Attachment 1. 86

REVIEW BOARD ON THE USE OF HUMAN VOLUNTEERS Date_____
CRL

Principal Investigator David A. Sack Trainee investigator(if any)_____

Application No 77-11 Supporting Agency(if Non-CRL)_____

Title of study [illegible] 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 N/A)

1. Source of Population:
   a) Ill subjects (Yes) No
   b) Non-ill subjects Yes (No)
   c) Minors or persons under guardianship Yes (No)
2. 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 possibly damaging to subject or others Yes (No)
3. 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
4. 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 N/A
   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
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).
   X Protocol (Required)
   X Abstract summary (Required)
   X 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)
   X Informed consent form for subjects
   ___ Informed consent form for parent guardian
   X 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 for review:
1. A description of the areas to be covered in the questionnaire or interview which could be considered of a 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 committee for review.

We agree to obtain approval of the Review Board on Use of Human Volunteers before changes involving the rights and welfare of subjects before making such changes.

David A. Sack

Principal Investigator

Trainee

Attachment 1a

INFORMATION TO INCLUDE IN ABSTRACT SUMMARY

The Board will not consider any application which does not include an abstract summary. The abstract should summarize the purpose of the study, the methods and procedures to be used, by addressing each of the following items. If an item is not applicable, please note accordingly:

1. Describe the requirements for a subject population and explain the rationale [illegible] using in this population special groups such as children, or groups whose [illegible] to give voluntary informed consent may be in question.

[illegible]s any potential risks - physical, psychological, social, legal [illegible] the likelihood [illegible]s of such risks. If methods [illegible], if any, that were

[illegible] protecting against or minimizing potential risks and an assessment of their likely effectiveness.

4. Include a description of the methods for safeguarding confidentiality or protecting anonymity.

5. When there are potential risks to the subject, or the privacy of the individual may be involved, the investigator is required to obtain a signed informed consent statement from the subject. For minors, informed consent must be obtained from the authorized legal guardian or parent of the subject. Describe consent procedures to be followed including how and where informed consent will be obtained.

   (a) If signed consent will not be obtained, explain why this requirement should be waived and provide an alternative procedure.

   (b) If information is to be withheld from a subject, justify this course of action.

6. If study involves an interview, describe where and in what context the interview will take place. State approximate length of time required for the interview.

7. Assess the potential benefits to be gained by the individual subject as well as the benefits which may accrue to society in general as a result of the planned work. Indicate how the benefits outweigh the risks.

8. State if the activity requires the use of records (hospital, medical, birth, death or other), organs, tissues, body fluids, the fetus or the abortus.

The statement to the subject should include information specified in items 2,3,4 and 7 as well as indicating the approximate time required for participation in the activi[illegible]

Rcd. 09 Aug 197

77- 011

SECTION I - RESEARCH PROTOCOL

1) Title: Local Immune Response in Cholera

2) Principle Investigator: David A. Sack, M.D.

3) Starting Date: May 1977

4) Completion Date: May 1979

5) Total Direct Cost: $74,471

6) Abstract Summary:

Using several assays of immunologic function, recently adapted for use with cholera, we plan to quantitate the local intestinal as well as systemic immune response to cholera in humans. Acquired immunity to cholera is one of the defense mechanisms against reinfection with V.cholerae, and both local intestinal immunity (IgA) and systemic immunity are likely stimulated by the disease. While rises in serum anti-bacterial and antitoxic are well documented following the disease, the local immune response, which may be more important in protection, is less well understood. Various cholera vaccines have been developed for human use; however, the best and longest lasting protection is afforded by a previous episode of the disease, which gives excellent protection for at least one year. Documentation and quantitation of the local immune response to cholera would seem crucial to the evaulation of new cholera vaccines, especially if they are to be administered locally. Since antitoxic and anti-bacterial immunity may act

synergistically, both types of antibodies would need to be measured. Assays which will be used include the following: Antitoxin - neutralization, hemmaglutination, enzyme-linked immunoabsorbent (or radio immune assay) hemolytic plaque assay, and flourescent antibody; Antibacterial-vibriocidal, enzyme-linked immunoabsorbent and vibriolytic plaque assay. The assays will be done using the following clinical apecimens: serum, peripheral lymphocytes, duodenal secretion, and breast milk. These studies should form the basis for quantitating the local antitoxic and antibacterial immune response in human cholera and provide a basis for comparing the response in different population and nutritional groups, as well as providing a basis for the evaluation of potential new cholera vaccines designed to stimulate local immunity.

7) Reviews:

a) Research Involving Human Subjects: _____

b) Research Committee: _____

c) Director: _____

d) BMRC: _____

e) Controller/Administrator: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective: The long range goal of these studies is the control of enterotoxigenic diarrheal diseases through the use of effective vaccines. Primary emphasis will be placed on quantitating the local immune response, both antitoxic and antibacterial, in naturally acquired cholera in Bengali adults. Secondary emphasis will be the development and/or adaptation of techniques which will reflect gut immunity and would be applicable to larger study groups. These studies should provide a basis for evaluating the local immune response to new cholera vaccines, especially those designed to be administered locally, and for determining the optimal route and dosages of the antigens. It is also anticipated that these studies will enhance basic understanding of the local intestinal immune response in humans which will be applicable to other intestinal antigens and bacterial infections.

2. Background: Cholera is a disease characterized by severe watery dehydrating diarrhea and is caused by the bacterium Vibrio cholerae. The V.cholerae are first ingested; they then colonize and multiply in the small intestine, where they secrete an exotoxin. This toxin, a protein (MW 84,000) binds irreversibly to the surface of the mucosal cells (to a GM_1 ganglioside

receptor) and stimulates adenylate cyclase which leads to an increase in intracellular cyclic AMP which mediates the secretion of electrolytes and water into the lumen of the gut[1]. The clinical syndrome of cholera is due to the massive outpouring of fluids and electrolytes with resulting diarrhea and dehydration. The bacteria do not invade the mucosa, and physiologically significant amounts of toxin are not absorbed; rather the pathogen and its toxin cause disease from the lumen of the gut or at the surface of the mucosa.

Humans have several defense mechanisms against enteric infections (including cholera), and these include sanitation and public health measures, gastric acid,[2] nonspecific intestinal factors such as mucus and motility, bacterial competition, and local and systemic immunity.[3] The exact nature of the local immune response to cholera is not yet known. Much more is known of the systemic immune response because of the ease in obtaining serun specimens and because of the diagnostic usefulness of serum antibodies. Patients with cholera regularly develop rises in antibody to both the bacteria (vibriocidal antibodies)[4] and the toxin (antitoxic abtibodies).[5,6] The measurement of these serum antibodies has been useful in confirming the diagnosis of cholera in suspected cases of cholera, in detecting sub-

clinical infection and in performing seroepidemiological studies.[7]

Patients with cholera also have an immune response as measured by protection from reinfection. Cash, et. al. demonstrated protection for one year after an episode of volunteers in American adult volunteers[8] and Woodward's study suggested homologous serotype protection for one year among Bengali subjects who had had a naturally acquired cholera infection.[9] It seems clear that immunity, as measured by serum antibacterial and antitoxic antibodies, and by protection from reinfection, developes following the disease. The local immune response to the disease is much less well understood, although rises in vibriocidal titer in intestinal secretions from convalescent cholera patients have been observed.[10]

A parenteral vaccine is commercially available for cholera using bacterial cell wall as the antigen. This vaccine which stimulates serum vibriocidal antibody does give some protection ($\sim$ 50%) for a relatively brief duration ($\sim$ 6months).[11,12] Clearly an improved vaccine is needed which will give increased protection for a longer duration. Several approaches have been suggested for an improved cholera vaccine. These include: 1) using a toxoid vaccine,[13] 2) combining a toxoid with cell wall antigen,[14]

3) changing the route of administration of antigen to stimulate local immunity,[15] 4) using a live non-pathogenic Vibrio cholerae, which produced either no toxin or produces a natural toxoid.[16, 17]

A toxoid vaccine given systemically has shown a 100% protection for 4 months in the dog model.[13] A similar toxoid given to humans stimulated only a poor antitoxic response and provided only a low level of protection for a few weeks in a cholera vaccine field trial.[18] Combining a toxoid and cell wall antigens appeared to protect synergistically in the rabbit loop model but has not been tested in either the whole dog model or in a field trial. Changing the route of administration to stimulate local immunity has also been shown to provide protection. Dogs primed systemically and boosted orally are highly protected for up to 1 year following the booster.[15] Killed whole vibrios generally also induce local antibacterial antibody production in both man[19] and animals;[20] however, the protection produced by this vaccine schedule is not known.

Studies are now underway in animals to assist in defining the mechanism of IgA antibody stimulation from a soluble antigen

in the gut,[21] and is based on the following model. In the presence of antigen, primed lymphoblasts migrate from peyers patches, through the thoracic duct, the circulation, then selectively "home" nack to the lamina propria of the gut. (Figure 1). From the lamina propria the now mature plasma cells secrete IgA antibody which combines with secretory piece and diffuses into the lumen. In rats which have been primed with

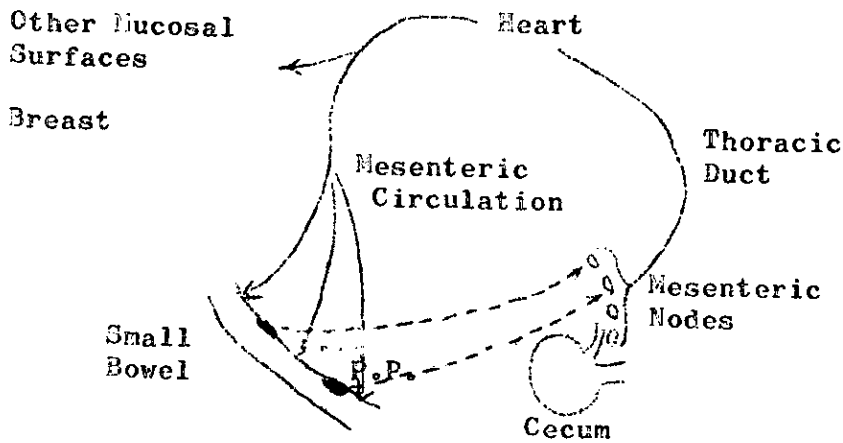

Fig. 1. Schematic circulation of enteric immunocytes. Immunoblasts arise in Peyer's patches (P.P.) and migrate as shown, eventually homing to intestinal lamina propria and the breast.

either intraperitoneal or intraduodenal toxoid, and intraduodenal boost will produce a marked increase in the numbers of antibody containing cells in thoracic duct lymph as measured by flourescent antibody technique. This increase begins within 60 hours and peaks by 72 hours, then falls to baseline in 6 days. Antitoxin plasma cells then begin appearing in the gut lamina propria reaching a peak by day 5 after boost. (Figure 2).[22] This is correlated with an increase in antitoxin in intestinal secretion in dog loops wihich peaks at day 7.[15] However, the immunologic response as measured by flourescent cells in thoracic duct lymph or lamina propria varies greatly with the antigen used, route of administration of both the primary and booster vaccine, and the species of the test animal,[23] so that optimal antigen

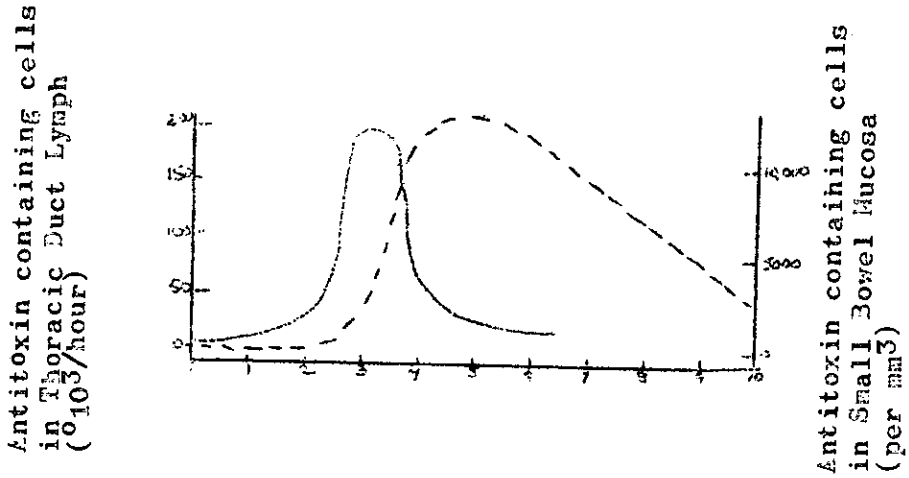

Fig.2 Appearance of antitoxic-containing cells after intraperitoneal priming and intraduodenal boosting. Rats were primed with 100 ug toxoid in FCA, intraperitoneally and boosted with 1 mg toxoid given intraduodenally on day 14. Data on antitoxin-containing cells among TDL are from 11 rats; on small bowel mucosa from 20 rats.

preparations, and dosage schedules will have to be determined in humans in order to obtain maximum effectiveness.

The class of antibody which is active in protection from infection is somewhat controversial. Clearly, IgG antitoxin in high titers are protective as demonstrated in dogs passively immunized with IgG antitoxin.[24] The serum titer required however, for protection was approximately 300 units, a level not easily sustained by present vaccines. IgA is also protective as illustrated by dogs boosted locally with toxoid who were protected at a time when serum levels of antitoxin were low.[15] It seems that whatever the class of antibody, because of the pathophysiology of the disease, the immunoglobulin must be present in sufficient quantities in the lumen or at the mucosal surface of the gut. IgG is capable of diffusing into the gut lumen;[25] however, as previously mentioned, high serum titers are required. Secretory IgA would seem to be the more likely mechanism of prolonged immunological protection resulting from the naturally acquired disease.

Another controversial area is the relative protection afforded by antibacterial vs. antitoxic immunity. Antibacterial serum titers in seroepidemiologic and vaccine studies have correlated

with protection,[26] though this need not be a cause-effect relationship. In animals, as previously mentioned, a toxoid vaccine gave excellent protection without increasing vibriocidal titers.[15] It would seem likely that both types of antibody might be effective, one by blocking colonization, the other by neutralizing the toxin. The two effects should be at least additive and perhaps synergistic,[14] though this has not yet been shown conclusively in either whole animals or humans.

Several problems have existed in studying local immunity. One has been the availability and adequacy of specimens. Intestinal secretion and feces have been collected from cholera patients to measure anticholera antibodies.[19,27] The fluid collected from an intestinal washing, however, may not reflect the true concentration of the antibody at the mucosal surface because of a variable diffusion gradient into the lumen associated with mucous and proteolytic enzymes. Also, this type of specimen collection, requiring intubation, is not applicable to large study groups.

Recently several assays have been developed which may be useful in studies of local gut immunity. These include 1) a flourescent antibody assay for antitoxic antibody containing

cells,[22] 2) a helolytic plaque assay for antitoxin secreting cells,(unpublished data) 3) an enzyme-linked immunoabsorbent assay, for both antitoxin[28] and anti-lipopolysaccharide 4) a toxin neutralization assay using Y1 adrenal cells,[29] 5) a vibriolytic plaque assay for antibacterial-antibody secreting cells.[30] These assays should be applicable to one or more of the following tissues or fluids: peripheral lymphocytes, milk lymphocytes, and intestinal secretions.

The study of human milk in lactating mothers may be particularly enlightening. As mentioned earlier, stimulated lymphocytes preferentially "home" to the gut.[21] A secondary homing site, however is to the breast where the lymphocytes are secreted into the milk (Figure 1). The mechanism and regulation of this homing is not well understood; however, recent studies have demonstrated that nursing mothers who ingest unique bacterial antigens begin secreting specific IgA antibodies and cells into the milk shortly after the exposure.[31,32] If confirmed in cholera, the study of breast milk antibody and antibody secreting lymphocytes might be an extremely useful method of assessing local gut immunity.

Peripheral blood lymphocytes also produce antibody and may be helpful in studying local immunity. The hemolytic vibriocidal

plaque assays, which are Ig class specific, or the flourescent antitoxin assay, may detect a peak of IgA antitoxin or anti-bacterial antibody secreting cells as they course through the circulation in response to cholera infection. These assays would require approximately .001% of the lymphocytes to be specific antibody secreting/containing cells to be detected. If this level of antibody secreting or containing cell is found in the circulation, these assays would offer a means of assessing the local immune response by sampling peripheral blood.

A study of local immunity will also include sampling of intestinal secretions for antitoxic and antibacterial antibody determination for correlation with the plaque and flourescent antibody milk and peripheral lymphocytes, and with antibody titers in serum, milk and of stool.

3. Rationale: We propose to document and quantitate the local immune response to clinical naturally acquired cholera infections in persons who are admitted to the hospital in Dacca, Bangladesh. These studies are proposed for several reasons. First, the immunologic events occuring in the lumen or the mucosa of the small bowel are important to patients in terms of recovery from

the infection and of protection from reinfection, regardless of Ig class of antibody. This would seem evident from the pathophysiology of the disease in which neither the bacteria nor the toxin penetrates the mucosa. Secondly, the best protection with the longest duration against cholera is a previous episode of cholera. This is superior to protection from any vaccine now available, and it would seem reasonable that future vaccines, especially those that act by stimulating local immunity, be compared to the immunologic response of the disease itself. Thirdly, the techniques, and assays for quantitation of the local response need to be established in humans so that potential vaccines which are given orally can be evaluated and dosage regimens can be optimized. Fourthly, the immunologic response to cholera can be used as a model for studying other enteric bacterial infections, especially enterotoxigenic E.coli and perhaps Salmonella, and Shigella.

4. Preliminary Studies: As mentioned in the background section, many of the assays to be used in this study are established techniques, e.g. the adrenal cell assay for toxin and antitoxin, the hemagglutination assay for antitoxin, the vibriocidal assay for antibacterial antibodies. Other assays are established techniques

in other laboratories and will be initiated in our laboratories, e.g. the Elisa assay (or radioimmune assay) for antitoxin and anti LPS antibody. One assay which has not previously been described is the adaptation of the hemolytic plaque assay to detect antitoxin producing cells in tissues and fluids. The methods and preliminary results of this assay are therefore described.

The hemolytic plaque assay for antitoxin producing cells was adapted from the method of Norden[34] utilizing an hemolysin in-agar technique adapted to microscopic slides. Briefly, a single cell suspension of lymphocytes is mixed with cholera toxin coated sheep red blood cells, agarose, and minimal essential media and placed on a precoated glass slide (75 x 50 mm). The slides are then incubated for one hour, followed by a second hour incubation with guinea pig complement. The slides are then rinsed, dehydrated fixed and dried, and the plaques are counted using a dissecting microscope. The fixed slide can be saved and stored indefinitely, thereby constituting a permanent record of the assay). Each plaque represents one IgM antitoxin producing cell and the results are expressed as plaque forming cells (PFC's) per 10^5 lymphocytes. IgA and IgG PFC's are detected by incubating the slides with anti IgA or anti IgG

(developing serum) for one hour prior to the addition of complement. Plaques formed with the developing sera are "indirect plaques" and represent the total of IgM plus IgA or IgG antitoxin producing cells. Numbers of IgA or IgG cells are then determined by the difference between indirect and direct plaques.

Coating of the red cells is accomplished by combinations of equal volumes of packed twice-washed sheep red cells and cholera toxin (100 ug/ml) and incubating for 30 minutes at room temperature. The cells are washed again x 2 and the concentration, adjusted to 14% in PBS. Costing of the red cells and lysis by antitoxin can be confirmed in a microtiter antitoxin hemolysis assay using serial dilutions of Swiss serum institute anticholera serum with coated red cells

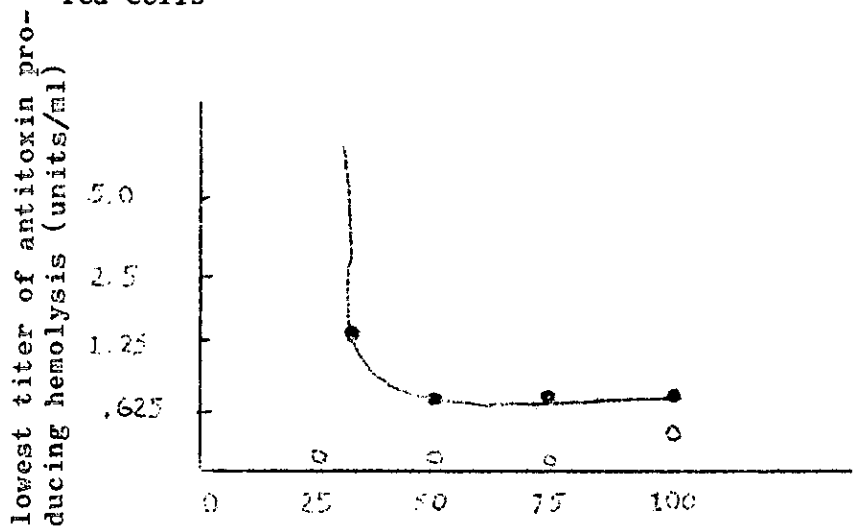

Fig. 1 - Concentration (ug/ml) of cholera toxin used to coat sheep red blood cells.
● - closed circles indicates with the addition of complement
O - open circles indicates no complement added

In one experiment, (Figure 1) red cells were coated with dilutions of choleragen and then added to the serially diluted antitoxin and complement. Complement dependent lysis was demonstrated with an end point at 0.625 units of antitoxin in cells coated with 50, 75 or 100 mg/ml but at 1.25 units at 25/mg/ml. No hemolysis occured in the absence of complement or with uncoated red cells.

Next we determined that antitoxin plaque forming cells could be detected in immunized rats. In one experiment we immunized six rats with two intraduodenal injections. The primary antigen contained 40 mg cholera toxoid plus 1.5 mg cholera toxin (lot No. 001); the booster, given 2 weeks later, contained 40 mg cholera toxoid without the toxin. Spleens were disrupted by gentle rubbing against a stainless steel wire mesh, filtered and washed with MEM. The direct PFC's detected in this groups of rats is shown (Figure 2).

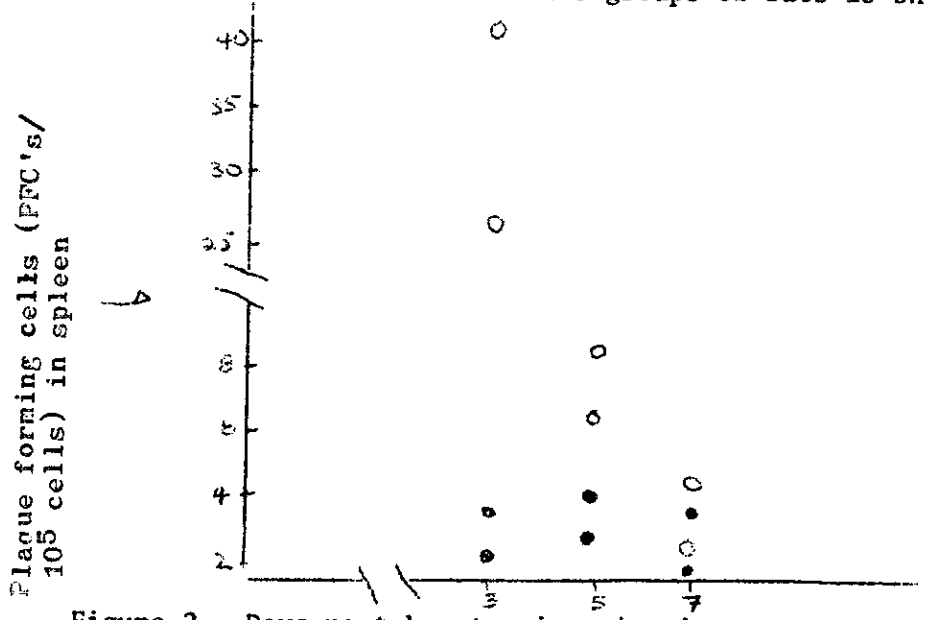

Figure 2 - Days post booster immunization
O - open circles indicate choleragen coated red cells
o - closed circles indicate uncoated red cells

Shown on page 14, figure 2 is the small number of PFC's detected against sheep red blood cells not coated with CT. These "background" PFC's although low in number illustrate the need for this uncoated red cell control with each assay. Flourescent antibody stain on one of the spleen cell suspensions on day 5 showed that approximately 40 times as many cells flouresce than are detected by the plaque assay. This difference illustrates the difference in the two assays: flourescent positive cells indicate antibody containing cells while PFC's indicate antibody secreting cells.

B. SPECIFIC AIMS

Specific aims of this proposal are to:

1. Adapt and develop assays related to local immunity for use in humans. These assays include the hemolytic plaque assay for cholera antitoxin producing cells in milk and peripheral blood, the vibriolytic plaque assay for antibacterial antibody producing cells in milk and peripheral blood, an enzyme linked immunosorbent assay for antitoxin and anti LPS antibody in milk, serum intestinal secretions, and the flourescent antibody for antitoxin containing cells in milk and peripheral blood. The toxin neutralization assay for antitoxin in serum and intestinal secretions is already established.

2. Document and quantitate the local immune response to naturally acquired cholera in Bengali adults. This would include determining the peak response, the duration between antigen exposure to peak response and the duration of measureable response.

3. Document the local antitoxic antibacterial immune response to cholera at a second secretory aite (breast milk), and determine the reliability of the breast milk as an indication of gut local immunity.

4. Determine if a peak of IgA antitoxin or antibacterial antibody producing cells can be detected in peripheral blood in patients with cholera.

5. Determine the correlation between the measurement of local and systemic immunity.

6. Compare the local immunologic response to the protein antigen (toxin) to the response of the lipopolysaccharide antigen with respect to Ig class, time course and peak response.

C. METHODS OF PROCEDURE

Materials and Methods

Purified choleragen will be obtained from Swartz-Mann, crude cholera toxin will be lot No. 001 from N.I.H. Purified cholera toxoid will be obtained from Wellcome Research Laboratories, Beckenham, Dent, England. This toxoid is purified from crude formalinized toxoid by means of affinity chromatography using purified equine antitoxin coupled to sepharose.

Purified flourescent anticholera toxin is prepared by repeated immunization of rabbits with purified toxoid plus Freunds complete adjuvant. The serum is passed through a 5 ml column of sepharose 4B previously coupled with purified toxoid. Saturation of the column is confirmed by titration of antitoxin in serum alequots coming off the column. After washing the column, the antitoxin is eluted with 0.1 M propionic acid, neutralized, dialyzed and concentrated by ultra filtration. It is then conjugated with FITC; unbound FITC is removed by gel filtration, and the final product stored frozen in multiple small alequots.

Purified antibuman IgG, and IgA will be obtained from Norden. Standard anticholera serum will be the Swiss Serum Institute

anticholera serum (4470 units/ml).

Sheep red blood cells will be obtained fresh and stored refrigerated in Alsevar's solution. Choleragen coated sheep red cells will be prepared by mixing equal columes of choleragen (100 ug/ml) and packed twice washed red cells, incubating for 30 minutes, and washing the red cells again x 2.

One Ogawa and one Inaba V.cholerae will be used in the vibriolysis plaque assay and vibriocidal antibody assay. The hemologous strain, relative to the patients stool culture will be used.

Collection of specimens

1. Milk - Milk will be collected using a breast pump, and will be immediately cooled to $4^{o}C$. A concentrated suspension of mononuclear cells will be obtained by centrifugation (1200 RPM x 10 min.). These cells will be washed once in minimal essential media, resuspended in a small volume of media, and the cell concentration adjusted to 2×10^{6} cells/ml. These cells will be used immediately in plaque assays. The milk (supernatant from initial centrifugation) will be defatted

and frozen for future antibody assays.

2. Peripheral Blood - Blood will be drawn by venapuncture, with anticoagulant. The lymphocytes will be separated using Ficol-Paque (Pharmacia), and washed once in minimal essential media, counted in a hemocytometer andused immediately in a plaque assay. Serum will be separated by centrifugation and frozen for future antibody assays.

3. Intestinal Secretions - Duodenal aspirate will be obtained by passing a tube into the duodenum. Position of the tube will be documented by appearance of luid obtained, pH and by X-ray if necessary. Duodenal fluid will be collected on ice, the inactivated by heating to $56^{o}C$ x 1/2 hr, then frozen.

Subjects

Initial studies of naturally acquired cholera will utilize adult lactating female patients admitted to the Cholera Hospital because of severe diarrhea. Patients with bacteriologically confirmed V.cholerae infections as well as patients with enterotoxigenic E.coli and "no pathogen" diarrhea, (for control) will be studied.

Informed written consent will be obtained from all patients. Antibiotics will not be administered to those with the cholera syndrome, but all will be treated with appropriate fluid and electrolyte replacement. Patients will be hospitalized for 10 days during their acute illness, and will be studied as outpatients following discharge.'

Techniques - the following assays will be utilized as measurements of immunologic function.

1. Toxin neutralization in Y, adrenal cells-microtiter technique. This assay is based on the ability of antitoxin to block the morphologic change in adrenal cells caused by cholera toxin. Y adrenal cells maintained in Hams F10 media, supplemented with 15% horse serum, 2.5% fetal calf serum, in a 5% CO_2 atmosphere, are grown either in flasks, dishes or miniplates. When exposed to cholera toxin or E.coli heat labile toxin the cells change their appearance from a flat cell to a round cell. In the antitoxin assay, serial dilutions of sera (diluted using microtiter technique in a transfer plate (Cooke) are incubated with a constant amount of cholera toxin (500 ug/ml 001 corde-cholera toxin) for 1 hour and then the contents of each well are transferred to the

corresponding well of the tissue culture miniplate. The highest dilution which neutralizes the toxin is the "end point" and is compared to the standard sera which has 4470 units/ml.

This assay has been successfully used for both serum and intestinal secretions and should be applicable to milk. Its sensitivity is approximately 0.5 to 1 unit antitoxin/ml.

2. The microtiter hemagluttination antitoxin assay will be used for serum specimens. Serial dilutions of serum are imcubated with choleragen coated formalinized sheep red cells and the highest titer during hemagglutination is the end point and is compared to the standard serum run with each assay.

3. Hemolytic plaque assay for antitoxin producing cells was described in the preliminary results.

4. The Vibriolytic plaque assay for vibriocidal antibody producing cells utilizes live vibrio organisms rather than red cells as the indicator. Eighteen hour BHI cultures of vibrios, are centrifuged and resuspended in sterile saline to a concentration of 5×10^8. To tubes containing agarose and

minimal essential media are added the collected lymphocytes and vibrios. These are mixed and passed onto a petrie dish prepared previously with BHI agar. After the upper layer has solidified the plates are imcubated for 90 minutes,then complement is added and reincubated for 2 hours. The complement is then discarded and the plates are again incubated for 6 hours until a lawn of bacteria is apparent. Plaques are seen as discrete zones of "no growth" against the background of microcolonies. The plaques are counted and expressed as PFC/10^5 cells. Indirect vibriolytic plaque forming cells are performed by adding anti IgA or anti IgG and incubating for one hour prior to the addition of complement in a manner similar to the indirect hemolytic plaque assay.

5. Enzyme-linked immunosorbent assay for cholera antitoxin is described by Holmgren.[35] Choleragen is forst bound to polystyrene tubes, the tubes are then rinsed with saline to remove unbound toxin. Serum is then added to the tube, incubated anddecanted. Antitoxin remains bound to the tube; unbound antibody is washed off. Antihuman IgG (or IgA or IgM) is then added, to which is linked alkaline phosphatase. After incubation this is decanted leaving the toxin-antitoxin-anti

Ig - alkaline phosphatase sandwich which is bound to the tube. Substrate for alkaline phosphatase is then added and the color developed, as determined by the colorimeter, are related to the antitoxin in the serum.

6. Enzyme linked immunosorbent assay for lipopolysaccharide antibodies is similar to the above assay except that LPS is bound to the tubes rather than choleragen.

Specific experiments

a. Studies of local immunity in naturally acquired cholera. These initial studies will be primarily a descriptive study of the local and systemic immune response to cholera measured by the assays as outlined. Ten to fifteen patients with documented cholera, and a matched group of patients with non-cholera diarrhea (including both enterotoxigenic E.coli and "no pathogens" diarrhea) will be studied during the 1977 fall epidemic. Specimens of blood (serum and lymphocytes), milk (for lymphocytes and whey) will be collected on the day of admission to the hospital and daily thereafter through 6 days, on day 8 and 10 weekly for a total of 6 weeks following discharge. In addition, duodenal aspirates will be

collected on the day of admission, day 5 and day 9 and day 17. (Depending on initial results, the schedules may be altered somewhat). The patients will all be studied in the clinical research center ward of the Dacca Cholera Hospital. Treatment will consist of maintenance of hydration, but routine antibiotics will not be used. The follow-up out-patient visits will require the reimbursement to the subjects for expenses of travelling to the hospital and for their cooperation.

The objectives of this study can best be summarized as a table which would be completed on each patient studied, on each dayycollections are made.

	Blood	Milk	Duodenal
ANTIBODY			
Antitoxin			
Neutralization			
Hemaglutination			
Elisa (IgG, IgM, IgA)			
Antibacterial			
Vibriocidal			
Elisa (IgG, IgM, IgA)			
LYMPHOCYTES			
Antitoxin PFC (IgG, IgM, IgA)			
Antitoxin Flourescing			
Vibriolytic PFC (IgG, IgM, IgA)			

From the results of this initial series of patients conclusions will be drawn as to (1) the suitability of peripheral blood IgA lymphocytes and/or milk IgA lymphocytes as indicators of intestinal immunity; (2) the magnitude, time course and duration of the measureable local immune response; (3) the variability of the immune response in patients; (4) the relative magnitude of theantitoxic vs. antibacterial immune response; (5) the Ig class(es) of antibody stimulated locally and systemically; (6) the correlation between the local immune response and the cessation of symptoms.

A major problem for the study would exist if the IgA response was not detectable in either the milk or peripheral blood. This would not be expected since this response has been documented with other bacterial enteric antigens. If, however, a peak in plaque forming cells, flourescent cells or antibody is not found, the commited lymphocytes might be detected upon culture of the lymphocytes might further differentiate and be detectable after one to three days. Also, if true, the lack of a breast response would be important to document. The study of duodenal secretions would also likely give the needed information in the absence of the milk data; however, this appreach would not be useful for future large groups.

A final problem deals with cooperation of the patients and follow up after discharge. Because this study involves a relatively small group of patients, each is very important. Patient selection will have to be careful, and some financial compensation will very likely be needed. Assistance from the epidemiology section will help in locating patients after discharge and if necessary help in transporting the patients back to the lab for follow-up visits.

D. SIGNIFICANCE

The development of a cholera vaccine which would stimulate local immunity depends on the ability to measure and quantitate the local immune response. This study will form the basis for measuring local immunity in cholera.

E. FACILITIES REQUIRED

1. Office Space: Office space for the principle investigator will be required.

2. Laboratory Space: Laboratory space will be required for processing of specimens. Specific requirements include a tissue culture laboratory, a flourescent microscope room, and bench space for performing the other immunologic assays, 400 square feet would be adequate.

3. Hospital Resources: Patients will reamin in the hospital for 10 days. 30 patients x 10 days = 300 patient days. In addition patients will return for follow-up weekly for 6 weeks. 30 patients x 6 visits = 180 follow up visits.

4. Animal Resources: Patients with "non Vibrio Cholera" who are admitted to the study will be screened for enterotoxigenic E.coli. This will require 300 infant mice.

5. Logistic Support: Follow-up visits will require pick-up of patients by transport in most cases. 20 patients x 6 pick-ups x 10 miles/pick-up = 1200 miles.

6. None.

7. Specialized Requirements: In some cases, in order to encourage cooperation from patients (especially from mothers with other small children) we may have to provide special facilities or support e.g. expenses for an ayah, V.I.P. room where small family can stay. Each case however, will have to be assessed so that we can accomodate reasonable requests.

F. COLLABORATIVE ARRANGEMENTS

Dr. Nathaniel Pierce will be coming to Dacca in October 1977 to assist with the study, especially with the flourescent antibody studies. Although not specifically for this protocol, Dr. Jan Holmgren will be coming to Dacca in winter 1978 and it is anticipated that he will be helpful in this protocol.

SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

Name	Position	% Of Effort	Annual Salary	Taka	Dollers
Dr. David Sack	Investigator	50%	$34,750		17,375
Dr. Ahmed	Co Investigator	30%	Tk 62,280	18,684	
Dr. Asma	Co Investigator	50%	Tk 27,084	13,542	
Mr. Hudda	Technician	100%	Tk 19,248	19,248	
Mr. Kibriya	Technician	20%	Tk 35,820	7,164	
Epidemiology	Field Assistant	30%	20,484	6,145	
Secretary		50%	24,000	12,000	
			Sub Total:	76,783	17,375

2. SUPPLIES AND MATERIALS

Antisera, Media, Supplies — Sub Total: 10,000 | 5,000

3. EQUIPMENT - None

4. PATIENT HOSPITALIZATION

Number of patient days - 300 patient days — Sub Total: 40,500 | -

5. OUTPATIENT CARE

Number of outpatients -]80 patient visits — Sub Total: 9,000 | -

6. CRL TRANSPORT

Mileage - Dacca:]200 miles 1,680
Mileage - Other (specify) None
Hours - Water Transport: None

Sub Total: 1,680 | -

		Project Requirement	
		Taka	Dollars
7. TRAVEL AND TRANSPORTATION OF PERSONS			
LOCAL TRAVEL			
Transport: None			
Per Diem/Expenses: None			
INTERNATIONAL TRAVEL			
Transport: 1 meeting			2,000
Per Diem: 10 days			490
	Sub Total:	-	2,490
8. TRANSPORTATION OF THINGS			
Import of Supplies:			1,500
Import of Equipments: None		-	-
Local Shipments: None		-	-
	Sub Total:	-	1,500
9. RENT, COMMUNICATIONS & UTILITIES			
Postage:			20
Telephone: None			
Cables: None			
Rent, etc. None			
	Sub Total:	-	20
10. PRINTING AND REPRODUCTION			
Printing forms:		1,000	-
Special Reproduction: None			
Publication Costs:			600
Xerox costs:		1,000	-
	Sub Total:	2,000	600
11. OTHER CONTRACTUAL SERVICES			
Consultant fees: NOne			
Patient payments		8,250	-
Others:		-	-
	Sub Total:	8,250	-
12. CONSTRUCTION, RENOVATION, ALTERATIONS: None			

B. BUDGET SUMMARY

Category	Year 1 Taka	Year 1 Dollars	Year 2 Taka	Year 2 Dollars	Year 3 Taka	Year 3 Dollars
1. Personnel	76,783	17,375	77,000	17,500		
2. Supplies	10,000	5,000	10,000	5,000		
3. Equipment	-	-	-	-		
4. Hospitalization	40,500	-	40,500	-		
5. Outpatients	9,000	-	9,000	-		
6. CRL Transport	1,680	-	2,000	-		
7. Travel Persons	-	2,490	-	2,500		
8. Transportation Things	-	1,500	-	2,000		
9. Rent/Communication	-	20	-	40		
10. Printing/Reproduction	2,000	600	2,000	600		
11. Contractual Service	8,250	-	9,000	-		
12. Construction	-	-	-	-		
Total:	148,213	26,985	149,500	27,640		
Total $	36,865		37,606			

Conversion Rate $ 1.00 = Tk. 15.

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LOCAL IMMUNITY IN CHOLERA - ABSTRACT SUMMARY

1. Only adult lactating mothers with cholera will be included in this study.

2. The risks from this study are minimal and consist of the discomfort of passing a duodenal tube for acquiring duodenal aspirates. All patients will be treated with appropriate fluid therapy to correct and prevent denydration.

3. The dehydration will be treated optimally.

4. All patients will be identified by patient number and all records will be kept locked in the investigator's office.

5. All subjects will sign a consent form. (See enclosed form).

6. N.A.

7. The individual will accrue no personal medical benefit other than treatment for her cholera which she would receive regardless of the study. She will, however, receive payment for participating in the study. Society will benefit if an effective oral vaccine can be developed for cholera. This study of local immunity in cholera

is directed toward that goal.

8. Blood, breast milk, stool, and duodenal secretions will be collected for this study.

CONSENT FORM

LOCAL IMMUNE RESPONSE IN CHOLERA

The Cholera Research Hospital is carrying out research to better understand how to protect people from cholera and other diarrheal diseases. We would like you to participate in a study, under the direction of Dr. David Sack to determine the immune (protective) response which occurs when a person develops cholera. We hope that the information we gain will be helpful in developing a new cholera vaccine which will be much more effective in preventing cholera. if you agree to participate in this study, you can expect the following:

1. You will need to stay in the hospital for 10 days; also you will need to return to the hospital for one day each week after you leave the hospital for 6 weeks. We will pay you for the cost of transportation plus 15 Taka for each day of study.

2. During your hospitalization we will collect blood and breast milk daily. Also on 3 occasions we will pass a tube into your intestine to collect a specimen of intestinal juice.

3. After your discharge from the hospital we will collect a blood and breast milk specimen each week for 6 weeks and an intestinal juice specimen once (a week after discharge).

4. None of the tests are harmful to your health. Drawing blood and passing the intestinal tube are somewhat uncomfortable; they do not have any serious side effects.

5. Your medical records will be kept confidential.

6. You do not have to participate in the study. Your decision concerning the study will not effect your medical treatment while in the hospital. If you do enter the study, you are

free to leave the study at any time without jeopardizing your medical care. We will answer any questions you have concerning the study.

If you agree to participate in this study, please sign your name here.

_____

_____ _____ Date

Investigator's signature