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ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date 9/11/84

Principal Investigator DR. ANSARUDDIN AHMED

Trainee Investigator (if any) 84

Application No. 84-045

Supporting Agency (if Non-ICDDR,B)

Title of Study : IDENTIFICATION OF PROTECTIVE SHIGELLA ANTIGENS BY WESTERN-BLOT ANALYSIS

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).

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 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)
   (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 Ctte. for review.

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

A Ahmed
14/11/84

Principal Investigator

Trainee

NOV 19 1984

84-045
19/11/84

SECTION I - RESEARCH PROTOCOL

1. TITLE : Identification of protective Shigella antigens by Western-blot analysis

2. PRINCIPAL INVESTIGATOR : Dr. Ansaruddin Ahmed, ICDDR,B

CO-INVESTIGATORS : Dr. I. Ciznar, Dr. M. M. Rahaman } ICDDR,B

Dr. J. Hackett, Dr. J. T. Labrooy, Prof. D. Rowley } The University of Adelaide, South Australia

3. STARTING DATE : December 15, 1984

4. COMPLETION DATE : December 14, 1986

5. TOTAL DIRECT COST : US$ 50,593

6. SCIENTIFIC PROGRAM HEAD : Dr. Ivan Ciznar

This protocol has been approved by the Host Defense Working Group

Signature of the Program Head : _____

Date : _____

7. ABSTRACT SUMMARY :

The main goal of this study is to identify the putative protective antigenic determinants in Shigella dysenteriae 1 and Shigella flexneri 2a that may be relevant to induce immunity to individuals against shigellosis caused by these two etiologic agents (these two strains are most frequently isolated from shigellosis patients in Bangladesh).

This goal will be achieved essentially by the Western-blot analysis of protein and lipopolysaccharide antigenic components of the bacteria by

convalescent sera and intestinal juice from patients recovering from diseases caused by the same strains.

The EDTA-lysozyme treated antigen extract of the bacterial strain will be separated into individual bands on a gel slab by SDS-PAGE; the bands will be transferred electrophoretically on a nitrocellulose sheet (Western-blotting) and analyzed by a visualized double-antibody-system immuno-enzyme reactions using convalescent sera and intestinal juice from recovering shigellosis patients. Finally, the relevance of protective antigen(s) will be assessed by qualitative and semi-quantitative studies of the coloured reaction bands obtained against serum and/or intestinal juice material from convalescing patients in reference to those obta ned from subjects recoveering from other diarrhoeal diseases like cholera, enterotoxigenic E. coli and normal subjects from non-endemic countries, like Australia and America. The protective values of the relevant antigenic determinants will be further bolstered by in vivo animal experimentations using oral challenge in rabbits and Sereny test on guinea pigs.

As antibody materials, sera from bacteriologically defined shigellosis patients of Teknaf and Dhaka stations will be taken by venepuncture and finger-tip puncture, respectively, for adults and minors. Intestinal juice from children (4 to 8 years), as a source of specific secretory IgA, will be taken with the help of polyethylene tube, perforated at the lower end, where a small latex bag containing mercury will be attached. Intestinal juice from adults will be collected by the standard lavage collection method, allowing the patient to ingest large volume of isomatic saline solution.

(The knowledge obtained regarding the protective antigen(s) will allow us, by "gene manipulation," to develope a live-oral-vaccine strain, into the body of which, the relevant antigenic determinants will be focussed. This will be done by a separate, extended and collaborative study with Prof. D. Rowley's group in the University of Adelaide, South Australia.)

8. REVIEWS:

a. Research Involving Human Subjects : _____

b. Research Review Committee : _____

c. Director : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective

Identification of the important antigen(s) of Shigella dysentariae 1 and Shigella flexneri 2a relevant for inducing protective antibody using immuno-blot (Western-blot) analysis by convalescent sera and concentrated intestinal juice from patients recovering from shigellosis. These two strains are selected for antigen-analysis, because they are the most frequently isolated ones from shigellosis patients in Bangladesh

2. Background

The possibility of prolonged immunity, following the first attack of gastroenteritis caused by a particular species of microbial pathogen, to a second attack of the same pathogen, has gained considerable support from volunteer studies in cholera and in shigellosis and with vaccine trials of streptomycin dependent strains of Shigellae. Also, the assumption that to achieve protection against diarrhoeal diseases, the antibody must primarily be present in the gut lumen, in the mucus layer or on the epithelial surface, has now been universally accepted. It stands on the evidence of an extensive research on the subject of local immune response and its role of protection against diseases like cholera and enteretoxigenic E. coli diarrhoea. In these diseases, the bacteria simply adhere to the mucosal epithelium, multiply on the surface and elaborate the diarrhoeagenic exotoxin without actually invading the tissue. Whereas, in addition to these features, the

shigellae possess an additional pathogenetic attribute of invasiveness. This has two steps: (i) penetration of epithelial cell barrier, and (ii) development of infective foci in host tissue by subsequent multiplication inside and across the epithelium, as well as in the lamina propria (1,2).

The attribute of exotoxin production by Shigellae has questionable association with virulence, because an invasive non-toxigenic Shigella dysenteriae 1 hybrid strain (3) (later proved actually to be one hundred times less toxin-producing 'hypotoxigenic' strain) was able to produce full-blown clinical shigellosis (4). In addition to enterotoxin activity of inducing net secretion of water and electrolyte in small gut of experimental animals, the Shigella exotoxin has other activities of neurotoxin and cytotoxin. The cytotoxic factor can produce histological abnormality like epithelial cell death, micro-ulcer formation, inflamatory infiltration in the lamina propria and lethality on in vitro cell culture. This activity is supposed to contribute to the formation of intestinal ulcer in shigellosis (5).

So, it is generally believed that the secretory IgA (and to some extent secretory IgM) is likely to form the first line of defense in the lumen/in the mucus layer by the process of antigen-exclusion probably by forming antigen-antibody complex followed by degradation/prevention of absorption (6,7) or by prevention of the offending microbes to reach and adhere to the mucosal surface (8,9). The later phenomena, perhaps the most important mechanism for protection, was observed in cholera in early 70's. It was found that anti-Vibrio cholerae antiserum or the

specific purified secretory IgA, while protecting experimental animals, reduced bacterial adherence at the mucosal surface and inhibited the multiplication of the locally adhered bacterial population without affecting the overall Vibrio multiplication in the gut lumen (9,10). The evidence that this mechanism may apply to shigellosis was further bolstered by Reed (11) in finding out the capacity of the antibody to inhibit the adherence of Shigella flexneri 2a on isolated epithelial cells in vitro, obtained from large intestine of germ-free mice. It was shown that organisms precoated with specific secretory IgA adhered to the cells in significantly less numbers than the organisms not coated or coated with IgG or IgM. In addition, it was shown that specific secretory IgA, and not IgG or IgM, antibodies protected guinea pig/rabbit against Keratoconjunctivitis induced by Shigallae (12,13). Thus, it is expected that the putative Shigella antigen may be found by immuno-blot analysis using intestinal juice (containing IgA) from convalescent patients. On the other hand, a purified Shigella antigen(s) may be checked for its protective values on this Keratoconjunctivitis model.

The mechanisms of complement-mediated bacteriolysis or opsonising phagocytosis cannot occur at the intestinal epithelial surface, because the secretory IgA, the dominiating local antibodies, does not possess the capacity to activate complement. The locally available complement level is low and the intestinal milieu is inimical to the self-life of complement. But, once the bacteria reach deeper tissue, the process of bacteriolysis promptly sets in by complement activation

in classical or in alternative pathways. Heat-liable opsonization and phagocytosis by polymorphoneuclear neutrophils require the presence of IgM along with complement, whereas heat-stable opsonin has been shown to be IgG (14). The 11S secretory IgA never takes part in these bacteria-eliminating phenomena. The very effective killing by these mechanisms in the deeper intestinal tissue probably keeps bacteremia or sepsis in shigellosis at a very low level of frequency in healthy, well-nourished individuals, even if they possess very low levels of specific antibodies or none.

On the basis of the foregoing discussions regarding diarrhoeas of other etiologies, it is pertinent to suggest that any successful attempt for prophylaxis in shigellosis should aim the development of protective immune responses that would be focussed locally at the mucosal and submucosal layers of the gut in response to some as yet unidentified protective antigen(s). Towards this end, the technology to generate, by gene-manipulation, a live vaccine in which relevant antigens from a pathogenic bacterial strain may be transferred,--has been developed rapidly in the recent past with good confidence. But the choice of which of the many antigens of the pathogen would provide greatest protection when carried by the vector vaccine strain, must be made before the relevant clones can be selected from the gene-bank. The problem may be solved by an empirical approach considering the hypothesis that, as a solid immunity usually follows natural infection, antibodies to the relevant (as well as too many other irrelevant) bacterial antigens are likely to be found in the convalescent serum

and intestinal juice of patients recovering from natural disease. Also, there would be some individuals in the community who are more protected than the majority due to immune response to these relevant antigen(s), whilst others may get repeated attack due to inadequate response to these particular antigen(s). Thus, among many antigens from a pathogenic Shigella strain extracted by lysozyme-EDTA and separated on high-resolution polyacrylamide gel electrophoretogram, the relevant antigens can be identified by the convalescent sera and intestinal lavage fluid. This will be done by a replica-transfer of the seperated antigens on a nitro-cellulose sheet, followed by visualization of the relevant antigen bands by an Ig-type restricted specific immuno-enzyme technique using peroxidase-labelled anti-human IgS,--the so called Western-blot analysis. The concentrated intestinal lavage fluid will serve as the source of locally produced specific secretory IgA antibodies, considered to be crucial for identifying the putative protective antigen(s).

It is to be mentioned here that the particular class of non-lipopolysaccharide outer-membrane protein antigens (OMPs) of Shigellae will be favoured in the analysis. This is, in our opinion, desirable too. The relevant lipopolysaccharide antigens, though will not be stained by Coomassie blue, will be recognized by antibodies on the immuno-blot and if needed, will be visualized by silver-staining.

The reasons for favouring OMPs in the antigen-analysis should be mentioned here. Until mid 70's the somatic lipopolysaccharide (LPS) of a Gram-negative enteric pathogen was believed to be the most important antigen fraction related to the host defense against the

disease caused by the organism. The belief was based upon the facts that (i) LPS is connected very frequently with serological classifications of bacteria, (ii) it has been shown to participate in easily done early serologic assays for antibody titration like agglutination, passive hemagglutination, complement mediated bacteriolysis, etc., (iii) it remains stable when conventional bacterial vaccines are detoxified by drastic steps like boiling, alcohol/acetone-treatment, etc., so that whatever protection follows are thought to be more due to LPS than other easily degradable components like membrane-proteins.

But, contrary to the above-mentioned popular belief, a large body of evidence have recently accumulated to indicate that the immunity conferred by various non-LPS bacterial membrane components are in fact more convincingly associated with protection from enteric diseases than the immunity to LPS. The relevant components are OMPs/Porins, special proteins like K-88, K-99 of E. coli in porcaine and bovine enteritis, flagella-associated protein of V. cholerae, lectins, pilli, etc. The search for the possible protective antigen(s) in the outer membrane (OM) of enteric pathogens, however, is very logical because the OM forms the interface between a particular pathogen and the gut mucosa of the diseased host. Thereby, important biologically active molecules capable of attaching to the mucosa and stimulating the gut immune apparatus are very likely to be present in the OM. Studies implicating the role of membrane components in the disease-protection are not being discussed here for the sake of brevity. Only one interesting experiment done by Romanowska's group (15), right

in the field of protective immunity in shigellosis deserves special mention. They showed that OMPs from Shigella flexneri 3a and Shigella sonnei Phase I protected, on active immunization, guinea pig and rabbits against keratoconjunctivitis shigellosa induced with the homologous or hetrologus strain. Protection was also conferred to rabbits by passive immunization with anti-OMP immune serum. There was no protection in rabbits in this keratoconjunctivitis model following active immunization with LPS derived from S. flexneri 3a.

The major OMPs in most enteric pathogens have been found to possess molecular weight varying between 40-50,000 daltons. Other minor protein bands are also shown in the SDS-PAGE Electrophoretograms having a wide range of molecular sizes. By Western blotting and immunoautoradiographic techniques to determine the antigenicity of OMPs, it has been found that additional bands may appear during infection when acute and convalescent sera are analyzed and compared (16). Protection afforded by OMP may often be non-specific and broadbased to have activity against homologous as well as hetrologous strains of bacteria. So, protective antigens like these may be more desirable to be introduced in a living vaccine candidate. OMP in many bacteria has been reported to play a role in attachment, invasion, serum resistance, chelation of iron, and resistance to phagocytosis (17).

From late 70's to date there have been quite a number of investigations characterizing OMPs in various gram-negative organisms from the standpoints of biochemical characters, antigenicity in humans and/or experimental animals, implication of protective role to the extent of

considering vaccine candidates (18,19) etc. These organisms are Shigellae (15), Campylobacter jejuni (16,20), Salmonellae (21,22), Escherichia coli (23,24), Pseudomonas aeruginosa (25), Haemophilus influenzae (26), Neisseriae (27,28,29), etc. of which the first four generally belong to the group of enteropathogens.

3. Rationale

Acute diarrhoea can claim half of the death toll in the first 5 years of life and one-third in all ages, in developing countries where satisfactory sanitation and health care are lacking. Shigellae have been shown to be the most common etiologic agent associated with diarrhoeal episodes by a community-based ICDDR,B study in Bangladeshi village when 5 most prevalent bacterial and viral infections were screened by rectal swab examination guided by intense field surveillance (30). In a recent case-control analysis of indoor patients' data for one year in ICDDR,B hospital to identify factors predicting death in diarrhoea, it was revealed that shigellosis is the single most important cause of death (S. Islam, 1983, paper under preparation). This is a killer disease having unique combination of malnutrition and complications like hypoproteinimia, leukemoid reaction and hemolytic-uremic syndrome.

This protocol will enable us to identify the putative protective antigenic determinants(s) of S. dysenteriae type 1 and S. flexneri 2a. A live oral vaccine will then be constructed by gene manipulation that will express the same determinants as surface components for stimulating the gut immune apparatus of the vaccinees to achieve protection from the diseases. This will be done as a future collaborative work with the Microbiology Department of the University of Adelaide, South Australia.

Though the presence of various sero- and sub-sero types in the genus Shigella poses problem in contriving any immunoprophylaxis against shigellosis, this putative vaccine is expected to largely prevent the morbidity and mortality of shigellosis in Bangladesh; because by far the largest number of shigellosis patients here suffer from the attack of S. dysenteriae type 1 and S. flexneri 2a species. Moreover, by earlier experimental evidence (22), the protective activity achieved by this vaccine may be expected to spill over against diseases caused by other related hetrologous strains of Shigellae.

B. SPECIFIC AIM

To identify the putative protective antigenic determinants of S. dysenteriae type 1 and S. flexneri 2a by analyzing all the determinants by Western blotting with the help of specific antibodies obtained in convalescent sera and intestinal juice of patients recovering from clinical disease.

C. MATERIALS AND METHODS

All types of patients' material will be collected with prior informed consent of the patients or their parents to comply with the usual ethical practice of the ICDDR,B.

1. Sera and intestinal juice as source of antibodies

(a) Sera from patients convalescing (day 14 after the onset) after bacteriologically defined attacks of S. dysenteriae type 1 and S. flexneri 2a would be taken from Teknaf and Dhaka stations (hospital follow-up cases). Two ml blood will be taken from adults by venepuncture; 100/200 microliter blood from children and adults unwilling to be venepunctured will be taken by

finger-prick in a capillary tube containing dried anticoagulant. Plasma seperated either by centrifugation or by keeping the capillary tubes vertically one-end sealed in refrigerator will be diluted 10X with sterile saline and transported in 1 dram vials inside ice-box.

-- Number of sera 25 against each of the strains; 10 from adults, 15 from children in each group (total 50)

(b) Acute and convalescent (paired sera, the 2nd sample on day 14 after onset) from patients will also be taken from the same areas to pinpoint: (i) the antigens against which rising titres are found, and (ii) additional antigen bands that may appear during convalescence which were not present in the acute-phase serum. The 'onset' will have to be determined by case-history obtained from the patients or their attendants.

-- 10 paired sera against each strain

(c) Serial samples of sera also be tried to collect from patients to determine longitudinally whether persistence of antibody to any of the Shigella antigens is particularly prolonged.

-- 5 serial sets of sera against each strain. Each set will comprise day 1-, day 14-, 6 week- and 6 month-serum samples. Serial samples may better be taken from Teknaf, because Dhaka population are often mobile.

In practice, collection of paired and multiple samples will be attempted from the same series of patients. Patients showing non-compliance following the 2nd collection would yield the paired sets of sera.

(d) Sera from healthy children or children who continue to remain healthy after one attack of shigellosis being member of the family that experienced many attacks, will be interesting to examine whether they consistently contain particular antibodies recognizing particular antigen band(s)--proving their importance from the protection point of view.

-- 10 sera against each strain

(e) Control sera

(i) Sera from patients convalescing from other forms of bacterial gastroenteritis like cholera and ETEC diarrhoea. These sera will be taken from Matlab rural and Dhaka urban area. Alternatively, minute quantities of cholera convalescent sera from the oral vaccine field trial may be used with permission.

(ii) Sera from healthy adults residing in non-endemic regions, e.g., from Australia, America.

-- 15 sera that would be applicable as control materials against both the strains

(f) Intestinal juice as a source of specific secretory IgA antibodies

(i) Intestinal juice from the jejunum or the jejuno-ileal segments of the gut will be taken with the help of a long polyethylene tube, perforated at the lower end, where a small latex bag containing mercury will be attached. The method will be similar as was done in the "Gastric Emptying Time in Children" Protocol No. 81-019, year 1981, P.I. - Dr. P. Bardhan. The usual procedure of guiding the tube by fluoreocopy will not be adopted for avoiding X-ray exposure to the participants. Instead the pH of the aspirates will be checked frequently to note the time of conversion of the pH from acid to alkali, signifying the presence of the tube-end in the jejunum. Except a little inconvenience and tickling sensations in the throat, this collection method is perfectly safe for the children.*

(ii) As high antibody concentration is not needed for antigen-identification by Western-blott analysis, it is hoped that non-concentrated jejunal juice will be good enough as specific IgA antibody source. In that case, about 25 ml

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* An elaborate discussion regarding this collection was done with Dr. A. M. Molla, who performed with his own hands one of the largest collection of jejunal biopsy material in the world. He fully agreed to the safety of this procedure in minor patients.

of juice from a participant will be sufficient; but if concentration of the antibody is needed for optimum visualization of antigen bands 250 to 500 ml of intestinal juice will be collected by standard intestinal lavage collection methods by allowing the patient to ingest large volume of isotonic saline solution (31). This method will be used in case of adult patients. (The method of collecting intestinal juice by polyethylene tube is also excellent for the adult patients. Actually, it would be a matter of choice for the participant which of the methods of collection will be applied for him.)

-- Number of samples 5 - 10 against each strain

2. Bacterial strains in which protective antigen will be searched

(a) Freshly isolated smooth strains of S. dysenteriae type 1 and S. flexneri 2a will be taken from Teknaf area. The serologic identification of the strains will be done critically with the help of standard set of diagnostic Shigella aggulutinin sera from Burrows Wellcome. Antigens of the above two Shigella species, homologous against the respective sets, a sera collected from a particular patient will be analyzed initially and most critically. Species from heterologous sub-sero types of the genus Shigella will also be included later in the analysis, to study the nature of cross-identification of particular antigen band(s) by the convalescent sera or intestinal juice, etc.

3. Technique Proper

In general, the discontinuous SDS-PAGE and the Western-blotting will be performed according to the methods described bu U. K. Laemmli (32) and H. Towbin (33), respectively, with necessary modifications.

Following are the main steps:

a) Antigen preparation from the Shigella strains

A selected Shigella strain will be grown in brain-heart infusion (BFI) broth (BBL or DIFCO) for 6 to 8 gours of shaking, washed in sterile saline, antigen extraction done by treatment with EDTA-lysozyme, followed by osmotic shock. Though BHI broth is expected to be a good start, the best medium, of course, would have to be selected by studying the antigen-pattern obtained on SDS-PAGE.

b) Antigen separation by SDS-PAGE (only main steps)

The EDTA-lysozyme-extracted crude antigen would be separated primarily on the basis of their molecular weights by polyacrylamide gel electrophoresis in presence of sodium dodycyl sulphate (SDS). The electrophoresis run will be in a one-dimentional vertical gel slab. The span of the protein movement will be guided by a dye-front. The molecular weight (mol. wt.) of a protein under investigation will be determined by comparing its electrophoretic mobility with those protein standards of known mol. wt. Protein standards ranging in their mol. wt. between 14.4K (14,400) and 200K will be used for the comparison.

c) Blotting by electrophoresis

The whole pattern of the separated antigenic bands, including the protein standards, will be replica transferred to a sheet of nitrocellulose (blotting) electrophoretically. This transfer process will be done very easily and efficiently by a Trans-Blot apparatus (Bio-Rad Laboratories) within 2 hours in Tris-HCl buffer containing Glycine and Methanol.

d) Staining for protein

The blot may be stained in Coomassie blue in 45% methanol/10% acetic acid and de-stained with 90% methanol/5 to 6% acetic acid, for direct visualization.

e) Immunological identification of immobilized protein antigen on the nitrocellulose sheet

The blot (usually not stained by Coomassie blue) will be soaked in Tris HCl buffer saline containing 3% BSA to saturate additional protein binding sites. Following thorough rinse in saline the blot will be incubated in suitably diluted convalescent serum/ secretory antibody-containing intestinal juice. The antibody-containing buffer here will also contain 3% BSA for prevention of non-specific protein binding.

f) Visualization of the immune complex

The sheets, following sufficient washings in saline will be incubated in a horseradish peroxidase-conjugated second (indicator) antibody (anti-human Igs, diluted 1 in 1,000 BSA-buffer) directed against the immunoglobulins of the first antiserum i.e. antibodies

from convalescent patients or control population. The immunoglobulin type-specific nature of the first antibody (the materials from convalescing patients) will be ascertained by the selection of the different isotypic anti-human Igs as the second antibody raised in rabbit or goat. Finally, colour is developed by 15 min treatment in the substrate, 3, 4, 3', 4'-tetra-aminobiphenyl hydrochloride, 0.5 mg/ml in 50 mM Tris-HCl, pH 7.4, containing 0.01% hydrogen peroxide, washed in water to terminate the reaction and dried between sheets of filter paper. Control sheets having treatment with conjugate but not serum, will be included in each run. It has been calculated, by a spot-test using known graded doses of immunoglobulin standards, that test serum containing a minimum of 5 ng/ml of specific IgG and 30 ng of specific IgA (29) may be detected by this method.

If required, the blots may be stored carefully either before the application of the first or the second (conjugated) antibody.

g) Interpretation of the results

The interpretation will depend primarily on identification of type-specific and species-specific antigens to which the convalescent serum and intestinal juice antibodies bind. Qualitative inspection, such as, how many major or minor bands of what mol. wt., whether a limited number of antigenic bands are pinpointed most of the times by majority of the convalescent antibodies, whether new bands appear during disease and convalescence, whether there is indication of

rising titre by increasing optical density restricted to some important band(s), etc., are of significance.

Although quantative estimations of specific protein antigen bands is possible by measurement of absorption using a gel scanner and a planimetric estimation of the antigen from the area under the absorption peak is possible, this quantitation when SDS is used as the dissociating reagent is not very good. So, towards assessing a rising titre, probably a semi-quantitation may only be possible. But, if the quantitation is needed precisely, urea, in place of SDS, may be used.

The LPS antigens will also be considered in this immunoblot analysis. If needed, they will be stained by silver staining method for visualization, because Coomassie blue does not stain LPS. However, all the immunogenic LPS antigens, in addition to proteins, will be identified in any way on the blot by the same immunoenzyme method that would be applied for the protein antigens.

Once a gel band would be identified as possibly protective, cell fractions with or without it would be tested for protection by in vivo animal assay or in vitro cell adherence blocking assay (11). Small amount of the relevant cell fraction (antigen band) may also be eluted out by cutting the relevant band from a preparative SDS-PAGE electrophoretogram. This eluted portion may be injected in guinea pig or rabbit to register its protective capacity by the sereny test or the antibody may be used for showing passive protection in the mouse assay. This relevant cell fraction may be

isolated in a sizeable amount, in its pure or semipure form, by modern separation methods like ultrafiltration, preparative gel electrophoresis, chromatography, including fast protein liquid chromatography (FPLC). We may get help in this isolation process from Prof. D. Rowley's group in the Department of Microbiology and Immunology, the University of Adelaide, South Australia.

D. SIGNIFICANCE

Similar as has been discussed under the heading 'Rationale.' One additional point is setting up the method of immunoblotting in ICDDR,B. The method may profitably be used in other investigations.

E. FACILITIES REQUIRED

Special facilities required, over and above those routinely obtainable in ICDDR,B Microbiology and Immunology Laboratories, are as follows:

1. Vertical slab gel electrophoresis tanks (three) - to be fabricated locally or bought from abroad

2. 'Transblot' apparatus from Bio-Rad Company for rapid electrophoretic transfer of antigenic bands from gel slab to nitro-cellulose sheets.

3. Nitrocellulose membranes

4. Ultracentrifuge

5. Farmacia Fast Protein Liquid Chromatographic system of separation apparatus and special reagents for purification, characterization, etc., of bacterial membrane proteins.

6. Expensive immunochemical reagents like Peroxidase-conjugated antihuman immunoglobulins from Western biologic houses.

F. EXTENSION AND COLLABORATIVE ARRANGEMENT

The important antigen fraction(s) identified by the pattern study, followed by immunological experiments implicating its protective value, will facilitate efforts to select-out relevant DNA segments from the genome of Shigella species for cloning and transfer to the vector bacteria which will be used as the live oral vaccine. This genetic engineering work is considered to be a logical extension of the present protocol. It will be done as a collaborative research project with Prof. D. Rowley's group, Dr. J. Hackett, being the primarily responsible scientist. If the Host Defense Working Group of ICDDR,B can extend him proper facilities and support, he may come to do the job in Dhaka. Thereby, junior scientists and technologists in ICDDR,B may learn the very much needed current technology of gene-manipulation. He will, of course, write a separate protocol for the study.

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ABSTRACT SUMMARY

The purpose of this study is to identify the putative protective antigenic determinant(s) of the bacteria Shigella dysenteriae 1 and Shigella flexneri 2a that may be relevant to induce immunity in individuals against the attack of blood dysentery caused by these two etiologic agents (these two strains are the commonest bacteria to cause this disease in Bangladesh). Briefly, this will be done by the following steps in sequence:

a) Extraction of all bacterial antigenic body-components in a crude solution

b) Separation by electrophoresis of all the individual proteins and lipopolysaccharide components into single bands on a polyacrylamide gel sheet

c) Electrophoretic transfer of the bands on a nitrocellulose sheet by the process of im unoblotting (Western-blotting)

d) Analysis of the antigenic bands by visualized antigen antibody reactions using convalescent sera and intestinal juice from recovering shigellosis patients

e) Identification of the relevant protective antigens by qualitative and semi-quantitative specific antigen antibody reactions obtained with the use of serum and intestinal juice from convalescing patients and control subjects recovering from other diarrhoeal diseases, like cholera, enterotoxigenic E. coli and normal subjects from non-endemic countries, like Australia and America

f) Further immunologic animal experimentations to bolster the protective values of the recognized antigen determinant(s)

This knowledge will allow us, by gene-manipulation, to develope a live-oral-vaccine strain into the body of which the relevant antigenic determinants will be focussed. This will be done by a separate, extended and collaborative study in association with Prof. D. Rowley's group in the University of Adelaide, South Australia. Sera from adult and minor bacteriologically defined shigellosis patients from Teknaf and Dhaka stations will be taken in acute and convalescent stages. 25 ml blood will be drawn from adults by venepuncture; 100/200 ml blood from children will be drawn by finger-tip puncture in capillary tubes.

Intestinal juice, as a source of specific secretory IgA antibody from jejunum or jejuno-ileal section of the gut, will be taken from children with the help of a long polyethylene tube, perforated at the lower end, where a small latex bag containing mercury will be attached. The usual procedure of guiding the tube by fluoreocopy will not be adopted for avoiding X-ray exposure to the participants. Instead, the pH of the aspirates will be checked frequently to note the time of conversion of the pH from acid to alkali, signifying the presence of the tube-end in the jejunum. Except a little inconvenience and tickling sensation in the throat, this collection method is perfectly safe for the children.* (Suitable age group for taking intestinal juice may be 4-8 years.)

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* An elaborate discussion regarding this collection method was done with Dr. A. M. Molla, who performed with his own hands one of the largest collection of jejunal biopsy material in the world. He fully agreed to the safety of this procedure for children.

About 25 ml from children and about 250 ml from adults (adults not willing to participate in intestinal lavage method of collection) of jejunal juice will be taken from different individuals.

500 ml to 1 liter of intestinal juice from adults will be collected by the standard lavage collection method (David Sack, et al, 1980), allowing the patient to ingest large volume of isotomic saline solution. The participating adult patient may have a choice for the perforated polyethylene tube method as has been suggested for obtaining juice from minors.

The reason for taking materials from the group of children is because most of our shigellosis cases occur in this age group and their antibody forming pattern, especially in case of the crucial antibody IgA, may be different from those of adults. Also, the immunity pattern may be different by repeated attacks in this immunologically compromised groups. Except the inconvenience and a little discomfort, there is no potential risk in connection with the drawing of serum and intestinal juice samples.

In case of minors, informed consent will be obtained from the legal guardians at the time of drawing samples. Informed consent with signature on consent form will be obtained from the participating adults as per the ICDDR,B ethical procedure.

There is no direct, immediate benefit for the participants, but society in general will be benefitted by a successful oral vaccine against the two major eticlogic agents causing shigellosis.

SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

Name	Position	Time Effort	Project Requirement Taka	Dollar
Dr. Ansaruddin Ahmed	Investigator	50% (2 years)	320,000	-
Dr. Ivan Ciznar	Co-investigator	5% (2 years)	-	6,000
Dr. M. M. Rahaman	Co-investigator	2% (1 year)	-	1,400
Dr. A. Salam, Physican	Supporting	5% (1 year)	9,000	-
Dr. Asma Khanam, Physician	Supporting	5% (1 year)	9,000	-
Senior Research Officer (to be hired)		100% (2 years)	188,000	-
Laboratory Technician (to be hired)		100% (2 years)	88,000	-
Laboratory Attendant (to be hired)		100% (2 years)	41,180	-
Health Assistants (two), Teknaf Station (Level 3, VIII)		25% (1 year for each)	26,400	-
Health Assistant (two), Dhaka Station, 1 month's salary for each (Level 5, XIII)			17,000	-
			698,580	7,400

2. PATIENT'S MATERIAL

a)	Serum collection (about 175 @ Tk.165 per sample; transportation, m terial for collection, etc., excluding cost of time-effort of Health Assistants)	28,875	-
b)	Intestinal juice/lavage from 25 patients (hospital stay of average 7 days per patient, 175 patient-day, @ Tk.150 patient day + Tk.50 per day work-compensation and materials, excluding time-effort of two supporting Physicians)	28,000	-
	Work-compensation	8,750	-
		65,625	-

	Project Requirement	
	Taka	Dollar
3. SUPPLIES & MATERIALS		
- Ordinary laboratory glasswares and chemicals	-	500
- Bacteriologic medium	-	300
- Special chemicals for SDS-PAGE	-	300
- Immunochemical reagents	-	1,000
- Nitrocellulose membrane	-	500
- Rabbit @ Tk.140 x 15	2,100	-
- Guinea pig @ Tk.50 x 25	1,250	-
- Animal handling/care charge (approx)	2,500	-
- Bacteriologic media plates & slants	1,750	-
	7,550	2,600
4. NEW EQUIPMENT		
- Bio-Rad 'Protein' double-slab Electrophoresis cell	-	1,025
- Bio-Rad Trans-blot cell	-	415
- Automatic pH scanner	-	525
- Bio-Rad Model 1650 Transmittance/ reflectance Densitometer, 220V	-	1,650
	-	3,615
5. HOSPITALIZATION COST		
- 175 patient-day	Cost already included, Item 2(b)	
6. OUTPATIENT CARE	-	-
7. TRAVEL AND TRANSPORTATION OF PERSONS		
Local		
Teknaf-Dhaka-Teknaf, or the other way round, 3 trips	7,750	-
Teknaf-Dhaka-Teknaf, or the other way round, perdiem	1,500	-
+ what is included in Item 2(a)		
International		
Round-trip travel Dhaka-Adelaide-Dhaka	-	2,500
Perdiem $100 per day for 15 days	-	1,500

		Project Requirement	
		Taka	Dollar
8.	TRANSPORTATION OF THINGS (under items 3 & 4 @ 15% of total Dollar cost of material and equipment + transportation of control sera from Australia/America)	-	1,200
9.	RENT, COMMUNICATION & UTILITIES		
	Postage, stationery, etc.	500	-
10.	PRINTING AND REPRODUCTION	10,000	-
11.	OTHER CONTRACTUAL SERVICES		
	Patient fees and patients' own transportation	3,000	-
12.	CONSTRUCTION	—	—

B. BUDGET SUMMARY

			Taka	Dollar
1.	PERSONNEL SERVICES		698,580	7,400
2.	PATIENT'S MATERIAL (including transportation for collection, expenses for extra hospitalization and work-compensation)		65,625	-
3.	SUPPLIES AND MATERIALS		7,500	2,600
4.	NEW EQUIPMENT		-	3,615
5.	TRAVEL			
	Local, including perdiem		9,250	-
	International, including perdiem		-	4,000
6.	TRANSPORTATION OF THINGS		-	1,200
7.	RENT, COMMUNICATION & UTILITIES		500	-
8.	PRINTING AND REPRODUCTION		10,000	-
9.	OTHER CONTRACTUAL SERVICES (patient fees and patient's own transportation)		3,000	-
10.	CONSTRUCTION		-	-
	TOTAL	Taka	794,455	18,815
		Dollar	31,778	
	GRAND TOTAL: US$ 31,778 + 18,815	=		50,593

(conversion rate US$ 1 = Taka 25)

CONSENT FORM

The International Centre for Diarrhoeal Disease Research, Bangladesh, is carrying out research to better understand how to protect people from the attack of cholera, blood dysentery and other diarrhoeal diseases. We earnestly request you to participate in a research to find out what are the important components of a blood-dysentery-bacteria against which an individual can form immune (protective) response to protect himself from an attack of the same disease. We hope that the knowledge gained by this study will enable us to develop an effective vaccine against the disease.

If you kindly agree to participate in this study, you may expect the following:

1. You will need to stay in the hospital upto 14 days after the onset of your disease.

2. On the last day of hospitalization, we will take a sample of only 2 ml blood from your vein. Also, we will take some intestinal washing from you by the procedure of 'intestinal lavage.' You will be allowed to drink a large volume (upto 5 liters) of salty water in empty stomach. This will cause a temporary diarrhoea which will stop shortly after you stop drinking the salty water. During the lavage, you will feel a fulness in the abdomen; you may gain 1-3 Kg in weight, but you will feel no pain and there will be no serious side

(In case of 5 to 10 year old minor patients, 2 to 4 drops of blood will be taken by finger-prick. The intestinal juice or washing will be taken with the help of a thin plastic tube at the end of which a liquid metallic (mercury) weight will be tied in a tiny rubber bag to make the end heavy. The heavy lower end of the tube will be allowed to swallow by the child like a medicine-capsule, so that, within 2-4 of hours it will reach the upper part of the small intestine. With the help of a syringe, intestinal juice or washing with a light salty water will be drawn from the top end of the tube. Finally, the tube will be taken out gradually and carefully. This procedure is absolutely harmless and will not cause any pain except some ticklish sensation at the back of the throat.)

Collection of this blood and intestinal-washing samples for scientific tests will not have any bad effect on your health, except creating some discomfort.

3. Your medical records will be kept confidential.

4. You do not have to participate in the study. Your decision to join or not to join the study will not in any way affect your medical treatment while you are in hospital. Once you enter the study, you are free to leave the study at any time without jeopardizing your medical care. We will be ready to answer any of your questions concerning the study.