ্বারা অথবা মুখে "বি সাব ইউনিট এ্যান্টিজেন" দেওয়া হবে।

২) আমরা আগামী দুই মাসের মধ্যে কয়েকবার আপনার কাছ থেকে লালা, বুকের দুধ ও আঙ্গুল থেকে রক্ত নেবো। প্রত্যেক বারে রক্তের পরিমান ২ (দুই) সি.সি'র অধিক হবেনা।

৩) আপনাকে ৬ (ছয়) বার লবন পানি খেয়ে সাময়িকভাবে তরল পায়খানা করতে হবে (অর্থাৎ ইয়ুলেন)। আপনি লবনপানি খাওয়া বন্ধ করার কিছুক্ষনের মধ্যেই তরল পায়খানাও বন্ধ হয়ে যাবে। এই প্রক্রিয়ার সময় আপনার পেট একটু ভার লাগতে পারে এবং আপনার ওজন ১ সের থেকে ৩ সের পর্যন্ত বাড়তে পারে, কিন্তু এছাড়া অথবা অন্যকোন বিরূপ প্রতিক্রিয়া হবে না।

৪) "বি সাব ইউনিট" দেওয়ার পরে কোন প্রতিক্রিয়া হচ্ছে কিনা তা দেখার জন্য একজন "মাঠ কর্মী" প্রতিদিন আপনার সাথে সাক্ষাত করবেন।

৫) আপনি পরীক্ষাপ্রতি ৫০ টাকা অথবা সর্বসাকুল্যে ৩০০ টাকা পাবেন।

৬) আপনি এই গবেষণায় অংশগ্রহন না করলেও আপনার চিকিৎসা করা হবে। আপনি ইচ্ছা করলে যে কোন সময় গবেষণা পরিত্যাগ করতে পারেন, তাতে চিকিৎসার কোন তারতম্য ঘটবে না।

৭) আপনার চিকিৎসা সম্পর্কীয় সকল তথ্যাদি গোপন রাখা হবে।

আপনি এ গবেষণায় রাজী থাকলে এখানে আপনার স্বাক্ষর দিন।

তারিখ:

স্বাক্ষর/টিপ সহি

REACTION SURVEILLANCE FORM FOR INTRAMUSCULAR
ADMINISTRATION OF B SUBUNIT ANTIGEN

Name _____ Code Number _____ Census Number _____

Date of vaccination _____ Time _____

Primary (P) or Secondary (S) immunization? _____

If secondary, nature of primary immunication (IM or PO) _____

Pre-immunization temperature _____

Are there any objective and/or subjective symptoms of the following which can be referred to the oral intake of B subunit? _____

Local Reactions	Day 0 (Imm. Day)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Late Reaction
Pain	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Redness	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Tenderness	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Induration	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Skin Blister	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Glandular Enlargement	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Systemic Reactions											
Body Temp.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Urticaria	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other allergic Manifestations	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Decreased General Activity	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

* Date of reaction _____

** If yes, nature of symptoms _____
(Filled in with scoring criteria according to speerate list.)

Examiner: _____

REACTION SURVEILLANCE FORM FOR ORAL ADMINISTRATION
OF B SUBUNIT ANTIGEN

Name _____ Code Number _____ Census No. _____

Date of vaccination _____ Time _____

Primary (P) or secondary (S) immunization: _____

If secondary, nature of primary vaccination (IM or PO) _____

Preimmunization temperature _____

Are there any objective and/or subjective symptoms of the following which can be referred to the oral intake of B subunit? _____

Local Reactions	Day 0 (Imm. Day)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Late* Reactions
Abdominal pain	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Abdominal cramps	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Abdominal Distention	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Vomiting	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Diarrhea	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Systemic Reactions											
Body Temp.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Nausea	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Dizziness	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Exanthema	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Edema	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

* Date of reaction _____

** If yes, nature of symptoms _____

_____ _____
Examiner