Attachment 1.

FACE SHEET) ETHICAL REVIEW COMMITTEE, ICDDR,B. Date 10 - [illegible]

66

Principal Investigato: D. Islam, R. Raqib Trainee Investigator (if any) X

Application No. 96-013 Supporting Agency (if Non-ICDDR,B) SAREC/SIDA

Title of Study Further studies of systemic and local immuneresponses in shigellosis in order to establish a protective vaccine

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:
   No Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   Yes Protocol (Required)
   Yes Abstract Summary (Required)
   Yes 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)
   Yes Informed consent form for subjects
   Yes Informed consent form for parent or guardian
   Yes 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.

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

Dr. Zahrul Islam Rubhana
Principal Investigator

Trainee

ENTERED 2 1 JUN 1996

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

[Please tick (✓) the appropriate box]

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

   Yes [X]

   No [ ]

   If 'No', please clarify the reasons: _____

2. Has the proposal been peer-reviewed externally ?

   Yes [X]

   No [ ]

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

3. Has the proposal scope to address gender issues ?

   Yes [ ]

   No [X]

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

4. Has a funding source been identified ?

   Yes [X]

   No [ ]

   If the answer is 'YES', please indicate the name of the donor: _____

   SIDA/SAREC

5. Whether the proposal is a collaborative one ?

Yes [X]

No [ ]

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

Joint collaboration. Jan Andersson, M.D., PhD, Birger Christensson, M.D., PhD and Bengt Wretlind, M.D., PhD., Department of Immunology, Microbiology, Pathology and Infectious Diseases, Karolinska Institute at Huddinge University Hospital, S-14186 Huddinge, Sweden.

6. Has the budget been cleared by Finance Division ?

Yes [X]

No [ ]

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

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

Yes [ ]

No [X]

If 'YES', fill the necessary form.

10-7-96
Date

Dilara Islam Rubhana
Signature of the
Principal Investigator

B. Application for Project Grant

1. Principal investigators:

i) Dilara Islam, Ph.D. and Rubhana Raqib, Ph.D.
Laboratory Sciences Division (LSD), International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), G.P.O. Box 128, Dhaka-1000, Bangladesh. Telephone: +880 2 871751-60, Facsimile: +880 2 883116, 886050

ii) Jan Andersson, M.D, Ph.D., Birger Christensson, M.D., Ph.D. and Bengt Wretlind, M.D., Ph.D., Department of Immunology, Microbiology, Pathology and Infectious Diseases (IMPI), Karolinska Institute at Huddinge University Hospital, Stockholm, Sweden and Department of Immunology, Wenner-Gren Institute, Stockholm University, Stockholm, Sweden.

2. Co investigator:

Pradip Kumar Bardhan, M.D./Nurul Haque Khan, M. D.
Clinical Sciences Division, ICDDR,B, G.P.O. Box 128, Dhaka-1000, Bangladesh. Telephone: +880 2 871751-60, Facsimile: +880 2 883116, 886050

2. Consultant:

i) Adjunct Professor Alf A. Lindberg, M.D., Ph.D., IMPI, Karolinska Institute at Huddinge University Hospital, Stockholm, Sweden and Senior Vice-President Research, Pasteur Merieux Connaught group, 1541, Avenue Marcel Merieux - 69280, Marcy L Etoile-France. Telephone: +33 78873078, Facsimile: +33 78873976
ii) George Fuchs, M.D., Division Director, CSD, ICDDR,B.

3. Title of the Project:

Further studies of systemic and local immune responses in shigellosis in order to establish a protective vaccine.

4. Starting Date: September 1996

5. Completion Date: December 1998

6. Total Budget Requested: US$ 215,430

7. Funding Source: SIDA/SAREC

8. **Head of the Program:** Division Director, LSD, ICDDR,B

9. **Aims of the project:**

(a) General aim: To study the development of immune defense mechanisms during the course of natural *Shigella* infection in children and adults. Comparison of the host responses in individuals immunized with candidate *Shigella* vaccine and naturally infected individuals.

(b) Specific aim:

A) Kinetic studies of innate and T cell specific immune responses in children infected with shigellosis.

B) Detailed analysis of antigen-specific T cell responses in adults with shigellosis.

C) Local immune response and antigen-specific T cell responses to immunization with *Shigella* vaccine candidate (SFL 124).

(c) Significance: In the perspective of shigellosis being a predominantly pediatric disease, it is important to learn the difference in the pattern of the immune system of the growing children to that of the adults in an endemic area. This is specially pertinent in the setting of a vaccine development, suggested to be applied to the most susceptible infant population. Knowledge gained from the proposed project may help to better understand the development of the protective immunity in shigellosis.

10. Ethical Implication:

The proposed studies involved:

(i) Management of patients of different age groups with diarrheal diseases.

(ii) Sampling of blood, stool and rectal mucosa.

(iii) Administration of oral vaccines.

Approval of the ethical committee at Huddinge Hospital has been obtained.

For inclusion of patients and volunteers, informed consents will be required according to guide lines from the local ethical committee at ICDDR,B. For the children, the informed consent of the parents or guardians are required. Patients will be recruited among those seeking medical care at the outpatient section at Clinical Research Service Center (CRSC) of ICDDR,B. Included patients will receive clinical care and therapy free of charge and of the same type that they would have received had they not been enrolled in the study. Age and sex matched controls and volunteers will be recruited from the

same socioeconomic status as well as the same endemic areas. Patients and volunteers may discontinue their participation in the study at any time point. This decision would not have any influence on the clinical management or therapy of the patients. Repeated sampling of blood, stool and rectal mucosa will be performed from patients and healthy volunteers (as described below). All sampling procedures will be performed by clinically fully trained experts in the procedures concerned.

Patients and volunteer management, including repeated biopsy sampling has been done previously at ICDDR,B in conjunction with studies of immune responses in shigellosis. As has been previous occasions we consider that the potential scientific benefit obtained by the proposed studies well warrants the inconveniences and local pain associated with sampling. No serious side effects have been associated with sampling with previous studies and are not expected to occur in the proposed studies. Sampling of rectal mucosa from pediatric patients and volunteers in the vaccine studies may warrant extra considerations. The sampling in itself will be of no benefit to the child and will be associated with inconvenience and some pain. For children obtaining vaccines, a better resistance to natural *Shigella* infections, immunity though not absolute, may be expected justifying the procedure. Administration of the oral vaccine based on SFL 124 has been extensively tested for efficacy and safety both in adults and in children (13, 14, 27). At the indicated dose regimens (single dose) no side-effects have been recorded or expected to occur (27). Mucosal sampling from pediatric volunteers not receiving vaccines (controls) can only be justified by scientific reasons. We consider mucosal biopsies from these control group very essential unless the children that are to receive oral vaccine may be biopsied before vaccine administration. However, fecal and blood samples may still be needed as to monitor the constancy of the research parameters in the control population.

11. Background:

Shigellosis is one of the major causes of morbidity and mortality in many developing countries. It is estimated that *Shigella* species infect over 200 million people yearly. The world-wide mortality rate from acute shigellosis is estimated to be 650,000 per year. Persistent diarrhea, a common sequel to shigellosis accounts for an additional death toll in the hundreds of thousands annually. Children under the age of five years are most susceptible having a global mortality rate in excess of 500,000 per year (1-2). Out of the four pathogenic *Shigella* species, *S. dysenteriae* type 1 and *S. flexneri* are of major importance for dysentery in developing countries. In Bangladesh alone the yearly mortality rate from diarrheal diseases accounts for more than 250,000 deaths. In children below the age of four with severe dysentery, seen at medical centers such as ICDDR,B, the mortality rate is around 40% (3-5).

In endemic areas, prevention of disease spread could either be achieved by improvements in housing and sanitation or through vaccination. However, in many developing countries the costs for

efficient infrastructural improvements can not be met. Therefore the vaccination approach is seen as a rational alternative and there is a continuing great interest in the development of efficacious vaccines. For a rational vaccine program development, an in depth understanding of the immune response elicited in natural and induced shigellosis is required. *Shigella* being primarily a human pathogen, limits the possibilities to use experimental animal models (6). Therefore, *in vivo* and *in vitro* studies in humans or human cell lines have to be carried out.

Compared to infectious diseases where animal models are available, relatively little is known about the immunopathogenesis and development of immunity in shigellosis (6). Recently the development of genetic methods has allowed for dissection of the infectious process in shigellosis (7). Compared to other enteric pathogens, *Shigella* replicates more rapidly. Both experimental data and our clinical findings would favor this being a strictly intracellular process (6). The cytoplasmic localization of the micro-organisms would suggest that T-cell mediated host response mechanism are of major importance in disease defense. Epidemiological and experimental approaches have shown that serotype-specific immune protection develops after infection. There is epidemiological evidence to show that shigellosis peaks between 1-4 years of age. The finding of chronic *Shigella* infection in patients with HIV infection having low $CD4^+$ T cell counts but pre-exciting high serum antibody titers to *Shigella* antigens seems to support the notion that cell-mediated immune mechanisms are important in the host defense against shigellosis (8-10). *Shigella* being an enteric pathogen a major focus of attention has been on the potentially protective role of secretory (s) IgA molecules, and sIgA responses can be demonstrated following challenge in experimental systems (11). However, sIgA collected from ileal loops of rabbits failed to inhibit invasion of cultured mammalian cells by *S. flexneri* (7). Thus, although *Shigella* specific immune responses against polysaccharide and protein antigens can be identified in all IgG and IgA subclasses, a protective role of *Shigella* specific antibodies has yet to be clearly demonstrated.

Previous collaborative pilot trials with candidate vaccines in shigellosis have been performed in Vietnam and Sweden (13, 14, 27, 28). The strains SFL 124 and SFL 1070 have also been analyzed for immunogenicity, however only at the humoral level (12,13). Further knowledge of the local intestinal immune responses and specific T cell responses after immunization with such vaccine candidates is to be attained.

Our previous studies:

Recently we have published a series of papers elucidating cellular and humoral responses in naturally acquired shigellosis in adults. This work has also been summarized in two doctoral theses defended at the Karolinska Institute by two of the principal investigators (14-26).

During the acute stage of shigellosis, a marked inflammation in the rectum was associated with increased infiltration of granulocytes, T-lymphocytes, macrophages and NK-cells. Extensive

production of pro-inflammatory cytokines (IL-1α, IL-1β, TNFα, IL-6 and IL-8) was observed at the local site which persisted up to a month after the onset of disease (16). Analysis of cytokine producing cells at the single cell level showed that increased frequencies closely correlated to the histological grading of severity. A concomitant production of Th1 (IFNγ, TNFα, TNFβ) and Th2 (IL-4, IL-5, IL-10) types of cytokines was seen during at least 30 days after the onset of disease. In the early phase of the disease, 100 times higher cytokine concentrations were found in stool than in plasma (17). Increased stool concentrations of cytokines correlated to the severity of inflammation in the gut as well as to clinical markers of disease severity. A gradually increased deposition of extracellular IFNγ in the colonic mucosa was seen during recovery from shigellosis being entrapped to its receptor and being predominantly expressed on the mucosal surface epithelium (18). Several fold higher frequencies of cytokine mRNA expressing cells were observed for most cytokines than the corresponding protein producing cells at the local site during the course of *Shigella* infection. Possibly Shiga toxin, known to inhibit protein synthesis at the level of translation could account for the discrepancy.

Healthy controls constitutively expressed cell-surface cytokine receptors in the rectum. In contrast, during the acute stage of shigellosis a loss of cytokine receptors (IL-1 type I, TNF type I, IL-3, IL-4, IFNγ and TGFβ type I) was observed (14). These findings suggested that the loss may occur as a consequence of internalization and/or shedding of the receptors following ligand binding. In patients, the level of soluble cytokine receptor in plasma were 100 fold higher than the corresponding cytokines. In contrast, soluble receptors in stool were 4-6 folds lower than that of the corresponding cytokines at the acute stage. Counter-regulatory actions of soluble receptors at the local site were thus overcome by excessive local production of cytokines thereby promoting immune activation as well as tissue-damage. The expression and secretion of cytokines and cytokine receptors in the acute and convalescent stages were differentially regulated in order to modulate systemic immune activation (14, 16).

A method based on immunomagnetic bead DNA-separation (IMS) and polymerase chain reaction (PCR) detection was developed for the direct isolation and identification of *S. dysenteriae* 1 and *S. flexneri* in fecal samples. This method showed a 15% higher sensitivity than the conventional culture method, and shortened the time of diagnosis by up to two days (20, 27). Analysis of *Shigella* antigens (lipopolysaccharide (LPS), invasion plasmid associated antigens (Ipa) and Shiga toxin) specific serum IgA and IgG antibody responses at the subclass level showed a differential response pattern against individual antigens over a time period of 30-35 days after the onset of disease. The levels of antigen-specific antibodies correlated to the clinical disease severity (21, 22).

Using triple-color flow cytometry, a sequential activation of peripheral blood lymphocytes (PBL) from the *Shigella* infected patients was observed during the course of the disease, suggesting recirculation of gut activated cells. The activation was most pronounced in the acute phase but was still evident upto 30 to 40 days after onset (23, 24). Flow cytometric analysis of the T cell receptor (TCR) variable (V) β repertoire of blood T cell subsets showed a skewed usage of TCR Vβ segments

particularly in helper T cells. The kinetic of the TCR Vβ repertoire changes followed that of the *in vivo* T cell activation during shigellosis. The enhanced expression of Vβ 2, 3, 5.1 and 17 both *in vivo* and *in vitro* after stimulation of PBL, suggests that in shigellosis antigens or superantigens are presented to the immune system which preferentially activate certain TCR Vβ types within the T cell subsets (25).

Immunohistochemical analysis of the local gut mucosal inflammation showed a proliferation associated upregulation of p53 expression in crypt epithelium. Inducible nitric oxide synthase was found in surface epithelium and an extensive apoptotic cell death was observed (26). Analysis of repeated biopsies obtained during the disease showed a prolonged inflammation in the rectal mucosa in shigellosis indicating that *Shigella* induced cellular reactions may persist long after the resolution of clinical symptoms (26).

All these studies indicated that cell-mediated immune response mechanisms were important for local disease development in adults infected with *Shigella*. In the adults studied, however we are probably analysing a secondary immune response pattern in previously *Shigella* exposed individuals, as indicated by serological evidence. The high morbidity and mortality rate seen in children indicate that in primary infection, the type of immune response may be quite different. The type and extent of local and cellular immune response seen in the naive host at primary *Shigella* infection needs to be further characterized. Although our findings indicate that antigen-specific T-cell mediated immune responses do take place in shigellosis but the nature of the involved antigenic structures is virtually unknown.

Research plan:

A) Kinetic studies of innate and T cell specific immune responses in children infected with shigellosis.

Specific aims:

We aim to assess the immune responses in children (5-10 years of age) with shigellosis. Characterization of inflammation at the local site, the effector mechanisms responsible for tissue damage and for eradication of *Shigellae*, as well as the general immune responses in the acute phase and at convalescence will be made by assessing:

1. Cytokine and cytokine receptor secretion in repeated stool and serum samples.
2. Expression of cytokine and cytokine receptor at the single cell level in rectal biopsies.
3. The humoral *Shigella* specific antibody responses both in serum and in stool. Toxin-specific neutralizing capacity of serum antibody.
4. The phenotypic characteristics of effector cells in peripheral blood and in rectal tissue.

Study populations:

Shigella infected children age 5-10 years (n=30), with naturally acquired shigellosis (*S. dysenteriae* 1 or *S. flexneri*) will be finally recruited from a group of patients initially selected from the outpatient ward. The diagnosis will be based upon identification of the microorganism by routine culture procedure and the recently developed PCR method (20). Patients who are enrolled in the study and later develop complications will be excluded from the study due to overlapping with another protocol (submitted for funds; P.I. Dr. Tasnim Azim). Patients with other concomitant gastrointestinal infections will also be excluded. Control groups will be included in the study: i). Age matched healthy children living in the same endemic area (n=30), ii). Age matched children with diarrhoea caused by other organisms (*Salmonella, Escherichia coli, Vibrio cholerae* and *Campylobacter jejuni*) other than *Shigella* (n=30). All patients with diarrhea will be treated according to the current therapy protocols at CRSC, ICDDR,B for their respective diseases. Patients and controls may be invited to stay in the hospital to facilitate disease monitoring and sampling. Wage loss due to hospitalization associated with the study may be reimbursed.

Study materials:

All samples (blood, saliva, stool, urine and rectal biopsies) will be collected following informed consent. Children admitted because of diarrhea caused by *Shigella* or other pathogens will be sampled accordingly as shown in the "flow chart for sample collection" and "scheme for sample collection".

Methods:

The techniques to be used for these studies have been developed at Huddinge Hospital and will be transferred to ICDDR,B to be applied for current investigations.

1. Assessment of the production of cytokines and their corresponding receptors in repeated stool and serum samples will be performed by ELISA technology. We will use cytokine specific mAb's directed against non-neutralizing epitopes on the cytokine in order to measure the total amount. Analyses of IL-1α, IL-1β, TNF-α, IL-1ra, TGFβ, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ and G-CSF plus GM-CSF cytokines will be performed. The corresponding cytokine receptors will be identified with mab's directed against non-cytokine binding epitopes.

2. Assessment of cytokine producing cells (IL-1, Il-1rα, IL-2, IL-4, Il-6, Il-8, IL-10, IL-12, IFNγ, G-CSF, GM-CSF, TNFα and TNFβ) will be performed at the single cell level by imunohistochemical technique. Semiquantification will be assessed by use of computerized image analysis technique. Cell surface expression of the corresponding cytokine specific receptors will be performed by immmunohistochemical technique in cryopreserved rectal tissue.

3. Analysis of *Shigella* antigen specific antibodies (Ipa, LPS, Shiga toxin); Consecutively obtained serum and fecal samples at indicated periods after onset of disease will be assessed by ELISA for *Shigella* specific IgA subclass 1 and 2, IgM, and IgG subclass 1-4 antibodies. The serum level of *Shigella* specific antibodies in the initial Samples from children will identify individuals previously unexposed to *Shigellae*. The neutralizing capacity of antibodies eluted from stool or serum of patients with shigellosis will be analyzed in vitro. Cultured polarized colonic epithelial cells (Caco -2) and peripheral blood mononuclear cells from healthy blood donors will be used as targets following exposure to attenuated whole *Shigella* bacteria or to purified *Shigella* antigens. Inhibition of cytopathic effects on target cells by *Shigella* will be compared in presence or absence of different concentration of eluted antibodies to assess their nutralizing capacity.

4. Characterization of phenotypes of peripheral blood mononuclear cells will be performed by Flow cytometry assessing colonic homing as well as memory markers (previously carried out in adults (24)).

5. Assessment of Ig-producing cells at the local site for IgM, IgA and IgG including subclasses will be performed by immunohistochemical and ELISA techniques. Cellular surface expression of activation, memory and homing associated markers on immunocompetent cells will be performed by use of immunohistochemistry.

B) Detailed analysis of antigen-specific T cell responses in adults with shigellosis.

Specific aim:

Our aim is to isolate and clone *Shigella* antigen specific T cells from PBL and rectal mucosa to:

1. Analyse phenotypic characteristics of isolated antigen-specific T cells with respect to clonotypic TCR expression by sequence analysis of V-D-J junctional diversity and preferential utilization of particular TCR Vβ or Vα types.

2. Characterize cytokine production and cytotoxic effector function in response to antigen stimulation induced by different types of antigen presenting cells.

Study population:

Adult males (n=30) with naturally acquired shigellosis (*S. dysentriae* 1 or *S. flexneri*). Inclusion and exclusion criteria are described above.

Study materials:

1. Multiple (10-12) colonic biopsies obtained at colonoscopy, taken around days 0, 15 and 60 after disease onset.
2. Peripheral blood samples obtained as specified in "scheme for sample collection".

Methods:

1. Isolation of mononuclear cells from peripheral blood by conventional density gradient separation.

2. Isolation of rectal mucosal T cells
   a) Enzyme digestion of isolated mucosal fragments with dispase, collagenase, DNase to release lymphocytes.
   b) Isolation of T cells by anti CD3 coated magnetic beads
   c) *In vitro* expansion of T cells in the presence of IL-2 and feeder cells.

3. *In vitro* activation and expansion of *Shigella* antigen specific T cells by cultivation of T cells and irradiated autologous antigen presenting B cells together with heat killed *Shigella* or *Shigella* antigens (Ipa, LPS and Shiga toxin) and IL2.

4. Selection of antigen activated T cells by dilution and cloning in feeder layer culture systems.

5. Phenotypic and functional analysis of isolated T cell clones with respect to:
   a) T cell subset phenotype.
   b) Expression of clonotypic TCR Vβ type by flow cytometry and RT-PCR and sequence analysis of V-D-J junctional diversity.
   c) Analysis of preferential utilization of particular TCR Vβ or Vα types by flow cytometry.
   d) Phenotypic characterization including the expression of mucosal homing receptors and pattern of co-receptor expression by flow cytometry.
   e) Antigen mediated induction of cytokine and cytokine receptor profile by immunoenzyme methods.

C) Local immune responses and antigen-specific T cell responses to immunization with *Shigella* vaccine candidates.

Specific aim:

This project aims at analysing the local and cellular immune responses obtained after oral vaccination

with a well tested vaccine in children and adult. These findings will be compared to the response seen after natural infection.

Vaccine:

The attenuated *Shigella* strains to be used for this study were constructed by deletion of *aro*D gene from wild type *Shigella flexneri* strains, thereby making the vaccine strains auxotrophic for aromatic compounds not available in cultured mammalians cells. The strains SFL124 (*Shigella flexneri* Y) that has been extensively tested for efficacy and safety and previously used in field trials will initially be used in the current study (12, 22, 28). Children will be immunized with single dose (10^8) of SFL 124. As it has been shown previously that with single dose immunization protocol there was no side-effect (27). Adults will also be immunized with single dose (10^9) of SFL 124 or SFL1070. These vaccine strains have reduced intracellular multiplication capacity in cultured cells and unable to cause Keratoconjunctivitis in guinea pigs. The excretion of vaccine strain fecal sample will be monitored by both routine culture and IMS-PCR procedure (20). Orally vaccinated monkeys are protected when orally challenged with virulent *Shigella flexneri*. In initial pilot trials these candidate vaccines have been shown to induce IgA response against LPS and Ipa by ELISPOT assays. In addition, IFN-γ responding cells were also found when stimulated with polysaccharides from S. *flexneri* strains. Optimal response was found at day 7-9 after immunization (13, 27, 28). Vaccines to be used in the study will be produced under certified conditions according to GMP standards in commercial production facilities and will be provided free of charge through the courtesy of professor Alf A. Lindberg (Pasteur M rieux connaught group, 1541, Avenue Marcel M rieux - 69280, Marcy L toile - France).

Study population:

Healthy children (age 5-10 years; n=10) and healthy male adult volunteers (n=10) will be immunized following informed consent (from parents or guardians with regards to children). Corresponding numbers of individuals will serve as controls, receiving placebo.

Study materials:

Blood, feces and rectal biopsies will be obtained before immunization. Peripheral blood samples and feces will be obtained as specified in "scheme for sample collection" following immunization. Rectal biopsies will be obtained at days 15 after immunization.

Methods:

Serological analysis of pre vaccination and postvaccination (repeated) serum samples will reveal the presence of *Shigella* specific antibody titers and the kinetics of the humoral response.

1. Mucosal sIgA responses in isolated cells will be assessed in ELISPOT assays.
2. sIgA in stools will be assessed by ELISA as described above (A).
3. Cytokines and cytokine receptor responses in stool and serum will be assessed by ELISA as described above (A). Analysis of consecutive samples will allow assessment of reaction kinetics.
4. Generation of antigen-specific T cell responses will be analysed as described above (A & B).

List of assays to be performed in the project:

Some of the methods mentioned below have been standardized by the investigators during earlier studies and references are provided for all procedures including new techniques. Hence, detailed procedures are not included.

1. **Routine assays**: (a) blood, stool, urine- R/E (b) stool- microbiological tests (c) blood- total protein, albumin, creatinine, glucose, C-reactive protein and electrolytes (d) stool- total immunoglobulin and albumin (e) urine- creatinine, glucose and osmolarity. All routine tests will be performed in the Clinical Services Department at ICDDR,B.

2. **Immunological assays**:

Blood:

a) PBMC- (i) cell surface markers, analysis by flow cytometry (23, 24, 25) (ii) *in vitro* stimulation studies for studying cytokines and respective receptor expression, TCR Vβ expression (17, 19, 25).
b) Plasma- (i) Soluble cytokines and respective receptors (14, 17) (ii) *Shigella* antigen specific antibodies and immunoglobulin subclasses (21, 22).

Stool:

i) *Shigella* identification by IMS-PCR (19, 20) (i) Soluble cytokines and respective receptors (14, 17) (ii) *Shigella* specific sIgA (21, 22).

Rectal biopsies:

i) Morphological studies (15,.16)
ii) Cellular surface expression of various markers for activation, memory and homing (15, 26).
iii) Assessment of Ig- producing cells (26)
iv) Cytokines and respective receptor expression (14)
v) Inflammatory mediators (16, 26)

Isolated immunocytes from colonic biopsies:

i) *in vitro* stimulation studies for studying cytokines and respective receptor expression, TCR Vβ expression (29, 30, 31).

ii) ELISPOT assay for cytokines and immunoglobulin secreting cells (13, 27).

Statistical methods

Data will be processed using Softwares- Excel 4.0 (Microsoft, Redmond, WA, USA), JMP 3 (SAS Institute, Carey, NC, USA), and STAT View 4 (Abacus Concepts, Berkeley, CA, USA). Statistical analysis will be done using Student's T test, Wilcoxon, Kruskal-Wallis, Multiple analysis of variance tests and other tests where necesarry.

Scheme for sample collection

1) Scheme for collection of samples from adult patients and controls

Days from the	Samples collected from each individual				
enrolment	blood	saliva	biopsy	stool	urine
Day (D)-0	+	+	+	+	+
D-3	+	+	-	+	+
D-7	+	+	-	+	+
D-11	+	+	+	+	+
D-30	+	+	-	+	+
D-60	+	+	+	+	+
Control	+	+	+	+	+

2) Scheme for collection of samples from pediatric patients and controls

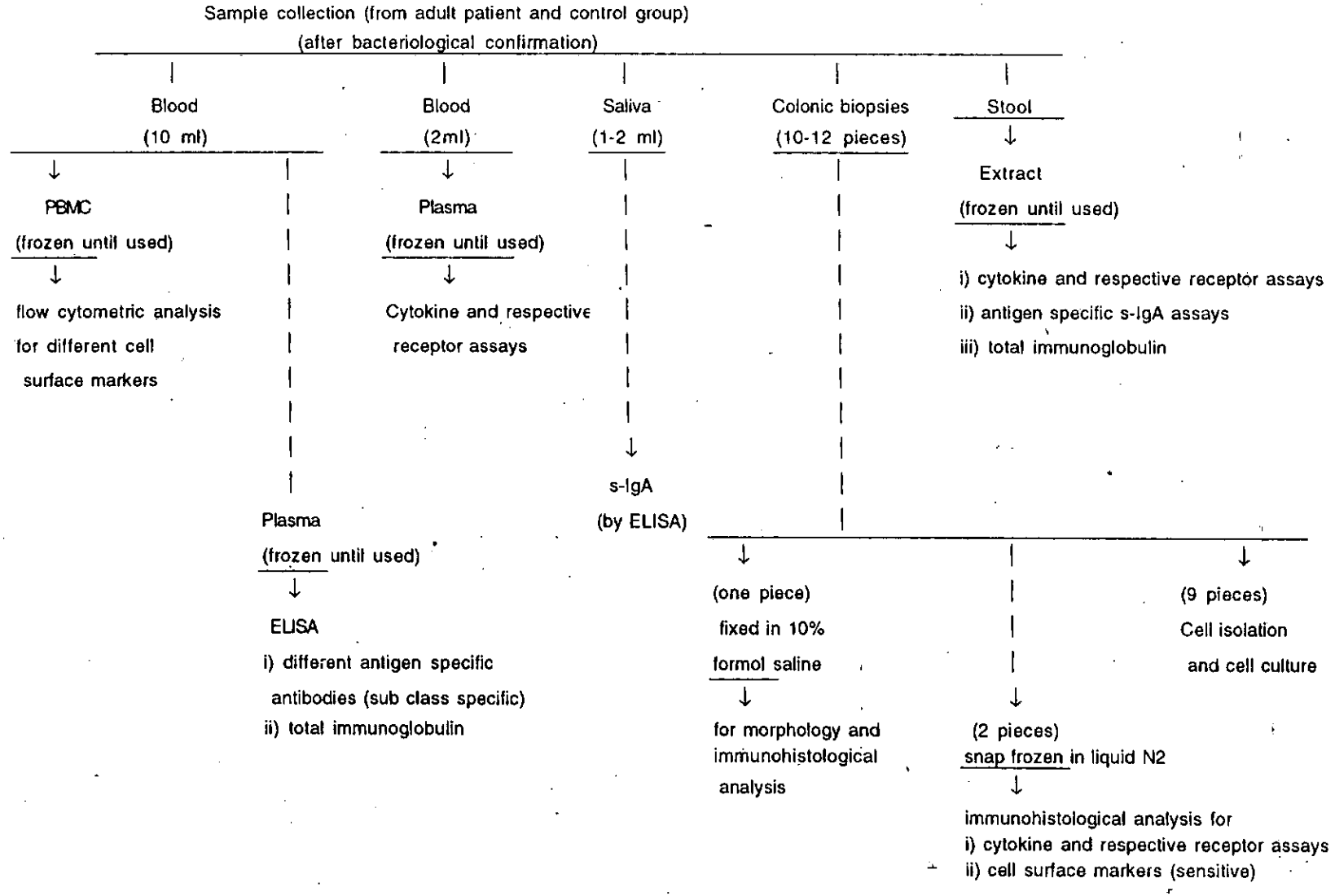
Days from the	Samples collected from each individual				
enrolment	blood	saliva	biopsy	stool	urine
Day (D)-0	+	+	+	+	+
D-5	+	+	-	+	+
D-11	+	+	-	+	+
D-30	+	+	+	+	+
D-60	+	+	-	+	+
Control	+	+	+	+	+

3) Scheme for collection of samples from volunteers

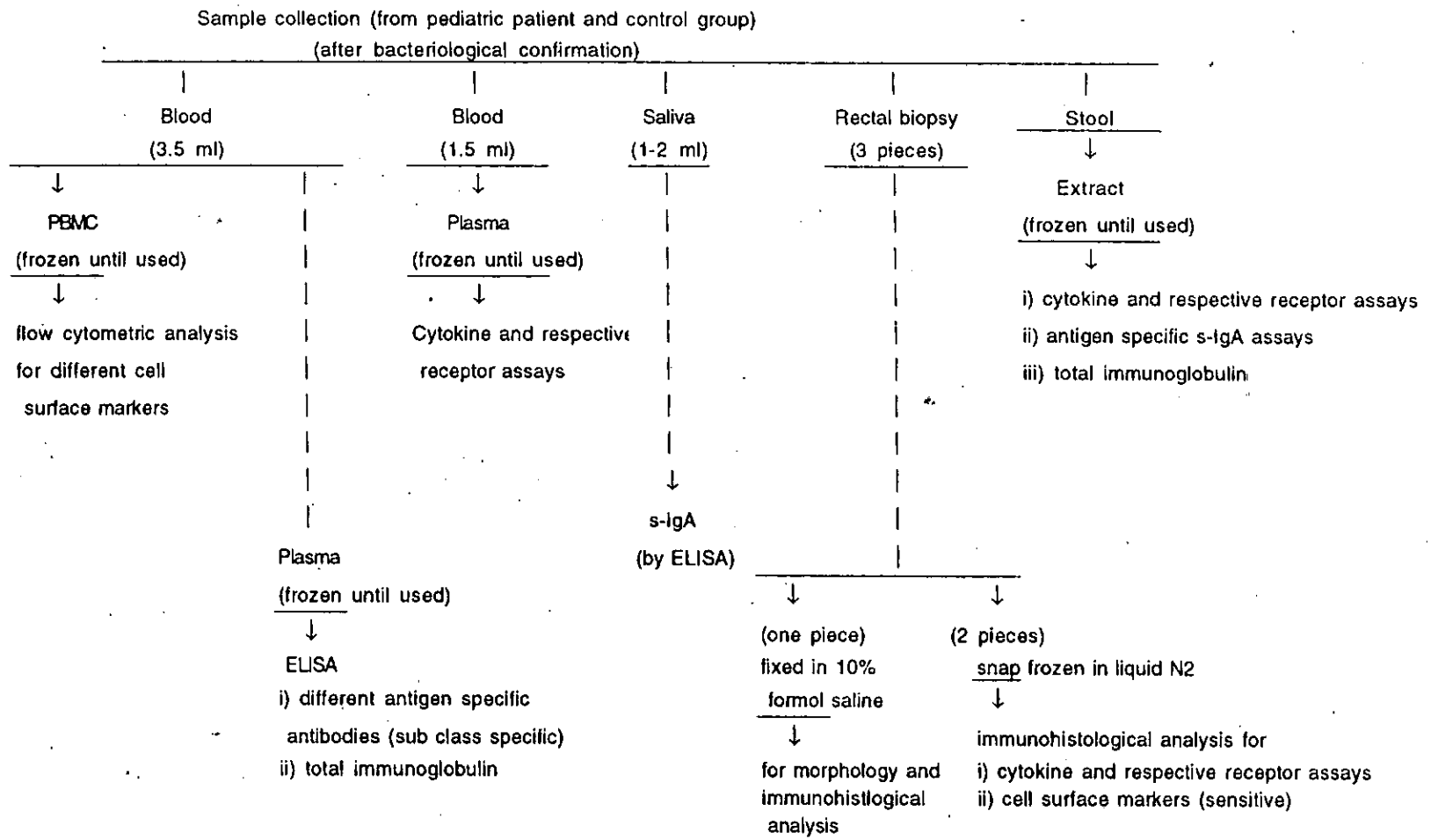
Days of sample	Samples collected from each individual				
collection	blood	saliva	biopsy	stool	urine
before [a]	+	+	+	+	+
after [b]					
D-3	+	+	-	+	+
D-7	+	+	-	+	+
D-11	+	+	+	+	+
D-30	+	+	-	+	+
D-60	+	+	-	+	+

before[a] immunization, after [b] immunization

Flow chart: Sample collection and sequence of work



Flow Chart: Sample collection and sequence of work



15. BUDGET PROPOSAL

PROJECT TITLE: Further studies of systemic and local immune responses in shigellosis

NAME OF THE DONOR: SIDA/SAREC

PROJECT DURATION: 2 & 1/2 years

STARTING DATE: 1/7/1996

CLOSING DATE: 31/12/1998

NAME OF PI: D. Islam, R. Raqib (ICDDR,B) and J. Andersson, B. Christensson and B. Wretlind (KI, Sweden).

RRC APPROVAL DATE:

ERC APPROVAL DATE:

Amount in US$

Line item	Year			TOTAL
	Six month	One year	One year	US$
	A	B	C	D=A+B+C
PERSONNEL LOCAL: SALARIES				
_Local employees salaries and benefits	2x4,000	2x9,000	2x10,000	
SUB-TOTAL	8,000	18,000	20,000	**46,000**
LOCAL TRAVEL:				
_Travel	100	105	110	315
SUB-TOTAL	100	105	110	**315**
INTERNATIONAL TRAVEL:				
_Travel	0	2,300	2,415	4,715
_Perdiem	0	1,600	1,680	3,280
SUB-TOTAL	0	3,900	4,095	**7,995**
SUPPLIES & MATERIALS:				
_Drugs	0	500	50	550
_Hospital supplies	0	100	105	205
_Office suppplies	100	105	110	315
_Laboratory supplies	2,000	8,000	2,000	12,000
_Chemicals and Media	2,000	8,500	2,500	13,000
_Tools & Spares and Gasoline etc.	50	100	120	270
_Wage loss	200	800	300	1,300
SUB-TOTAL	4,350	18,105	5,185	**27,640**

THER CONTRACTUAL SERVICES				
Repair, maintenance	200	50	53	303
Postage, fax,phone, electricity bill etc.	800	840	882	2,522
Allowance for volunteers, daily wager,				
short term employees, workshop	500	2,000	1,000	3,500
Printing & publication expenses	0	2,000	5,000	7,000
Legal & audit fees etc.	40	42	44	126
ICDDR,B guest diet & lodging	100	500	500	1,100
UB-TOTAL	1,640	5,432	7,479	**14,551**
TAFF DEVELOPMENT SERVICES				
Training fees & materials,				
scholarship to employees	400	200	210	810
UB-TOTAL	400	200	210	**810**
NTER-DEPARTMENTAL SERVICES				
Computer charges, E-mail	100	100	105	305
Transport; land, water	50	53	55	158
Medical illustration	100	105	110	315
Photocopy, Library services	100	105	110	315
Lab. & Pathological tests	615	1,000	200	1,815
Maintenance charges	100	105	110	315
Staff clinic charges	200	210	221	631
Patient study	1,000	3,000	500	4,500
UB-TOTAL	2,265	4,678	1,411	**8,354**
APITAL EXPENDITURE:				
Equipment	0	1,000	1,050	2,050
UBTOTAL	0	1,000	1,050	**2,050**
TOTAL PROJECT COST	**16,755**	**51,420**	**39,540**	**107,715**

Expenses of reagents and equipment will be shared by Dr. F. Qadri.
art of the expenses will also be borne by the Karolinska Institute.

No provision could be made for o/H. 100% of the P.I.s salary is budgeted
SG
25/4/96

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14. Raqib R, Lindberg AA, Bj rk L et al. Down-regulation of gamma interferon, tumor necrosis factor type I, interleukin-1 (IL-1) type I, IL-3, IL-4, and transforming growth factor b type I receptors at the local site during the acute phase of *shigella* infection. Infect Immunity 63: 3079-3087, 1995.

15. Raqib R, Reinholt F, Bardhan PK, et al. Immunopathological patterns in the rectal mucosa of patients with shigellosis: expression of HLA-DR antigens and T-lymphocyte subsets. APMIS 102: 371-380, 1994.

16. Raqib R, Lindberg AA, Wretlind B, et al. Persistence of local cytokine production in shigellosis in acute and convalescent stages. Infect Immun 63:289-296.

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

18. Raqib R, Ljungdahl , Lindberg AA, et al. Local entrapment of IFN- γ in the recovery of *Shigella dysenteriae* type 1 infection. GUT 38:328-336, 1996.

19. Raqib R, Ljungdahl , Lindberg AA, et al. Dissociation between cytokine mRNA expression and protein production in *Shigella dysenteriae* type 1 infection. Eur J Immunol 26:1130-1138, 1996.

20. Islam D, Lindberg AA. Detection of *Shigella dysenteriae* type 1 and *Shigella flexneri* in faces by immunomagnetic isolation and polymerase chain reaction. J Clin Microbiol 30:2801-2806, 1992.

21. Islam D, Wretlind B, Ryd M et al. Immunoglobulin subclass distribution and dynamics of *Shigella*-specific antibody responses in serum and stool samples in shigellosis. Infect Immun 63:2054-2061, 1995.

22. Islam D, Wretlind B, Bardhan PK, et al. Semi-quantitative estimation of *Shigella* antigen specific antibodies: correlation to disease severity during shigellosis. (In press) APMIS, 1996..

23. Islam D, Lindberg AA, Christensson B. Peripheral blood cell preparation influences the level of expression of leukocyte cell surface markers as assessed with quantitative multicolor flow cytometry. Cytometry (Communications in Clinical Cytometry) 22:128-134, 1995.

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12. Publications of PI (ICDDR,B)

List of Publications of Dilara Islam

1. Islam, D., Wretlind, B., Lindberg, A. A. and Christensson, B. Changes in the peripheral blood T cell receptor Vβ repertoire *in vivo* and *in vitro* during shigellosis. Infect Immune. 64:1391-1399, 1996.

2. Islam, D., Bardhan, P. K., Lindberg, A. A. and Christensson, B. *Shigella* infection induces cellular activation of T and B cells, and distinct species-related changes in peripheral blood lymphocyte subsets during the course of the disease. Infect Immune. 63:2941-2949, 1995.

3. Islam, D., Wretlind, B., Ryd, M., Lindberg, A. A. and Christensson, B. Immunoglobulin subclass distribution and dynamics of *Shigella*-specific antibody responses in serum and stool samples in shigellosis. Infect Immune. 63:2054-2061, 1995.

4. Islam, D., Lindberg, A. A. and Christensson, B. Peripheral blood cell preparation influences the level of expression of leukocyte cell surface markers as assessed with quantitative multicolor flow cytometry. Cytometry (Communications in Clinical Cytometry). 22:128-132, 1995.

5. Islam, D., Tzipori, S., Islam, M. and Lindberg, A. A. Rapid isolation of *Shigella dysenteriae* and *Shigella flexneri* in faeces by O-antigen specific monoclonal antibody coated immunomagnetic beads. Eur J Clin Microbiol Infec Dis. 12:25-32, 1993.

6. Islam, D. and Lindberg, A. A. Detection of *Shigella dysenteriae* 1 and *Shigella flexneri* in feces by immunomagnetic isolation and polymerase chain reaction. J Clin Microbiol. 30: 2801-2806. 1992.

7. Islam, D., Wretlind, B., Bardhan, P. K., Hammarstrom, L., Christensson, B., and Lindberg, A. A. Semi-quantitative estimation of *Shigella* antigen specific antibodies: Correlation to disease severity during shigellosis. (In press), APMIS, 1996.

List of Publications of Rubhana Raqib

1. Raqib R, Tzipori S, Islam M and Lindberg A. Immune responses to *Shigella dysenteriae* type 1 and *Shigella flexneri* lipopolysaccharide and polysaccharide antigens in Bangladeshi patients with shigellosis. Serol Immun Infect Dis. 1:37-45, 1993.

2. Raqib R, Reinholt F, Bardhan PK, et al. Immunopathological patterns in the rectal mucosa of patients with shigellosis: expression of HLA-DR antigens and T-lymphocyte subsets. APMIS 102: 371-380, 1994.

3. Raqib R, Lindberg AA, Bj rk L et al. Down-regulation of gamma interferon, tumor necrosis factor type I, interleukin 1 (IL-1) type I, IL-3, IL-4, and transforming growth factor β type I receptors at the local site during the acute phase of *shigella* infection. Infect Immun 63: 3079-3087, 1995.

4. Raqib R, Lindberg AA, Wretlind B, et al. Persistence of local cytokine production in shigellosis in acute and convalescent stages. Infect Immun 63:289-296, 1995.

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

6. Raqib R, Ljungdahl , Lindberg AA, et al. Local entrapment of IFN- γ in the recovery of *Shigella dysenteriae* type 1 infection. GUT 38:328-336, 1996.

7. Raqib R, Ljungdahl , Lindberg AA, et al. Dissociation between cytokine mRNA expression and protein production in *Shigella dysenteriae* type 1 infection. Eur J Immunol 26:1130-1138, 1996.

13. Flow Chart (sequence of tasks within time frame)

August-December '96

i) Procurement of supplies and standardization of methods

↓

August '96-December '97

ii) Recruitment of patients and controls in the study

iii) Processing and storage of samples

↓

January-June '98

iv) Multiple samples from an individual required to be studied at a time will be analyzed after completion of sample collection

↓

January-December '98

v) Completion of assays , compilation and analysis of data and reporting the outcome of study in several articles

14. Itemized specific tasks for each listed investigator:

At the moment it is difficult to define which part of the work will be done in ICDDR,B and which part at KI. Based on the availability of the major equipment (flow cytometer and image analyzer) at ICDDR,B, the work can be carried out in ICDDR,B. Otherwise, sample (PBMC and biopsies) processing and storage will be carried out at ICDDR,B. When sample collection will be completed, part of the analysis will be done at ICDDR,B and the rest will be done at KI. For this purpose, samples will have to be transported to KI for further analysis.

- Dilara Islam (100%)

  A:3, A:4 (page 6), B:1, B:2 (page 8), C (page 9)

- Rubhana Raqib (100%)

  A:1, A:2 (page 6), B:1, B:2 (page 8), C (page 9)
  Both D. Islam and R. Raqib will supervise work in the lab and coordinate specimen collection from patients
  Data compilation and analysis, and writing up of the results obtained

- Jan Andersson, Birger Christensson, Bengt Wretlind (KI), Alf A. Lindberg (France) and George Fuchs, Division Director, CSD, ICDDR,B.
  Scientific and academic feedback

- Division Director, LSD, ICDDR,B

  Coordination

- P. K. Bardhan/ N. H. Khan (CSD) and George Fuchs

  Overall in-charge of patient treatment, to carry out colonoscopy and proctoscopy

- Debasish Saha* (Medical officer), (50%)

  Patient enrolment and clinical management and follow-up

- Research officer (100%)

  Carry out tests specified for the protocol involving both microbiological and immunological techniques

- Lab. attendant* (50%) and Ward attendant* (50%)

  Patient recruitment and others.
  *Will also be involved (50%) in the project titled "Local and systemic immune responses in patients..... with *Vibrio cholerae* O139", protocol # 93-024.

REVIEWER I

Title: "Further studies of systemic and local immune responses in shigellosis in order to establish a protective vaccine"

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

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

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification ☒

- on technical grounds ☒

- on level of financial support ☐

I do not support the application ☐

Name o

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Instit

Detailed Comments

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

(Use additional pages if necessary)

Title: Further studies of systemic and local immune responses in shigellosis in order to establish a protective vaccine.

PI: Dilara Islam Ph.D. and Rubhana Raqib, Ph.D.

Reviewer:

This seems to me to be an interesting and well planned project concerning an important problem.

The applicants have a very good background in the field which gives promises that they will manage the study as planned.

I am somewhat concerned that they claim that they can study cytokines in the stool – even "the total amount". I would have expected that some of these compounds, which can be shortlived and sensitive, would be difficult to measure adequately in stool. However, they have given an interesting reference as to this subject (no 17).

With the good organisation and facilities at ICDDR, B it seems clearly feasible to do to clinical studies. The planned transfer of technology in this work is of course favourable.

In summary, this project seems realistic (including the budget), both as to planning and the availability of well trained scientists with fine experience in the field.

REVIEWER II

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

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

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification ☐

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

I do not support the application ☐

Name of Ref:

Signature:
Position:

Institution

Detailed Comments

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

(Use additional pages if necessary)

Title:

PI:

Reviewer: .....

TITLE - FURTHER STUDIES OF SYSTEMIC AND LOCAL IMMUNE RESPONSES IN SHIGELLOSIS IN ORDER TO ESTABLISH A PROTECTIVE VACCINE

GENERAL COMMENTS

This project is an extension of work which was carried out in the doctoral studies of the two principal applicants when in Sweden. Basically, the subject is of great importance, the long term aims are highly relevant to human health, the opportunities provided by ICDDRB to study the natural history of shigellosis and factors which influence disease severity and immunity, are unsurpassed. The opportunity also to investigate responses to vaccinees is highly appropriate.

Some parts of the application are potentially highly rewarding and although technically demanding, likely to produce positive results. I have some general concerns about methodology which are laid out below, and also some concerns about the applicants' interpretation of the results of their previous studies. This probably reflects their inexperience and the fact that the proposed project focussed on immunology. There would be benefit from significant input in design of the studies by knowledgable specialists in clinical disease expression and colonic tissue injury.

In general I am strongly supportive of the application but hope that my constructive comments laid out below can be borne in mind.

I consider the proposed work entirely ethical, if the specimens which are to be collected are properly used. It appears that a colonoscope is available to the investigators. It would be worth considering whether colonoscopic biopsies of the rectum would be less traumatic to the children than biopsies collected with a rigid protoscope.

Comments on the applicants' interpretation of previous studies, and proposed methodology

a) As the index of intestinal antibody production the Investigators have used antibodies prepared from faeces.

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

b) Knowledge of the very variable recovery of immunoglobulins and antibodies in faeces is also relevant to studies of cytokines, in fact probably more so. In the acute disease, faecal material passed will have been within the lumen of the rectum for minutes before collection; on the other hand in the convalescence phase, faeces if formed may have been stored in the descending colon and rectum for many hours, up to 24. It is inconceivable that these two different materials equally reflect phenomena taking place in the bowel wall and the investigators do not appear to have considered this.

Although not debated in the medical literature, the general view of those in Europe who have discussed faecal cytokines in inflammatory bowel disease, is that the cytokines may well be contained within inflammatory cells which have migrated into the gut lumen but are still alive. Viability of faecal cells and recoverability of non-cell bound or cell contained cytokines, would need to be studied prior to the extremely costly range of ELISA techniques proposed by the applicants.

c) In the introduction the applicants discuss a proposed model whereby shigella invades the gut mucosa by being taken up through phagocytic vacuoles in M cells. The citation for this is their doctoral

Theses. The concept of shigella infection spreading laterally through the epithelium is well accepted. However, a postulate that it is only the M cells which are infected directly is highly controversial one and would need to be defended. There is no evidence that M cells in the colon differ from M cells in the small intestine where they are much more abundant. However, although there are some metabolic effects on small bowel function associated with shigellosis I am not aware that there is any evidence of epithelial cell damage. If it were that shigella invades M cells one would expect to have focii of infection spreading from lymphoid nodules and Payers patches and I do not think that this is observed clinically (although I have no direct knowledge of the pathology of shigellosis and the comment is based on my reading of the medical and bacteriological text books).

d) In the information given to the research subjects, it is stated that the biopsies will be 1-2mm across, the size of a mustard seed. Such biopsies are not suitable for immunohistochemistry and are completely unsuitable for any form of quantitative microscopy. This is an entirely different situation to proctoscopy and rectal biopsies taken with large forceps under direct vision.

Comments on individual sub-projects

Study A - I am concerned about the selection of cases for this study. It is indicated that 30 children with naturally acquired shigellosis will be investigated and patients with concomittant gastrointestinal infections will be excluded. I understand that it will be possible to exclude some of the common bacterial pathogens; however I envisage that it would take more time than 1 or 2 days to exclude rotavirus and some of the more unusual bacterial pathogens such as ETEC, and also giardia and other protozoa. Thus more than 30 cases will need to be recruited in order to obtain 30 relatively pure infections. A similar comment, of course, relates to the healthy age matched children.

I appreciate that at the population level, the presence of serum antibodies to shigella antigens broadly correlates with population exposure. However, other knowledge of the separation of mucosal and systemic antibody responses would argue against serum antibody titres being good predictors of previous exposure at the level of an

individual. Thus the suggestion that cases will be stratified by serum antibodies as previously exposed or likely primary infection needs to be defended and justified.

I have made my comments about the difficulties about interpreting faecal antibodies and faecal cytokines in the earlier section. On the other hand the group of methods described under Section 2 on Page 13 of the application are entirely appropriate as are some of the proposed studies on antibodies.

Neutralising capacity of antibodies eluted from stool is proposed. The applicants have already shown that early in infection but not later, there is abundant IgG in stool almost certainly derived from serum. They will therefore have to separate the faecal eluates by some prior immunochemical means if it is to be assumed that any neutralising capacity is a property of secretory IgA.

Sections 4 and 5 on Page 15 related to Aim 1 are technically possible and will provide useful general data. However, in addition to characterising gastrointestinal infections it will be important that the children are also well characterised with regard to diseases of the lung, skin etc. which would have parallel effects on circulating cells.

Section B

This work is very ambitious but I am sure should be encouraged and the proposed close collaboration with the Huddinge Hospital and Karolinska Institute will greatly increase the likelihood that the work will be successful. The applicants should recognise, however, that in attempting to generate T-cell clones from the gut the techniques which after all were developed for use in lymph node preparations and circulating cells, may preferentially select certain T-cell subsets and not others. Useful data on this is being generated from studies on small bowel T-cell clones sensitised to gluten which are generated from coeliac patients and I am sure that the investigators in Oslo working on this line would be able to offer considerable practical and constructive advice.

Essentially, this study will be measuring the effects, if any, on the intestinal IgA producing cells, intestinal antibodies and serum antibodies after administration of the vaccine strain. Children and adults will be compared.

It is not correct that this is a study of local inflammation. None of the techniques proposed will be assessing inflammation in any way. This is a study of local antibody and T-cell responses.

Throughout the application the applicants do not address non-antigen-specific immunity and its relevance. They have already shown in their earlier work on faecal antibodies that in fact faecal recovery of shigella antibodies is scarcely different from faecal recovery of IgA in general although the antibody repertoire of the antibodies directed to shigella antigens does evolve in the course of infection. This line of work is well worth pursuing but it is absolutely vital that the investigators recognise what can and what cannot be measured with the techniques they are proposing.

Response to the reviewers comments

REVIEWER I

No specific comments to address.

REVIEWER II

General comments

Rectal biopsies will be obtained from children at sigmoidoscopy, which is a flexible sigmoidoscope and is not a rigid proctoscope. A sigmoidoscope is chosen over colonoscope because it does not require prior bowel preparation and is easier and quicker to perform.

Proposed Methodology

(a). As shown by Ferguson et al (Clin Exp Immunol, 1995), detection of antibody in formed stool may give false negative results, i.e. these findings may not represent the true status of the gut immune system. However, we have always focused on looking at the immunoglobulins in fecal samples during the acute stage of shigellosis. During this phase stools are very soft containing blood and mucus and there was no difficulty in obtaining a true picture. It is equally true for the cytokine assays. We agree to the comments of the reviewer that in the convalescent stage recovery of cytokines and immunoglobulins will be difficult from formed stool. One possibility is to obtain gut lavage from these patients as suggested by the reviewer, which is not desirable after recently recovering from dysentery. Another alternative is to collect stool samples from patients in the convalescent stage, for three consecutive days in order to obtain representative samples from various parts of the gut. However, this is not feasible either because all these patients come from slums in and around Dhaka city or far away places. They are usually difficult to be convinced to come for follow-up check ups when they are clinically well. Moreover, they are difficult to be traced. Parallel studies using rectal biopsies will be carried out for the presence of cytokines and immunoglobulin secreting cells at the single cell level to obtain a better and more complete picture of the local immune status. When presenting findings these facts will be borne in mind.

(b) Answered above.

(c) From the comments of the reviewer, it is obvious that the reviewer got the impression that the hypothesis of spreading of *Shigella* infection via M cells was studied by the authors (Rubhana Raqib and Dilara Islam). Probably it was not clearly stated in the present protocol, but these findings of invasion through M cells have been studied by two different groups of investigators (Wassef et al, 1989, and Sansonetti et al, 1994). This part has been deleted from the protocol now to avoid confusion.

(d) Samples obtained at sigmoidoscopy are the size of a big rice grain (4-5 mm across). Biopsy samples of similar size have been used in the previous studies of the investigators involving immunohistochemistry as well as for in situ hybridization techniques.

Comments on individual sub projects

Study A- In the study, initially more than 30 patients will be selected and scrutinize for various diarrheal pathogens. Patients with other gastrointestinal infections will be excluded in order to finally recruit 30 patients with pure infections of *Shigella*. This has now been added in the protocol.

We totally agree with the reviewer that it is difficult to dissociate children with primary *Shigella* infection from primed adults with infection, based on the specific antibody titers. It is highly unlikely that children of the age group 5-10 yrs living in a *Shigella* endemic area will be unexposed to *Shigella*. It will be unethical to take multiple blood and biopsy samples from infected and sick child (under 5 yrs of age). Our observations will be limited to the study of development of immune responses in children and comparison with that of adults.

Only antibodies eluted from serum will be studied for neutralizing capacity. Specific antibodies from stool will be studied for humoral immune response. The lines have been in rephrased in the Research Plan.

A complete clinical check up of each patient will be carried out in order to rule out other diseases. This has already been mentioned in the text.

Section B

As suggested by the reviewer, the applicants will follow the techniques to separate cells from rectal biopsies adapted by Marie-Loiuse Hammerstrom and colleagues at Umea University, Umea, Sweden.

Section C

We agree to the the reviewer's comments that the third part (Specific aim C) is not a study of local inflammation. The investigators will only study the local immune response in gut after vaccination. Restructured in the text.

However, we do not agree to the comment that none of the techniques proposed can assess inflammation. Indeed in the earlier studies of the investigators, it has been shown that local gut inflammation is assessable with immunohistochemical techniques.

In response to the third comment, most of our previous studies have compared cellular immune responses of patients to that of healthy controls. The studies suggested that a part of the immunity developed after infection may be *Shigella* specific. Thus the current proposal aims to further study the *Shigella* specific responses.