FURTHER STUDIES OF IMMUNOPROTECTIVE AND IMMUNOPATHOGENIC MECHANISMS IN SHIGELLOSIS

RUBHANA RAQIB
JAN ANDERSSON

1998-037

Date:

Attachment 1

(FACE SHEET) **ETHICAL REVIEW COMMITTEE, ICDDR,B.**

Principal Investigator: Rubhana Raqib

Application No. 98-037

Title of Study: Further studies of immunoprotective and immunopathogenic mechanisms in shigellosis.

Trainee Investigator (if any): _____

Supporting Agency (if Non-ICDDR,B) SAREC/SIDA

Project Status: _____

[x] New Study

[ ] Continuation with change

[ ] No change (do not fill out rest of the 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) Minor or persons under guardianship — (Yes) No

2. Does the Study Involve:
   - (a) Physical risk to the subjects — (Yes) No
   - (b) Social risk — Yes (No)
   - (c) Psychological risks to subjects — Yes (No)
   - (d) Discomfort to subjects — (Yes)
   - (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 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 the study — (Yes) No
   - (b) Procedures to be followed including alternatives used — (Yes) No
   - (c) Physical risk — (Yes) No
   - (d) Sensitive questions — NA Yes (No)
   - (e) Benefits to be derived — NA 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 parents or guardian (if subjects are minor) — (Yes) No

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

7. Check documents being submitted herewith to Committee:
   - ~~NA~~ Umbrella proposal - Initially submit an with overview (all other requirements will be submitted with individual studies
   - ✓ Protocol (Required)
   - ✓ Abstract Summary (Required)
   - ~~NA~~ 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
   - ~~NA~~ 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. Example of the type of specific questions to be asked in the sensitive areas
3. An indication as to when the questionnaire will be presented to the Committee for review

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

Rubhana
Principal Investigator

Trainee

nternational Centre for Diarrhoeal Disease Research, Bangladesh

FOR OFFICE USE ONLY
Protocol No: Date:

RESEARCH PROTOCOL

RRCApproval: Yes/ No Date:
ERC Approval: Yes/No Date:

Title of Project (Do not exceed 60 characters including spaces and punctuations)
TUDIES IN SHIGELLOSIS: B. FURTHER STUDIES OF IMMUNOPROTECTIVE AND MMUNOPATHOGENIC MECHANISMS IN SHIGELLOSIS

1. Name of the Principal Investigator(s) (Last, Middle, First).	2b. Position / Title	2c. Qualifications
AQIB, RUBHANA AN ANDERSSON	NOB, Assistant Scientist Professor	PhD MD, PhD

Name of the Division/ Branch / Programme of ICDDR,B under which the study will be carried out.
Laboratory Sciences Division, Immunology.

Contact Address of the Principal Investigator at ICDDR,B
Office Location: Immunology,
boratory Sciences Division, Mohakhali
aka-1212, Bangladesh

4b. Fax No: +880-2-872529 / 886050 / 883116
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Use of Human Subjects 5a. Use of Live Animal

5b. If Yes, Specify Animal Species
Yes ☐
No ☐

Dates of Proposed Period of Support
Day, Month, Year - DD/MM/YY)
01.01.1999- TO 31-.12.2001

7. Cost Required for the Budget Period
7a. *1st Year ($)*: 68018US$ *2nd Year ($)*: 64,075 US$
3rd Year: 64,075 US$
7b. Direct Cost ($) 176,075 Total Cost ($): 196,194 US$

pproval of the Project by the Division Director of the Applicant

bove-mentioned project has been discussed and reviewed at the Division level as well by the external reviewers

ssor V. I. Mathan
e of the Division Director

Signature

8/10/98
Date of Approval

Certification by the Principal Investigator

ify that the statements herein are true, complete ccurate to the best of my knowledge. I am aware ny false, fictitious, or fraudulent statements or s may subject me to criminal, civil, or administra- enalties. I agree to accept responsibility for the ific conduct of the project and to provide the re- l progress reports if a grant is awarded as a result application.

10. Signature of PIs
1. Rubhana 2.

Date: 22-10-98 October 14- 1998

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PROJECT SUMMARY: Describe in concise terms, the hypothesis, objectives, and the relevant background of the project. Describe concisely the experimental design and research methods for achieving the objectives. This description will serve as a succinct and precise and accurate description of the proposed research is required. This summary must be understandable and interpretable when removed from the main application. (TYPE TEXT WITHIN THE SPACE PROVIDED).

Principal Investigators: RUBHANA RAQIB, JAN ANDERSSON

Project Name: FURTHER STUDIES OF IMMUNOPROTECTIVE AND IMMUNOPATHOGENIC MECHANISMS IN SHIGELLOSIS

Total Budget: 196,196 US$ **Beginning Date:** January 1, 1999 **Ending Date:** December 31, 2001

Shigellosis is one of the major causes of childhood morbidity and mortality in developing countries like Bangladesh. Although available antibiotics effectively treat shigellosis, prevalence of antibiotic resistant strains is very high. Shigellosis is associated with inadequate hygiene and sanitation, and overcrowded living conditions. Prevention of disease spread could be achieved by improvement of housing and sanitation. However, a radical improvement in infrastructures may not be feasible in many developing countries. The most appropriate approach of prevention would be through vaccination. At present, no safe and efficacious vaccine against *Shigella* species is available. Thus, a better understanding of the immune responses elicited in natural shigellosis is needed for the development of protective and efficacious vaccines. Recent studies in adults with shigellosis suggested that both the innate and specific immune responses were important for local disease development as well as for recovery from the infection. However, certain aspects of the immune response pattern seen in children were different from those in adults and were probably due to the immature state of the defense machinery. Further studies of specific and nonspecific humoral and cellular immune responses in pediatric and adult patients with *Shigella* infection need to be carried out.

The objective of the protocol is to study the development of specific and nonspecific immune defense mechanisms in children during the course of natural *Shigella* infection and to compare those in adults. A group of *Shigella* infected children (age 4-10 years; n=30) with age and socioeconomic status matched control group either healthy (n=30) or having diarrhea caused by other organisms (n=30) will be recruited for the study. In addition a group of *Shigella* infected adult patients (n=30) and age (age 18-45 years) and socioeconomic status matched healthy control group (n=30) will be recruited in the study. Blood and stool will be collected from each pediatric patient at the day of admission and 5, 11, 30 and 60 days after admission. Rectal biopsies (flexible sigmoidoscopy) will be obtained twice (0, 30) from pediatric patients. From adult patients, blood and stool will be collected at 6 time points (0, 4, 7, 11, 30, 60) and rectal biopsies will be collected thrice (0, 11, 30) after admission. All samples from healthy subjects will be collected once. Analysis of secretory IgA from feces and serum and lymphocytes isolated from peripheral circulation and gut may provide evidence of development of specific local immunity in gut including sIgA antibody mediated cellular cytotoxicity against *Shigella* species. To ascertain the functional significance of cells of the innate defense armory (mast cells, eosinophils and neutrophils), the cell-specific origin and release of various inflammatory mediators (defensins, lactoferrin, histamine, prostaglandin H synthase, major basic protein, stem cell factor and eotaxins) and key enzymes of the oxidative and anti oxidative pathways (myeloperoxidase and superoxide dismutase) will be studied by immunohistochemical techniques using rectal tissues from patients and healthy controls. Release of neuropeptides in plasma and feces will be analyzed by radioimmunoassays and immunhistochemical studies will be carried out to study semi quantitative localization of neuropeptides (Substance P, serotonin, vasoactive intestinal peptide and bradykinin) in rectal biopsies.

KEY PERSONNEL (List names of all investigators including PI and their respective specialties)

Name	Professional Discipline/ Specialty	Role in the Project
1. Rubhana Raqib	Assistant Scientist / Immunology	Principal Investigator
2. Jan Andersson	Professor / Infectious Diseases & Immunology	Principal Investigator
3. Firdausi Qadri	Senior Scientist / Immunology	Co-investigator
4. Dilara Islam	Assistant Scientist / Immunology	Co-investigator
5. Minnie Mathan	Research Histopathologist/ Histopathology	Co-investigator
6. Nurul Haque Alam	Clinician / Gastroenterology	Co-investigator

DESCRIPTION OF THE RESEARCH PROJECT

Hypothesis to be tested:

Concisely list in order, in the space provided, the hypothesis to be tested and the Specific Aims of the proposed study. Provide the scientific basis of the hypothesis, critically examining the observations leading to the formulation of the hypothesis.

The increased morbidity and mortality seen in children in comparison to adults are due to a lack of adequate specific and nonspecific immune response development in children during the course of natural *Shigella* infection.

Specific Aims:

Describe the specific aims of the proposed study. State the specific parameters, biological functions/ rates/ processes that will be assessed by specific methods (TYPE WITHIN LIMITS).

1. To study the localization and functional relevance of mast cells, eosinophils and neutrophils at the local site during natural *Shigella* infection in pediatric and adults patients.
2. To study the cell-specific origin and release of various inflammatory mediators (defensins, lactoferrin, myeloperoxidase, superoxide dismutase, prostaglandin H synthase, major basic proteins, stem cell factor and eotaxin) in the rectal mucosa.
3. To study the role of enteric neuropeptides in the immunity and immunopathogenesis of shigellosis
4. To study mucosal and systemic natural killer cell mediated cytotoxicity induced by s-IgA.

Background of the Project including Preliminary Observations

Describe the relevant background of the proposed study. Discuss the previous related works on the subject by citing specific references. Describe logically how the present hypothesis is supported by the relevant background observations including any preliminary results that may be available. Critically analyze available knowledge in the

field of the proposed study and discuss the questions and gaps in the knowledge that need to be fulfilled to achieve the proposed goals. Provide scientific validity of the hypothesis on the basis of background information. If there is no sufficient information on the subject, indicate the need to develop new knowledge. Also include the **significance and rationale** of the proposed work by specifically discussing how these accomplishments will bring benefit to human health in relation to biomedical, social, and environmental perspectives. **(DO NOT EXCEED 5 PAGES, USE CONTINUATION SHEETS).**

Shigellosis is one of the major causes of morbidity and mortality in many developing countries. It is estimated that over 200 million people are infected with *Shigella* species every year. The annual world-wide mortality rate from acute shigellosis is approximately 650,000. 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 of more than 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. Although available antibiotics effectively treat shigellosis, prevalence of antibiotic resistant strains are very high.

In endemic areas, prevention of shigellosis could be achieved by two basic strategies, i.e. by improvements in housing and sanitation thereby eliminating the possibility of ingestion of contaminated food and water and through vaccination. Preventive strategies to reduce exposure are important but difficult to implement, since in many developing countries the costs for efficient infrastructure improvements can not be met. Population burden makes it inadequate to effectively control shigellosis in these developing countries. 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. Due to the lack of convenient infection model of shigellosis, the immune mechanisms responsible for protection against the disease, immunopathogenesis, the relationship between host cell infection and inflammatory responses post infection are poorly defined. *Shigella* is primarily a human pathogen, thus there are limited possibilities to use experimental animal models (3, 4). The relative contributions of the elegant studies with *Shigella*-infected macrophages, neutrophils and epithelial cell lines are significant which pave the way to show that the host cells are partly responsible for the initiation of the inflammation (5, 6). There are various host inflammatory responses mediated by mast cells, eosinophils or neuropeptides which may play critical roles in the recovery and in the immunopathogenesis of shigellosis as are evident in inflammatory bowel diseases (7, 8). Therefore, *in vivo* and *in vitro* studies in humans with shigellosis need to be carried out.

The initial event in the infectious process in shigellosis is intracellular (9). It is therefore, considered that the protective immunity consists of cellular responses in the intestinal mucosa mainly T-cell mediated responses as well as the local antibodies produced in the gut. 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 (10, 11). With the development of genetic methods, the molecular and cellular basis of pathogenesis of shigellosis has been studied by using limited laboratory animal models and *in vitro* grown mammalian cell line susceptible to infection (3, 4, 5, 6, 12). Bacteria enter via induced phagocytosis, lyse the membrane-bound phagocytic vacuole, grow within cytoplasmic compartment and spread from cell to cell using host cell cytoskeleton as a motor (12).

Shigella being an enteric pathogen a major focus of attention has been on the potentially protective role of secretory (s) IgA molecules (13). The protection achieved by natural infection or vaccination is considered to be serotype specific where LPS is assumed to be the target antigen for protective immunity (14-16). Serotype specific immunity was demonstrated in a murine model of shigellosis by showing that mucosal antibodies directed against a single polysaccharidic surface epitope of *Shigella* could protect against the disease (3). However, sIgA collected from ileal loops of rabbits failed to inhibit invasion of cultured mammalian cells by *S. flexneri* (12). 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 in humans has yet to be clearly demonstrated (15-18). Relatively little is known about the immunopathogenic mechanisms in the host and the development of immunity against shigellosis.

Our previous studies:

We published a series of papers based on certain aspects of cellular and humoral responses in natural *Shigella* infections in adults. This work has also been summarized in two doctoral thesis defended at the Karolinska Institute by two of the investigators (17-34). After the completion of PhD studies, the principal investigator has joined ICCDR,B and collaborative studies on shigellosis has continued between the groups at Huddinge Hospital, Karolinska Institutet and at ICDDR,B. Outcome of this continued collaboration (since 1996): four papers have been published (23, 24, 25, 26), one manuscript has been submitted (27) and two manuscripts are in preparation.

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 (20). Extensive production of pro-inflammatory cytokines (IL-1-α, IL-1-β, TNF-α, TNF-β, IL-6 and IL-8) was observed at the local site which persisted up to a month after the onset of disease (21). 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 (IFN-γ, TNF-α, TNF-β) and Th2 (IL-4, IL-5, IL-10) types of cytokines was seen during shigellosis. In the early phase of the disease, 100 times higher cytokine concentrations were found in stool than in plasma (22). Increased stool concentrations of cytokines correlated to the severity of inflammation in the gut as well as to clinical markers of disease severity. Production of IFN-γ was significantly down-regulated during the acute stage of the disease and progressively increased during convalescence. In contrast, healthy controls had significantly higher concentrations of IFN-γ in stool and plasma than the patients reflecting a protective role of IFN-γ. A progressive entrapment and binding of IFN-γ to its specific receptor was observed at the local site during recovery from shigellosis which was comparable to the constitutive level of expression in the healthy subjects (23). 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 (24). 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, IFN-γ and TGF-β type I) was observed (19). 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.

A selective immunosuppression of IFN-γ protein and mRNA in peripheral circulation was observed during acute shigellosis, in line with the findings at the local site (26). These results were further strengthened by recently reported data by Sing Sing Way *et al* that showed that IFN- γ was essential for innate resistance to primary *Shigella* infection and IFN- γ deficient mice were highly susceptible to virulent *Shigella* strain (35). The source of IFN- γ was found to be NK cells. In adults with acute *S. dysenteriae* 1 infection, massive apoptosis was paralleled by an increased expression of Fas/Fas-L and a downregulation of Bcl-2 in the rectal mucosa (27). At the late recovery stage, a significantly enhanced production of perforin was accompanied by reduced numbers of *Shigella* antigen positive cells. Humoral mediators of the innate defense system were studied in stool and plasma of adult and pediatric patients with shigellosis and in healthy matched controls. Concentrations of prostaglandin E2, leukotriene B4, lactoferrin and myeloperoxidase activity in stool and plasma were significantly higher in both adult and pediatric patients during the acute disease in comparison to controls (manuscript in preparation). Superoxide dismutase (SOD) acitvity (one of the enzymes of the antioxidant defense mechanisms) in adult and pediatric patients was significantly lower in the acute stage in comparison to that in the healthy controls. In children, SOD activity remained significantly lower throughout the disease course, though in adults, the activity reached the control levels within 9-11 days of onset. The results indicated that in the acute stage of shigellosis, oxidative and nonspecific killing mechanisms were up-regulated, accompanied by enhanced inflammatory responses and lowered scavenging of oxygen radical leading to increased oxidative stress. Urinary nitrate levels in adult patients increased to peak levels after 6-8 days of onset and were significantly higher when compared to controls. However, nitrate levels in children were significantly lower than the healthy controls and gradually increased during recovery suggesting that killing by reactive nitrogen species is probably depressed in pediatric patients. The findings also suggest that some components of the congenital defense mechinary in children were not adequate in combating the disease. Cytokine responses in children were comparable to that in adults with high levels of pro-inflammatory cytokines in acute shigellosis and drastic decrease in the concentrations during convalescence (manuscript in preparation). The exception was evident for TNF-α and IL-1 β. TNF- α levels were significantly higher in children during acute shigellosis and IL-1β concentration in stool were markedly lower in children than in adults.

It is becoming increasingly evident that host inflammatory responses play an important role in *Shigella* pathogenesis. All these studies indicated that both the specific cell-mediated immune response as well as the non specific innate defense system were important in adults infected with *Shigella* for local disease development and recovery. In adults, a mature immune defense system operates where the innate defense mechanism is efficient in combating the pathogen. In addition, a secondary specific immune response pattern is seen in this primed adult population who have been previously exposed to *Shigella*. In children, however, the immune system is probably not fully matured/developed and thus the high morbidity and mortality rate seen in children. The nature of humoral and cellular (specific and nonspecific) immune responses seen in the naive host at primary *Shigella* infection needs to be further characterized.

CELLULAR AND HUMORAL (INNATE AND AQUIRED) ARMS OF THE IMMUNE SYSTEM

Recently, considerable attention has been focused on the interaction between the innate and the acquired arm of the immune system in the induction and the effector phase of the immune response. When microbes penetrate the mucosal barrier/surface, two types of defensive operations come into play. One is the destructive effects of soluble mediators and the other is phagocytosis. After phagocytosis, antigens are presented by macrophages or professional antigen presenting cells to cytotoxic T cells that recognize the antigens in association with MHC class I molecules and exert killing extracellularly. They also release IFN-γ which stimulates NK cells in extracellular killing.

CELLS OF INNATE IMMUNE SYSTEM

Various types of cells are involved in the innate immunity such as neutrophils, basophils, eosinophils, macrophages, mast cells and natural killer cells.

Natural killer (NK) cells are large granular lymphocytes known to play important role in the control of intracellular infections. NK cells and T cells share a number of biological functions including cytotoxicity and cytokine secretion but arise from related but distinct developmental lineages (36). Majority of NK cells in normal individuals express the $CD16^{+}CD56^{+}$ phenotype and these surface markers are used to determine the frequency of NK cells. NK cells have lectin-like nonspecific receptors for infected cells. They recognize antibody coated infected cells through their Fcγ receptor (for constant part of antibody molecule). The antibodies bring NK cells close to the target by forming a bridge and NK cell being activated by the complexed antibody molecule is able to kill the infected cells by its extracellular mechanisms (such as perforins and granzymes). This killing system is called antibody-dependent-cell-mediated cytotoxicity (ADCC). Secretory IgA at the mucosal surface first come in contact with the pathogen/bacteria and exert antibacterial activity. ADCC mediated by lymphocytes from murine gut-associated-lymphoid tissues (GALT) has been shown against *Shigella* strains (37). The natural antibacterial activity of these lymphocytes was enhanced by sIgA purified from intestinal secretion of rabbit Ileal Thiry-Vella loop model infected with the same *Shigella* strain. Similar studies need to be carried out in humans with shigellosis. Various studies have shown that interferon-gamma can enhance natural killer activity as well as ADCC of neutrophils (38). In patients with *Shigella* infection, mucosal as well as systemic IFN- γ levels are lower during the acute stage which increase during convalescence (22, 23, 26). Whether decreased IFN-γ levels in acute shigellosis depress the ADCC activity of NK cells or neutrophils at the mucosal surface is not known.

Polymorphonuclear neutrophils (PMN) and **macrophages (MΦ)** are the two main cell types which are professional phagocytes whose major effector function is killing microorganisms. PMN are short lived, respond to inflammatory stimuli within minutes to hours, rapidly appear within tissues in large numbers and are responsible for the acute phase of inflammation (39). However, MΦs have longer lives, are fewer in number, appear later in infection (after polymorphs) and participate in both acute and chronic inflammation (40). Interaction of MΦs with a pathogen may initiate inflammation by secretion of soluble mediators of the acute inflammatory response. These mediators up-regulate expression of adhesion molecules on endothelial walls for neutrophils, increase capillary permeability and promote chemotaxis. MΦs can act as antigen presenting cells in the amplification of either a humoral or a cellular immune reaction (41). MΦ can also influence a PMN-dependent inflammatory reaction by

releasing chemotactic factors such as interleukin 8 (IL-8), leukotriene B_4 (LTB_4), complement component C5a or tumor necrosis factor (TNF-α) (42). PMN on the other hand can reciprocally regulate subsequent activation and recruitment of MΦ by release of an array of cytokines (43).

Eosinophils reside predominantly in submucosal tissue and is recruited to sites of specific immune reactions. Eosinophils contain cytoplasmic granules that consist of major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil derived neurotoxin (EDN), which are released upon stimulation. These proteins contribute significantly to the host defense against helminthic parasites, and in tissue dysfunction and damage in eosinophil related inflammatory and allergic diseases. ECP, EPO and MBP can directly kill parasites (44). MBP exerts its toxic effect by interacting with lipid membranes leading to their derangement. MBP can also stimulate release of histamine from basophils and mast cells. EDN specifically damage myelin coat of neurons. MBP and EPO play a role in M_2 receptor dysfunction and bronchoconstriction. Eosinophils are capable of producing cytokines that include those with autocrine growth-factor activities for eosinophils (IL-3, IL-5 and GM-CSF), and those with activities in acute and chronic inflammatory responses (IL-1β, IL-6, IL-8, TNF-α, TGF-α and TGF-β). In bronchial tissues from asthmatic patients, IL-4 secretion by eosinophils was demonstrated (45). In mildly inflamed tissues, very few IL-4^+ eosinophils were seen. However, in severly inflamed tissues, 90% of the IL-4^+ cells were eosinophils. Probably, IgA immune complexes in the microenvironment stimulated the eosinophils into IL-4 production. The ability to synthesize cytokines by eosinophils suggests a regulatory role for these cells in the immune-inflammatory responses. Several mechanisms have been identified that cause eosinophil activation and degranulation and mediator production. Secretory IgA (sIgA) seemed to be the most potent stimulus for eosinophil degranulation as evident by release of EDN, EPO and ECP (46). Binding of secretory component to its receptor in eosinophil may cause release of ECP and EPO. High affinity IgE receptors on eosinophils was also shown to be involved in degranulation and defense against parasite. Eosinophils can produce a repertoire of mediators that are involved in a variety of aspects of the inflammatory response (44). These include eicosinoid mediators such as leukotriene LTC_4, neuropeptides such as SP and VIP, and platelet-activating-factor (PAF). These neuropeptides (SP, VIP) have potential role in the regulation of immune responses and in vascular permeability and smooth muscle contractility. Nevertheless, eosinophils also have beneficial activities. Acylsulphatase B released by eosinophils inactivate mediators of anaphylaxis (a mixture of LTC_4, LTD_4 and LTE_4), phospohlipase D destroys platelet lytic factor, histaminase degrades histamine and phosphalipase B inactivates membrane-active lysophosphatides. Eosinophils may also contribute to tissue repair process by producing TGF-β, and epidermal growth factor which are capable of stimulating epithelial cell proliferation and differentiation and fibroblast mitogenesis. Eotaxin, a CC chemokine is a powerful chemottractant for eosinophils and acts via CCR3 receptor (47). Eotaxin is constitutively expressed in most human organs at low levels, marked up-regulation of this protein or message has been detected in the intestine of patients with inflammatory bowel diseases accompanied by eosinophil infiltration (48). The major cellular source of eotaxin are thought to be epithelium, endothelium and activated infiltrating monocytes and eosinophils. A recent study have shown that eotaxin accumulation induced by IL-4 is partly mediated by a secondary generation of endogenous eotaxin (49). Ultrastructural studies by Mathan and Mathan revealed that during shigellosis in adults, persistence of dysentery and cell damage was probably due to the release of cytotoxic substances by degranulation of eosinophils and mast cells and cell-mediated cytolysis (50). Our ongoing studies using pediatric and adult patients

samples showed markedly increased numbers of eosinophils in the peripheral blood as well as in the rectal mucosa in the convalescent stage in comparison to the acute stage of shigellosis (unpublished data).

Mast cells are derived from the multipotential hematopoetic stem cell. Precursors of the mast cells leave the hematopoetic organs, migrate to the blood stream and invade the connective tissue or mucosal tissue (51). The precursors have few basophilic granules in the cytoplasm, express c-kit receptor tyrosine kinase on the surface and contain mRNA of mast cell-specific proteases. Precursor mast cells in circulation are committed and can differentiate to either mucosal mast cell or connective tissue mast cell (52). Mast cells are able to proliferate, even after degranulation and reproduce specific granules and restore their original morphology. Mast cells have the potential to produce a large spectrum of powerful mediators through which they are able to initiate and coordinate the host's immune and inflammatory responses against invading pathogens. Among the mediators released by mast cells some are stored preformed (serotonin, histamine, heparin, TNF-α) and some are produced *de novo* upon stimulation (PG, LT, thromboxanes). Some of these mediators are proinflammatory and may contribute to a number of chronic inflammatory conditions such as stress induced intestinal ulceration, rheumatoid arthritis, interstitial cystitis, scleroderma, and Crohn's disease (8, 52). Mast cells are also the main effector cells in the development of anaphylaxis.

Mast cells can exert their antimicrobial activities either by mediator release or by phagocytosis. They do not need direct physical contact and can be activated from a distance by various toxins, LPS or host derived proteins generated during bacterium-initiated inflammatory reactions. Defensins from neutrophils, cationic proteins from eosinophils are believed to activate mast cells. Stem cell growth factor (SCF) which is also a mast cell growth factor has a critical role in mast cell survival, development and function and promotes host defense by modulating mast cell function. SCF suppresses apoptosis of mast cells and there by maintains survival of mast cells. It is also an important mediator of mast cell chemotaxis and promote degranulation and secretion of mediators by mast cells. Studies by Klimpel et al (53) have shown that an early event in the intestinal tract response to bacterial infection is the enhanced production of SCF by intestinal epithelial cells which also enhanced the cells' resistance to invasion by *Salmonella typhimurium.* Mast cells were shown to phagocytoze and kill *Salmonella, E. coli, Enterobacter cloacae, K. pneumoniae.* Killing can be done either by oxidative or by nonoxidative methods. After phagocytic uptake of *Salmonella, E. coli* and *Shigella,* mast cells were shown to be able to present antigens through MHC class I molecules. The ability to process and present antigens, and the capacity to produce vital immunoregulatory cytokines indicate that mast cell may be one of the first inflammatory cells to be activated following invasion by pathogens. A recent ultrastructural study by Paulimood *et al* showed no statistically significant difference in the number of mast cells in the rectal mucosa during acute shigellosis and in healthy mucosa (54). However, mast cells were found to be activated as assessed by increased prevalence of lipid bodies which are the site of arachidonic acid metabolism, thereby indicating that there was increased synthesis and release of inflammatory mediators. Interestingly, adult patients with acute shigellosis exhibited a drastic decrease in mast cell numbers 11-15 days after onset in comparison to early acute stage (3-5 days after onset) as assessed by immunohistochemical staining (our preliminary observation).

ANTIMICROBIAL KILLING MECHANISMS

Killing of the microbes by neutrophil polymorphs and macrophages may be achieved by various means

such as by generation of reactive oxygen intermediates (ROI) (superoxide radical $O_2^{\bullet}$, hydrogen peroxide H_2O_2, hydroxyl radical $^{\bullet}OH$) and reactive nitrogen species (RNI) (nitric oxide NO,) and preformed antimicrobials. These reactive intermediates produced by stimulated phagocytes can act directly or in concert with other components to kill or damage ingested microbes. The antimicrobial molecules contained within the granules of polymorphs contact the ingested microbes when fusion with the phagosomes occurs and exert antibacterial effects. Primary azurophilic granules of neutrophils contain lysozyme, myeloperoxidase, defensins, bactericidal / permeability increasing factor (BPIF) and cathepsin G. The peroxidase negative secondary granules contain lactoferrin, lysozyme, alkaline phosphatase and cytochrome b_{558}. All these together generate a powerful antimicrobial system. The enzyme myeloperoxidase (MPO) is released upon degranulation of PMN during phagocytosis (39). It is able to catalyze the peroxidation of halides (Cl^-, Br^-, I^-) and the thiocynate ion to generate reactive products that have potent antibacterial properties. They can also protect the mucosal surfaces by preventing the accumulation of toxic products of oxygen reduction. MPO can regulate a number of MΦ functions, including enhancing bacterial phagocytosis, intracellular killing and inducing release of reactive oxygen species, TNF-α and IFN-γ (39). Nitric oxide (NO) is a highly reactive free radical synthesized by endothelial cells, neutrophils, macrophages, neurons and platelets. NO exerts both beneficial and detrimental effects on tissues. It participates in the neurotransmission, in the immune response, gut motility, blood pressure control and many functions in the kidney. When nitric oxide is produced in response to inflammatory stimuli, the biological role in such a case is defense against pathogen through oxidative toxicity (8). It plays the key microbicidal role in MΦ. However, very high levels of NO leads to formation of peroxynitrite by combining with superoxide anion and may cause destruction of cell membrane components and tissue damage.

PREFORMED AND DE NOVO SYNTHESIS OF ANTIMICROBIALS

Innate humoral factors include various proteins and metabolites that are secreted by different cell types onto the mucosal surface and play protective roles independent of the presence of specific antibodies. Lactoferrin, an iron binding protein is released by neutrophils upon stimulation. It is found in various secretory fluids such as milk, tears and intestinal mucus and has bacteriostatic and bactericidal effect. Lactoferrin also interacts with cells of the immune system and induces a regulated release of cytokines, maturation of T and B cells with regard to their phenotype and function (55). Thus, this protein has a positive role in the induction phase of the immune response. Human defensins (HD), are a large family of antimicrobial peptides identified by a conserved cysteine motif and are stored in the azurophilic granules of PMN and cytoplasmic granules of MΦ. They (HD1-3) are released into phagolysosomes where they contribute to killing of engulfed microbes (56). Paneth cells also produce defensins (HD5 and HD6) which are released in the lumen. These peptides have the capacity to insert themselves in the membrane of microbes and form destabilizing voltage regulated ion channels. Defensins exhibit broad range antimicrobial activities against a number of gram-positive and-negative bacteria, many fungi and some enveloped viruses (57). Presence of several enteric defensins in the epithelium and in the lumen, in intercellular clefts between PMN and their targets suggest that these peptides contribute to establishment of a local antimicrobial milieu. Because of the nature of action of defensins, high extracellular concentration of this protein may cause tissue injury at the site of inflammation (58) and may contribute to the pathophysiological consequences of inflammation in addition to their role in host defense (57). Eicosinoides such as prostaglandins (PG) and

leukotrienes (LT) are metabolites of the arachidonic acid pathway. They are synthesized de novo upon stimulation. PGE_2 is produced by epithelial cells, mast cells, endothelial cells, macrophages and follicular dendritic cells. Prostaglandins have anti-inflammatory, inflammatory and immunoregulatory properties. The key enzyme PGH-synthase (PGHS) of the arachidonic acid pathway was found to be present in both activated macrophages and eosinophils in ulcerative colitis (59). Leukotriene B_4 (LTB_4) is a 5-lipooxygenase product of arachidonic acid pathway. It is produced by mast cells, neutrophils, monocytes and macrophages. Activated neutrophils secrete LTB_4, which is an inducer of neutrophil chemotaxis, of neutrophil degranulation and of neutrophil-endothelial adhesion. LTB_4 was shown to be an important cytotoxic mediator in the mast cell-mediated cytotoxicity to Toxoplasma gondii (60). It has been suggested that it contributes to colitis through modulation of granulocyte infiltration, neutrophil degranulation, vascular permeability, smooth muscle cell contraction in the gastrointestinal tract and blood vessels.

ENTERIC NEUROPEPTIDES

There are close anatomical localization of mast cells and nerves in the mucosa. Studies of interaction between mast cells and the nervous system such as the role of substance P and other neuropeptides and endorphins in mast cell activation, regulation of nervous system function through mediators like PGD_2 show that the interaction is bidirectional. Accumulating evidence suggest that neurogenic inflammation are important in the intestine. **Enteric neurons** are abnormal in Crohn's disease, myenteric nerves are involved in transport changes in experimental mucosal inflammation (7, 8). Injury to cholinergic nerve terminals by oxidant NH_2Cl evokes release of acetylcholine (a secretagogue) from mucosal preparation. ROI-induced disturbances in vasoactide intestinal peptide (VIP)-ergic nerves in inflammatory bowel disease could play a role in stimulating electrolyte transport (7). VIP induces release of NO (61), and these two secretagogue can act synergistically. Elevated levels of substance P (SP) and increased expression of its receptor in tissues were observed during acute inflammation in IBD (62). *In vitro* studies showed that SP antagonist could reduce the impact of inflammation in mouse model. Mast cells are intimately involved with sensory nerves in the intestinal mucosa and may be involved in neurogenic inflammation. The secretory effects of SP in small intestine was found to be mediated in part, by mast cells. Histamine is considered as a major signal substance in neuroimmune communication in the intestine. It is stored in the cytoplasmic granules of mast cells and is released upon degranulation. Histamine signals the enteric nervous system to call action a special neural program of intestinal behavior (patterned secretion from the intestinal mucosa in concert with powerful propulsive contractile activity of the musculature) to eliminate the threat of a foreign substance (63). Histamine was shown to be involved in stimulation of acid secretion, contributing damage to proximal GIT. Therefore, studies of mast cell-enteric nerve interactions and their alterations during inflammatory states are of great importance in understanding the cognate immune response and the immunopathogenesis of shigellosis.

Research Design and Methods

Describe in detail the methods and procedures that will be used to accomplish the objectives and specific aims of the project. Discuss the alternative methods that are available and justify the use of the method proposed in the study. Justify the scientific validity of the methodological approach (biomedical, social, or environmental) as an investigation tool to achieve the specific aims. Discuss the limitations and difficulties of the proposed procedures and sufficiently justify the use of them. Discuss the

ethical issues related to biomedical and social research for employing special procedures, such as invasive procedures in sick children, use of isotopes or any other hazardous materials, or social questionnaires relating to individual privacy. Point out safety procedures to be observed for protection of individuals during any situations or materials that may be injurious to human health. The methodology section should be sufficiently descriptive to allow the reviewers to make valid and unambiguous assessment of the project. **(DO NOT EXCEED TEN PAGES, USE CONTINUATION SHEETS).**

I. STUDY POPULATION AND MATERIALS:

Patients attending the Clinical Research and Service Center (CRSC) of ICDDR,B will be screened for participation in the study according to the inclusion and exclusion criteria.

Inclusion criteria:

1. Children (4-10 yrs of age, n=30) of either sex and adults (18-45 yrs of age, n=30) male only.
2. H/O dysentric stool <72 hrs, confirmed *Shigella* infection by stool culture.
3. Controls of same age group with watery diarrhea <24 hrs; negative for *Shigella* infection by culture.
4. Age matched healthy controls, negative for any parasitic infestation by stool microscopy.
5. Informed consent by participants (adult patients and healthy subjects) and parents or legal guardians of children.

Exclusion criteria:

1. Extraintestinal infection
2. Severe malnutrition determined by <60% wt/age of NCHS (National Center for Health Science, USA) median.

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. Peripheral blood, stool and rectal biopsies will be obtained from these patients accordingly as shown in the table I and II and flow charts I and II (pages 34-36).

II. LOCALIZATION AND FUNCTIONAL RELEVANCE OF MAST CELLS, EOSINOPHILS AND NEUTROPHILS

The cytoplasmic granules of mast cells contain preformed chemicals such as serotonin, histamine, proteases, TNF-α etc and upon stimulation, are released by degranulation. After degranulation, mast cells secrete newly formed mediators such as PGD_2, LTE_4, IL-8 etc. Mast cell tryptase, a protein used for identifying mast cells, can be localized in intact cells or cells having undergone degranulation and nonactivated mast cells. We aimed to study mast cells by immunohistochemical staining using anti-mast cell tryptase (64), a protein used for identifying mast cells, which can be localized in intact cells or cells having undergone degranulation and non activated mast cells. Image analysis will be performed on immunohistochemically stained tissues to quantify positive cells in a total tissue area. The chloroacetal esterase staining method will be used for detecting granulated mast cells (by light microscopy) (65). Type of degranulation of mast cells and eosinophils may be evaluated by electron microscopy (54). Eosinophils and neutrophil polymorphs can be identified by hematoxylin and eosin staining of paraffin sections after

deparaffinization. These cells will be enumerated by using a stage micrometer and the total tissue area will be measured by image analysis. Eosinophils can also be identified by immunostaining using anti- major basic protein (MBP) as described below.

Brief Protocol: The rectal biopsies will be fixed in 10% buffered formalin, processed, embedded in paraffin, cut in sequential 3 μm sections and then will be used either for hematoxylin and eosin staining for histological grading or for immunohistochemical staining. Eosinophils and neutrophils will be enumerated from hematoxylin and eosin stained paraffin sections. Staining for mast cells will be done as previously described with slight modification (64). Deparaffinized sections will be digested with 0.1% trypsin in phosphate buffered saline (PBS) followed by washing and blocking of intrinsic peroxidase activity. Sections will be incubated with anti-human mast cell tryptase containing 1% normal goat serum for 60 minutes (min) in room temperature (RT) followed by washing and incubation with rabbit-anti-mouse antibodies for 30 min. After washing and incubation with peroxidase-anti-peroxidase (PAP) antibodies for 30 min in RT, substrate will be added to develop color.

For EM studies, biopsy will be fixed in 2.5% gluteraldehyde, post-fixed in osmium tetraoxide, and embedded in araldite. Ultrathin sections will be cut on LKB UM4 ultramicrotome (Bromma, Sweden) with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Philips EM201C electron icroscope (Eindhoven, The Netherlands).

III. CELL-SPECIFIC ORIGIN AND RELEASE OF INFLAMMATORY AND CHEMOTACTIC MEDIATORS

We aimed to study the localization of various protein and lipid mediators by immunohistochemical method in the rectal tissues and to identify the cellular source. Protein mediators (defensins, lactoferrin, major basic protein), chemokines / cytokines (eotaxin, stem cell factor) and enzymes (myeloperoxidase and superoxide dismutase) will be studied by immunohistochemistry using specific antibodies. For lipid mediator PGE_2, a product of enzymatic reaction of arachidonic acid, tissue expression of inducible form of prostaglandin H synthase (PGHS) a key enzyme, was selected to be studied. To identify the cellular source, phenotyping of cells (20) will be performed by double staining.

Brief Protocol: Staining for defensins (Human defensins 1-3 from neutrophils) will be done as described above for mast cells with minor modification using serially sectioned paraffin embedded tissues (64). Staining for myeloperoxidase, prostaglandin H synthase and major basic protein will be performed according to the published methods (59). Superoxide dismutase, lactoferrin, SCF

and eotaxin will be studied in the cryopreserved rectal tissues (snap frozen) by immunohistochemical method (59, 21) as follows: Frozen tissues embedded in OCT compound were sectioned in cryotome at 4 μm thickness. Sections will be fixed in 2% formalin in PBS (pH 7.4) for 15 min followed by washing in PBS and blocking of endogenous peroxidase activity by incubating in 3% hydrogen peroxide in PBS. After washing, sections will be reversibly permeabilized by incubating in 0.1% saponin in Earl's Balanced Salt solution (EBSS) for 5 min followed by incubation with 1% normal goat serum for 20 min. Antibodies (monoclonal or polyclonal) to specific markers will be diluted in EBSS containing 0.1% saponin at a concentration of 2-4 μg/ml and added to the sections and incubated for 18 hours at RT. After washing, biotinylated goat anti-mouse / rat / rabbit antibodies will be added to the sections for 30 min followed by treatment with avidin-biotin-horseradish-peroxidase complex for 30 min at 37° C. Lastly, after washing, substrate will be added for color development. All steps include 0.1% saponin in EBSS. Sections will be counterstained with hematoxylin and mounted in aqueous mounting media.

Eotaxin and SCF will be measured in stool and plasma by commercially available ELISA Kits (R&D Systems, Minneapolis, MN, USA).

IV. ENTERIC NEUROPEPTIDES

We aimed to localize the various neuropeptides functioning as secretagogues, having involvement in generation of NO, their cellular sources, and their involvement in the inflammatory changes that take place in the rectal mucosa during *Shigella* infection. The various neuropeptides (VIP, substance P, bradykinin), neurotransmitter (serotonin) and histamine, a mast cell mediator and a signal substance in neuroimmune system can be identified by immunohistochemical techniques using specific antibodies. The following method was used with some modifications (66).

Brief Protocol: Paraffin embedded tissue sections will be deparaffinized and microwave treatment will be given to the sections in citrate buffer (pH 5.3). Sections will be washed in 0.5% triton X-100 in PBS (pH 7.4). Sections will be blocked with 10% normal goat serum for 20 min and antibodies to specific neuropeptides (polyclonal antibodies, dilutions 1:100 to 1:1000) will be added and incubated for 24 hrs at 4° C. After washing in 0.5% triton X-100 in PBS, biotinylated goat anti-rabbit antibodies will be added (1:100) and incubated for 2 hours in RT. Sections will be quenched with 3% hydrogen peroxide in PBS for 30 min followed by washing and addition of avidin-biotin-horseradish-peroxidase complex for 1 hr at 37° C. Substrate is added after rinsing

for color development, counter-stained with hematoxylin and mounted with aqueous mounting media.

Concentrations of neuropeptides will be determined in stool and plasma by commercially available enzyme immunoassay kits (R&D Systems) and radioimmunoassay kits (Amersham Life Science, Buckinghamshire, UK).

V. NK CELL-MEDIATED CYTOTOXICITY INDUCED BY SECRETORY IgA

We aim to investigate (I) whether sIgA from stool of *Shigella* infected patients could mediate anti-*Shigella* ADCC when in contact with lymphocytes extracted from rectal biopsies of these patients at the acute stage of the disease. Similar investigations will be carried out with serum sIgA and NK cells from peripheral blood of these patients. (II) Whether addition and / or blocking of IFN-γ in the assay system has an influence in the ADCC activity of NK cells will also be studied.

Brief Protocol: s-IgA will be purified from serum and stool extracts (from days 1, 7, and 30 after admission) by published methods (62). Briefly, s-IgA will be isolated from serum by 50% ammonium-sulphate precipitation, followed by anion exchange chromatography on DEAE-Sephacel and gel filtration on a Sephacryl S-300 column. IgA will be separated by a mono Q column (Pharmacia LKB Biotechnology) with a gradient of 0-0.5 M Nacl. The IgA rich fractions will be freed of IgM and IgG impurities on anti-IgM and protein G HiTrap affinity columns (Pharmacia LKB Biotechnology). The IgA preparation will be concentrated to 3-5 mg/ml and purity will be assessed by sodium dodecyl sulphate ployacrylamide gel electrophoresis (SDS-PAGE).

NK cells from peripheral blood will be extracted and purified (67, 68). Peripheral blood will be fractionated over Ficoll-hypaque. The buffy coat will be harvested, washed and resuspended. Nonadherent lymphocytes, T and NK cells will be obtained after incubation in plastic tissue culture dishes followed by nylon wool column each for 1 hr at 37° C, that will remove most of monocytes. Purified NK cells will finally be obtained by negative selection using biotinylated anti-$CD3^+$. Similarly, NK cells from rectal biopsies will be extracted (69).

ADCC will be performed as follows: The infecting *Shigella* strain (10^4 bacteria) will be placed in conical tubes together with diluted sIgA antibodies (purified) and centrifuged at 1,300 g for 10 minutes at 4° C (37). Purified NK cells will then be added and the tubes will be centrifuged at 500 g for 5 min at 4° C. The experimental and control tubes (which contain bacteria but no cells) will be incubated for 2 hr at 37° C. After incubation, pellets will be vigorously resuspended

(without bacteria adherent to leukocytes as assessed by light microscopy) and diluted aliquots will be plated on agar plates. CFU will be counted after overnight incubation. The percentage of antibacterial activity will be expressed as an antibacterial index = 100-[100x(CFU of experimental tubes)/(CFU of control tubes without leukocytes)].

Recombinant IFN- γ will be added to the system before performing ADCC (as above) to study the effects of IFN- γ in NK activity. To examine whether neutralization of IFN-γ has any effect on NK cell activities, saturating concentrations of neutralizing anti-IFN- γ monoclonal antibodies will be added to the assay system (67).

Facilities Available

Describe the availability of physical facilities at the place where the study will be carried out. For clinical and laboratory-based studies, indicate the provision of hospital and other types of patient's care facilities and adequate laboratory support. Point out the laboratory facilities and major equipments that will be required for the study. For field studies, describe the field area including its size, population, and means of communications. **(TYPE WITHIN THE PROVIDED SPACE).**

Studies using immunohistochemical techniques, histology, radioimmunoassay and enzyme-linked immunosorbant assay will be carried out at ICDDR,B since the techniques have been standardized and the equipment needed for carrying out the experiments are available at ICDDR,B. However, for quantitative estimation of tissue expression of various markers, which is a major part of the research proposal, an automated image analysis system equipped with CCD video camera for image processing and analysis for quantitative microscopy is required. The facilities are available at the Division of Infectious Diseases, Huddinge University Hospital at the Karolinska Institutet. This part of the work will have to be carried out by the principal investigator at KI and for this purpose, stained slides will need to be taken.

The experimental know how such as collection of secretory IgA from stool, extraction of NK cells from biopsies has been set up at the Pasteur Institute, France in the lab of professor Philippe Sansonetti and techniques will be transferred to ICDDR,B and KI.

For electron microscopic studies, blocks of embedded tissues will be required to be sent to Christian Medical college, Vellore, India for further processing, sectioning, slide preparation, staining and analysis. Analysis will be done by Dr. Minnie Mathan.

Data Analysis

Describe plans for data analysis. Indicate whether data will be analyzed by the investigators themselves or by other professionals. Specify what statistical software packages will be used and if the study is blinded, when the code will be opened. For clinical trials, indicate if interim data analysis will be required to monitor further progress of the study. **(TYPE WITHIN THE PROVIDED SPACE).**

This is a descriptive type of study, therefore, using a mathematical formula for sample size calculation is not appropriate, as the primary outcome is not quantifiable. There will be three different groups of subjects (with shigellosis, with watery diarrhea and healthy controls). From each patient, samples will be collected on admission day, day-5 (day-4 and day-7 in adults), day-11, day-30 and day-60. Specific immune responses in patients will reach peak levels within days 7-11, and whether the responses will decrease or remain elevated can be assessed from day-30 and day-60 samples. Therefore, individual immune responses can be monitored within the same patient. In addition, immune responses in the group of patients with shigellosis will be compared to the watery diarrhea- patient group (children only) and healthy individuals.

In our present study, the sample size in each group is chosen based on the sample size selected for our previous and on going studies in shigellosis. In our previous study, a sample size of n=30, was sufficient for statistical differentiation of the humoral and cellular immune responses in patients with shigellosis compared to apparently healthy individuals. Therefore, in this study, the sample size for each group is chosen 30, which should be sufficient for the expected outcome of the study.

All data will be analyzed by: a) comparing data at different time points within each individual, ii) comparing data within groups. Statistical calculations will be performed using the JMP software (SAS Institute Inc., Carey, NC, USA) program.

Ethical Assurance for Protection of Human Rights

Describe in the space provided the justifications for conducting this research in human subjects. If the study needs observations on sick individuals, provide sufficient reasons for using them. Indicate how subject's rights are protected and if there is any benefit or risk to each subject of the study.

The ethical implications are outlined below.

1. For inclusion of patients and healthy subjects (controls), informed consents will be required according to the guide lines of the local ethical committee at ICDDR,B. For the children, the informed consent of the parents or guardians will be required.
2. Patients will receive clinical care and therapy free of charge. The study will not in any way interfere with the management and treatment of the children and adults. Patients may discontinue their participation in the study at any time point. This decision will not have any influence on the clinical management or therapy of the patients
3. The proposed study involves repeated sampling of blood, stool and rectal mucosa from patients. In case of healthy subjects, sampling will be done once. None of the procedures are harmful and none will result in permanent physical damage or injury. All samples from controls will be collected at one time point only, to monitor the constancy of the research parameters in the healthy children and adult population. Approximately 10 ml of venous blood (from median cubital vein) will be taken from adults (6 times, total volume of blood, 60 ml) and 5ml from children (5 times, total volume of blood- 25 ml). Age range of the children will be 2 to 10 years. Children two years old are expected to have a blood volume of over 400 ml, therefore drawing of 25 ml of blood over a span of 30 days will not be detrimental to the participating children. There may be a momentary pain and a very small chance of bruising at the site of insertion of the needles. To minimize the chance of infection, aseptic precautions will be taken and disposable, sterile syringes and needles will be used for drawing blood.
4. With a flexible sigmoidoscope seven tiny pieces of biopsies (about the size of mustard seeds, 2 mm across) will be obtained from the rectosigmoid area of adults. This instrument has a tube which will be passed through anus up to 10-12 cm and with a biopsy needle, pinches of rectal biopsies will be obtained. From pediatric patients and controls, three tiny pieces of biopsies (about the size of mustard seeds, 2 mm across) will be obtained from the rectum (10-12 cm). All sampling procedures will be performed by trained and well-experienced clinician in the procedures concerned. This procedure is safe and have been performed previously at ICDDR,B in conjunction with studies of immune responses in shigellosis. No serious side effects have been associated with sampling in previous studies and we do not expect any in the proposed studies. Patients and controls will be hospitalized during the time of sampling, if there is excessive bleeding or other complications following biopsy, patients can be promptly and appropriately managed.

Literature Cited

Identify all cited references to published literature in the text by number in parentheses. List all cited references sequentially as they appear in the text. For unpublished references, provide complete information in the text and do not include them in the list of Literature Cited. There is no page limit for this section, however exercise judgment in assessing the "standard" length.

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Dissemination and Use of Findings

Describe explicitly the plans for disseminating the accomplished results. Describe what type of publication is anticipated: working papers, internal (institutional) publication, international publications, international conferences and agencies, workshops etc. Mention if the project is linked to the Government of Bangladesh through a training program.

Research findings will be published in international journals to make the results available to all researchers in the relevant fields and will be presented in international conferences. Results obtained from this study may help in better understanding the role of protective immune mechanisms in shigellosis and the immunopathogenic processes involved in the development of disease. The study may provide information on better therapeutic interventions and better formulation of protective and efficacious vaccine against all *Shigella* infections.

Collaborative Arrangements

Describe briefly if this study involves any scientific, administrative, fiscal, or programmatic arrangements with other national or international organizations or individuals. Indicate the nature and extent of collaboration and include a letter of agreement between the applicant or his/her organization and the collaborating organization. (DO NOT EXCEED ONE PAGE)

The proposed study is based on the collaboration between Acting professor, Dr Jan Andersson, Head of the division of Infectious Diseases, Huddinge University Hospital, Karolinska Institutet, Sweden and the PI at ICDDR,B.

ICDDR,B

Patient recruitment, sample collection and processing and most of the experiments will be performed at ICDDR,B. Some of the techniques have been developed at Huddinge Hospital in collaboration with above party and have been transferred to and standardized at ICDDR,B.

Karolinska Institutet

A major equipment, the image analyzer, is not available at ICDDR,B but is available at the Division of Infectious Diseases, Huddinge University Hospital. The use of this analyzer will be required for quantitative determination of immunostaining in tissues obtained from patients which involves a substantial amount of work. Therefore, this part of the study will be carried out by the PI (ICDDR,B) at KI. In addition, two techniques developed at the Pasteur Institute will be learned by the PI and later transferred to KI and ICDDR,B by the PI.

Biography of the Investigators

Give biographical data in the following table for key personnel including the Principal Investigator. Use a photocopy of this page for each investigator.

Name: Rubhana Raqib **Position** Assistant Scientist, Immunology, LSD,

Date of Birth: 19 October, 1961 ICDDR,B, Dhaka, Bangladesh

Academic Qualifications

Institution and Location	Degree	Year	Field of Study
Karolinska Institutet, Sweden	PhD	1995	Immunology
Dhaka University	M.Sc.	1988	Biochemistry
Dhaka University	B.Sc.	1985	Biochemistry

Research and Professional Experience

Concluding with the present position, list, in chronological order, previous positions held, experience, and honours. Indicate current membership on any professional societies or public committees. List, in, chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. **(DO NOT EXCEED TWO PAGES, USE CONTINUATION SHEETS).**

1. For masters degree, research acitivities involved extraction, purification and study of the immunogenic properties of outermembrane proteins from *Shigella dysenteriae* type 1 and *Shigella flexneri* strains using immunoelectrophoresis, SDS-PAGE and Western blot.

2. For phD. dissertation, research activities were focussed on the study of the pathogenic mechanisms and immune responses in adult patients with shigellosis. Samples such as plasma, peripharal blood mononuclear cells, stools and rectal biopsies were collected from patients and healthy subjects and were analysed for cytokines (protein and mRNA), cytokine receptors and phenotypes of various cells and activation markers. The techniques used were ELISPOT, ELISA, immunohistochemistry, quantitative analysis of video microscopic images and *in situ* hybridization.

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

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DETAILED BUDGET

Project Title: Studies on shigellosis: B. Further studies of immunoprotective and immunopathogenic mechanisms in shigellosis.

Name of PIs: (1) Rubhana Raqib, Laboratory Sciences Division, International Centre for Diarrhoeal Diseases Research, Bangladesh
(2) Jan Andersson, Division of Infectious Diseases, Huddinge University Hospital, Karolinska Institutet.

Funding Source: SIDA/SAREC
Total Amount: 196,194 US$

Starting Date: January, 1999
Closing Date: December, 2001

BUDGET FOR ICDDR,B

Yearly Budget, 1 US$=8 SEK

Salary Support				**1st Year**		**2nd Year**		**3rd Year**	
Personnel	Position	Effort%	Salary	**US$**	SEK	**US$**	SEK	**US$**	SEK
1 Rubhana Raqib	NOB	27	11592	4,649	37,192	4,882	39,056	5,126	41,008
2 Nurul Haq Alam	Sr. Medi.Off.	5	16000	800	6,400	840	6,720	882	7,056
3 Research Officer	GS-5	100	4164	4,200	33,600	4,410	35,280	4,631	37,048
4 Lab Attendent	GS-1	100	170	2,040	16,320	2,142	17,136	2,250	18,000
5 Medical Officer	NO-A	25	6616	1,654	13,232	1,737	13,896	1,824	14,592
Sub Total				**13,343**	106,744	**14,011**	112,088	**14,713**	117,704
6 Consultant	Research Histopathologist		7,440	1,000	8,000	800	6,400	800	6,400
7 Local Travel				300	2,400	300	2,400	300	2,400
8 International Travel				1,400	11,200	1,400	11,200	1,400	11,200
Sub Total				**2,700**	21,600	**2,500**	20,000	**2,500**	20,000
Supplies and Materials									
9 Immunological Assays:									
Antibodies for immunostaining				1,000	8,000	1,500	12,000	1,200	9,600
Radioimmunoassay kits for neuropeptides				1,000	8,000	1,200	9,600	1,600	12,800
Assay kits for inflammatory markers				1,200	9,600	1,000	8,000	1,200	9,600
10 Laboratory Supplies:									
Plasticware, glassware, office supplies				800	6,400	500	4,000	600	4,800
Chemicals and media				471	3,768	555	4,440	500	4,000
Sub Total				**4,471**	35,768	**4,755**	38,040	**5,100**	40,800

	1st Year		2nd Year		3rd Year	
	US$	SEK	**US$**	SEK	**US$**	SEK
Interdepartmental Services						
14 Pathological Tests	800	6,400	600	4,800	600	4,800
15 Microbiological tests	800	6,400	600	4,800	600	4,800
16 Biochemistry Tests	500	4,000	600	4,800	600	4,800
17 Patient Study						
Study ward costs	3,000	24,000	2,500	20,000	1,250	10,000
Outpatient costs (10 US$)	400	3,200	300	2,400	200	1,600
Endoscopy (15 US$/endoscopy)	2,000	16,000	1,800	14,400	1,000	8,000
Traveller's clinic and utility	300	2,400	150	1,200	100	800
18 Wage loss for follow up & controls	800	6,400	700	5,600	600	4,800
19 Medicine	500	4,000	386	3,088	415	3,320
Sub Total	**9,100**	72,800	**7,636**	61,088	**5,365**	42,920
20 Capital Expenditure	**2,000**	16,000	**1,000**	8,000	**0**	0
Subtotal costs at ICDDR,B	**31,614**	252,912	**29,902**	239,216	**27,678**	221,424
Overhead, 10%	3,091	24,728	2,915	23,320	2,913	23,304
TOTAL COST AT ICDDR,B	**34,705**	277,640	**32,817**	262,536	**30,591**	244,728

Swedish Project Leader: Jan Andersson, Head, Division of Infectious Diseases, Karolinska Institutet, Huddinge University Hospital, Stockholm, Sweden

BUDGET FOR KAROLINSKA INSTITUTET

			Yearly Budget, 1 US$=8 SEK					
			1st Year		**2nd Year**		**3rd Year**	
Salary Support								
Personnel	Effort%	Salary	**US$**	SEK	**US$**	SEK	**US$**	SEK
1 Biomedical assistant	25		7,280	58,240	7,280	58,240	7,280	58,240
2 Laboratory attendent	15		3,560	28,480	3,560	28,480	3,560	28,480
Sub Total			10,840	86,720	10,840	86,720	10,840	86,720
Overhead 13.64%			1,479	11,829	1,479	11,829	1,479	11,829
Sub Total			**12,319**	98,549	**12,319**	98,549	**12,319**	98,549
International Travel								
3 Guest researcher to ICDDR,B			1,210	9,680	1,210	9,680	1,210	9,680
4 Guest researcher from ICDDR,B			1,380	11,040	1,380	11,040	1,380	11,040
5 Visit to ICDDR,B			1,500	12,000	1,500	12,000	1,500	12,000
Sub Total			**4,090**	32,720	**4,090**	32,720	**4,090**	32,720
Supplies and Materials								
6 Laboratory supplies for immunological studies Reagents to be used for immunohistochemistry, Immunofluorescent flow cytometry and computerized image assessments			4,000	32,000	4,000	32,000	4,500	36,000
7 Reagents for fixation, permeabilisation, cryosectioning, Enumeration of cells, quantification of specific immune reactivity (in situ hybridisation) PCR			4,000	32,000	4,000	32,000	4,400	35,200
8 Plastic disposals, glass ware, photo material, tissue culture material			1,500	12,000	1,500	12,000	1,050	8,400
Sub Total			9,500	76,000	9,500	76,000	9,950	79,600
Overhead 13.64%			1,296	10,366	1,296	10,366	1,357	10,857
Sub Total			**10,796**	86,366	**10,796**	86,366	**11,307**	90,457

	1st Year		2nd Year		3rd Year	
	US$	SEK	US$	SEK	US$	SEK
9 Purchase and shipment of reagents, bacterial cultures, biological material to ICDDR,B	3,491	27,928	2,300	18,400	2,900	23,200
10 Printing and Publication	1000	8,000	950	7,600	900	7,200
Sub Total	4,491	35,928	3,250	26,000	3,800	30,400
Overhead 13.64%	613	4,901	443	3,546	518	4,147
Sub Total	**5,104**	40,829	**3,693**	29,546	**4,318**	34,547
11 Capital Expenditure, small equipment	1,500	12,000	1,000	8,000	0	0
Overhead 13.64%	205	1,637	136	1,091	0	0
Sub Total	1,705	13,637	1,136	9,091	0	0
TOTAL COST AT KAROLINSKA INSTITUTET	**34,013**	272,100	**32,034**	256,273	**32,034**	256,273
Yearly Costs at ICDDR,B	34,705	277,640	32,817	262,536	30,591	244,728
Yearly Costs at KI	34,013	272,104	32,034	256,272	32,034	256,272
SUB-TOTAL	**68,718**	549,744	**64,851**	518,808	**62,625**	501,000

GRAND TOTAL FOR THREE YEARS **196,194 US$**
1,569,552 SEK

Budget Justifications

Please provide one page statement justifying the budgeted amount for each major item. Justify use of man power, major equipment, and laboratory services.

1. Full salaries of the research officer, lab attendant and 27% salary of the PI (ICDDR,B) is requested. Consultant's salary for histopathology associated work is requested. Expenses of medical officer's and consultant's salaries will be borne by two other projects (PIs. Firdausi. Qadri and Dilara Islam).
2. Charges for patients include costs for study ward, endoscopy, medicine and hospital supplies.
3. Costs for paying patients and controls for follow-up and wage loss.
4. Interdepartmental charges include costs of microbiological, pathological, histopathological and biochemical tests.
5. Costs for supplies and reagents required for various immunological assays, disposable plasticware, glassware.
6. Expenses for publication has been requested.
7. International travel is required per year for carrying out work at KI in Sweden, for analysis and group discussions of results and writing up of manuscripts at KI and at ICDDR,B.
8. Local travel is required for visiting patients / guardians to remind and encourage them to complete their follow-up visits.
9. Capital expenditure includes cost of a laser printer and a scanner. The computer that is being used in the PI's lab does not have a printer and it is essential for taking out print outs from image analysis work, for analyzing data, and for manuscripts. Costs for disposable knives , knife holder, and an analyzer are requested.

Continuation Sheet

Sequence of tasks within time frame:

	Year 1	Year 2	Year 3
Initial set up			
Patient enrolment with follow-up			
Laboratory assays			
Data analysis and writing up			

Itemized specific tasks for each listed investigator:

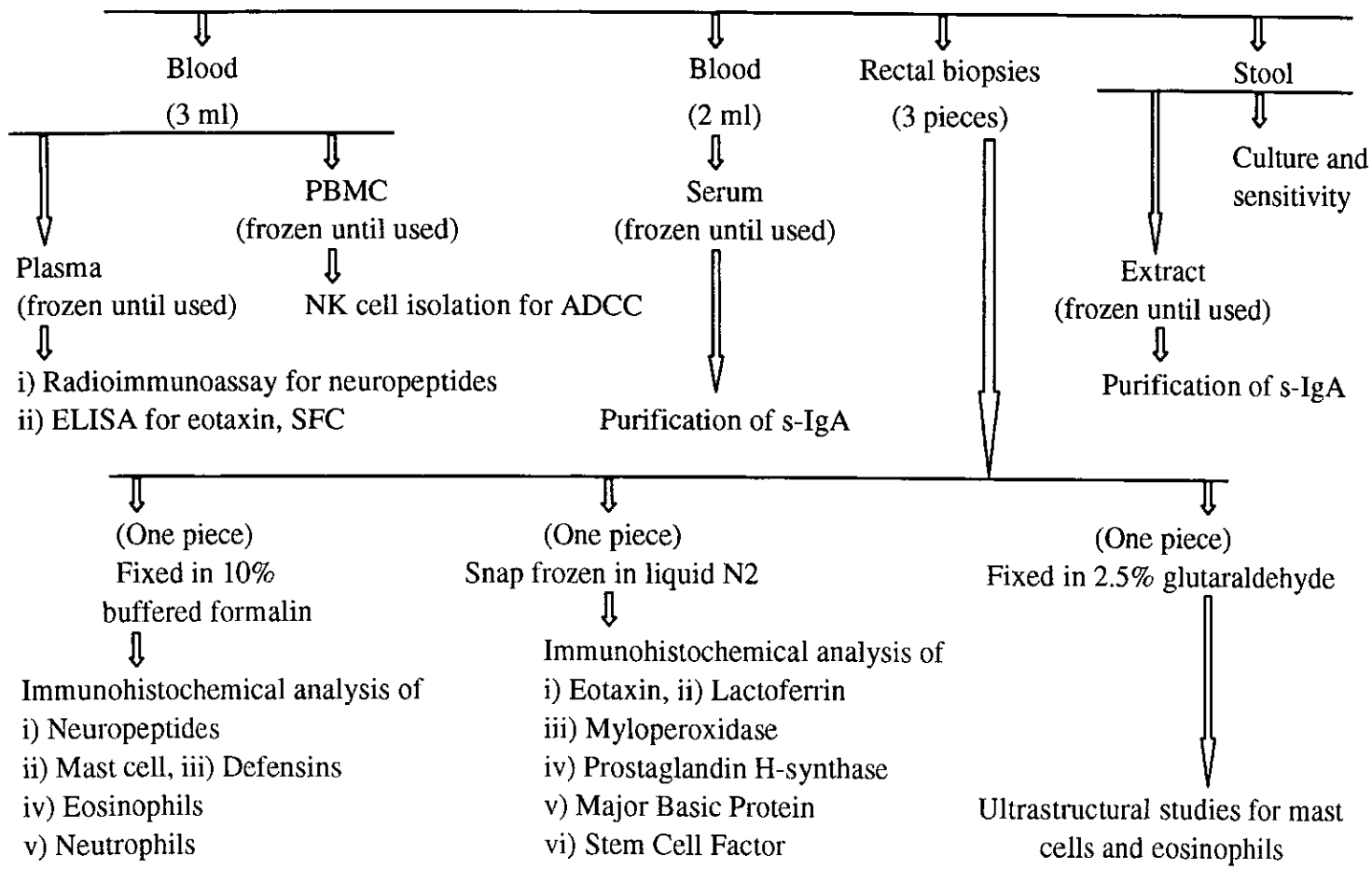
1. Rubhana Raqib (PI)
   Will standardize methods and supervise work in the lab at ICDDR,B. Coordinate specimen collection from patients. Carry out image analysis related work at KI. Data compilation and analysis. Writing up of results obtained.
2. Jan Andersson (PI)
   Scientific and academic feedback. Technical support and supervision for image analysis related work.
3. Firdausi Qadri
   Supervise work in the lab, scientific and academic feedback.
4. Minnie M. Mathan
   Supervise histopathological and electron microscopy related work Scientific feedback.
5. Dilara Islam
   Scientific and academic feedback.
6. Nurul Haque Alam
   Perform sigmoidoscopy
7. Medical officer
   Patient enrolment. Clinical management. Assistance in sigmoidoscopy.
8. Research Officer
   Carry out experiments specific for the protocol involving immunological and microbiological techniques.
9. Lab Attendent
   Motivate patients and healthy controls for enrolment. Sample collection from study population and processing for storage. Labeling and storing. Visiting patients at their respective homes to encourage patients to come to the Center for follow-up visits.

APPENDIX - I

Flow Chart: CHILDREN (AGE 2-10 YRS)

Sample collection and sequence of work

Patients infected with *Shigella*: Venous blood (from median cubital vein, 5 times), stool, urine (5 times) and rectal biopsy (by flexible sigmoidoscopy, 2 times)
Healthy control: Blood, stool and urine will be collected only once

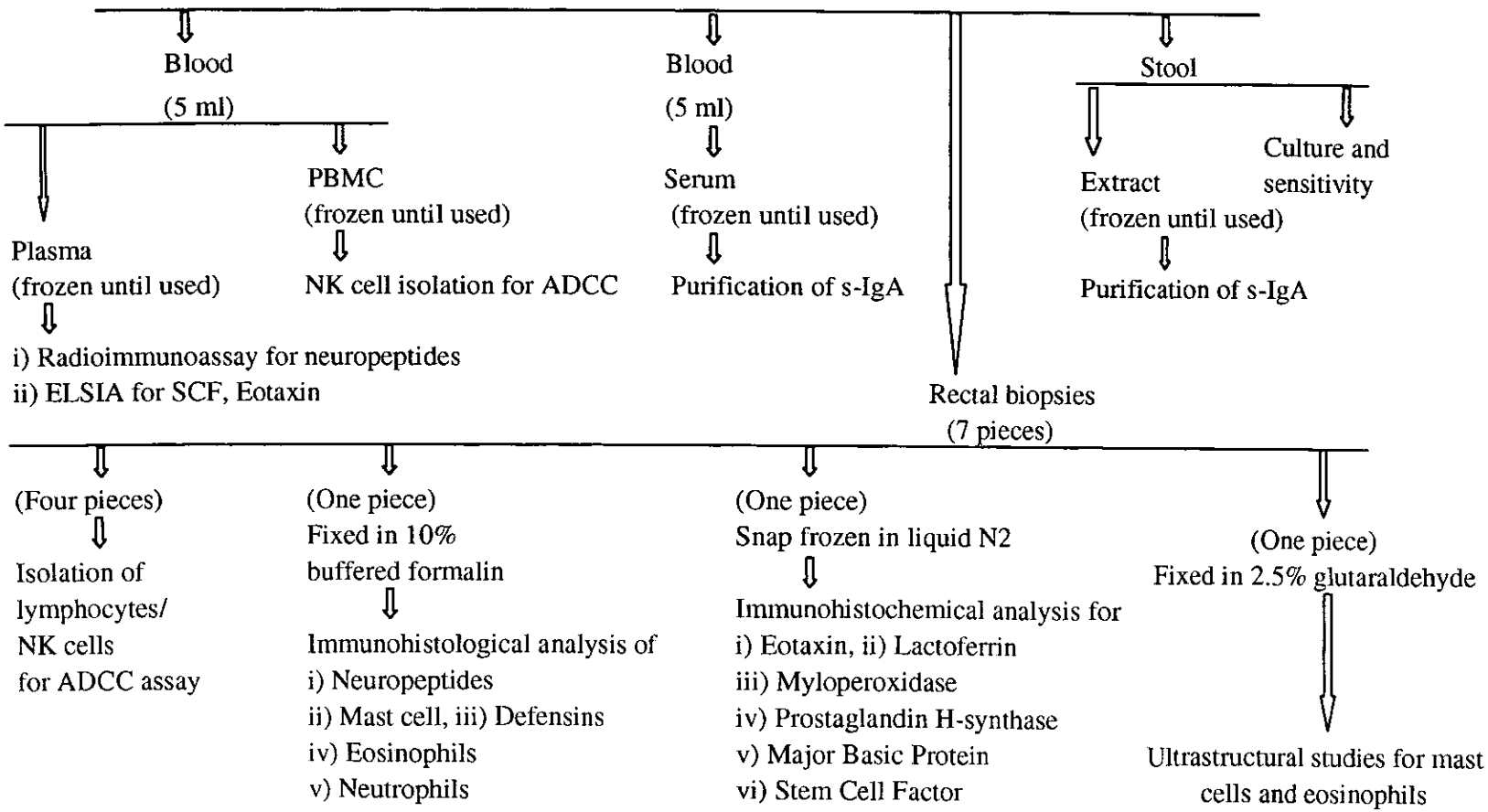


APPENDIX- II

Flow Chart: ADULTS (AGE18-55 YRS)

Sample collection and sequence of work

Patients infected with *Shigella* : Venous blood (from median cubital vein, 6 times), stool, urine (6 times) and rectal biopsy (by flexible sigmoidoscopy, 3 times)
Healthy controls: Blood, stool, urine and biopsy will be collected only once



APPENDIX III

Table I. Collection of samples from pediatric patients* and healthy controls**

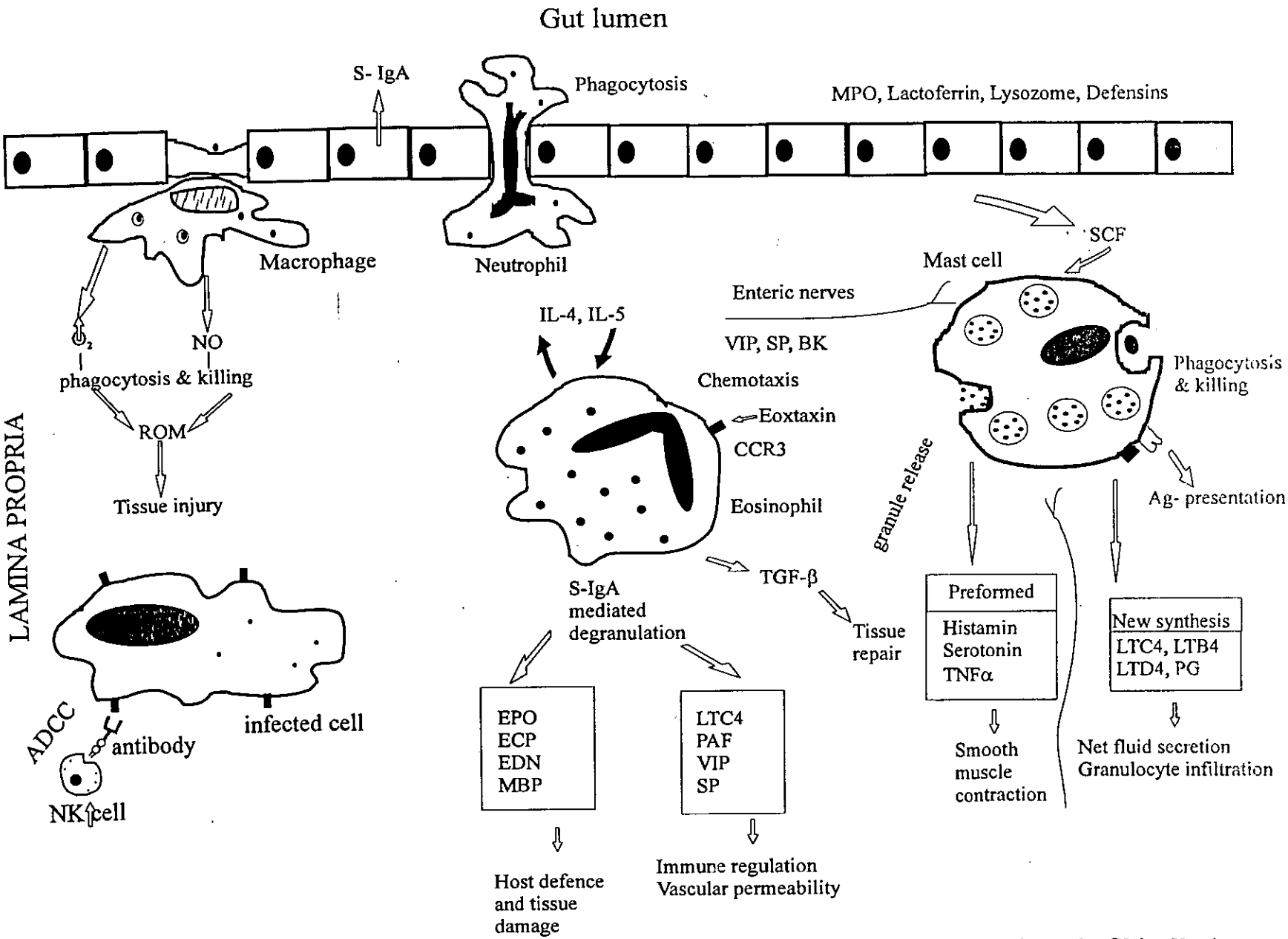
Days (D) from the enrollment	Samples collected from each individual		
	blood	biopsy	stool
D-0	+	+	+
D-5	+	–	+
D-11	+	–	+
D-30	+	+	+
D-60	+	–	+
Control**	+	+	+

Pediatric patients* 2-10 yrs old, include patients infected with *Shigella*.
Controls** are matched healthy subjects.

Table II Collection of samples from adult patients and controls†

Days from the enrollment	Samples collected from each individual		
	blood	biopsy	stool
D-0	+	+	+
D-4	+	–	+
D-7	+	–	+
D-11	+	+	+
D-30	+	+	+
D-60	+	–	+
Control	+	+	+

Adult patients infected with *Shigella* -18 to 55 yrs. Controls† - Matched healthy controls only.



Schematic diagram of the Immunoprotective and Immunopathogenic Mechanisms in Shigellosis

APPENDIX V

International Centre for Diarrhoeal Disease Research, Bangladesh

Consent Form

Title of the Research Project: Further Studies of Immunoprotective and Immunopathogenic Mechanisms in Shigellosis.

Principal Investigator: **Rubhana Raqib**

Before recruiting into the study, the study subject must be informed about the objectives, procedures, and potential benefits and risks involved in the study. Details of all procedures must be provided including their risks, utility, duration, frequencies, and severity. All questions of the subject must be answered to his/ her satisfaction, indicating that the participation is purely voluntary. For children, consents must be obtained from their parents or legal guardians. The subject must indicate his/ her acceptance of participation by signing or thumb printing on this form.

CONSENT FORM FOR PEDIATRIC PATIENTS

"SHIGELLA"

Your child has bloody diarrhea which is caused by a germ called *Shigella*. Very little is known about the immunopathogenic mechanisms of this disease. In order to understand more about this illness and how to increase immunity against this disease, we are conducting a study. Results obtained from this study may help to understand protective immunity and provide information on better therapeutic interventions and management of this disease. For this purpose, we would request for your permission to enroll your child in this study.

Your child will be examined thoroughly and will receive the necessary treatment of this hospital for the illness. For the purpose of the study, your child will be required to stay in the hospital for 4 days and will be discharged on the 5th day. You will be able to stay with your child in the hospital. The hospital costs and therapy will be free of charge. We will draw 5.0 ml of blood (about one tea spoon full) from a vein on his/her forearm (median cubital vein), and will also collect his/her stool and urine samples five times- on the day of admission and 5, 11, 30 and 60 days later. You will be requested to bring your child for follow-up visits on days 11, 30 and 60 after discharge. Other than momentary pain and a very small chance of bruising at the site of insertion of the needles, drawing blood will not cause any harm to your child. To minimize the chance of infection, we will take aseptic precautions and use disposable, sterile syringes and needles for drawing blood.

Your child will be examined by a narrow, flexible tube-like instrument called flexible sigmoidoscope which will be introduced into the rectum (10-12 cm from anus) to examine the rectosigmoid area. Your child will be sedated with a medicine Promethazine orally so as not to feel any discomfort or pain during this examination. Only three tiny pieces of biopsies (about the size of a mustard seed, 2 mm across) will be obtained from the rectum. Biopsies will be obtained from the child twice, on the day of admission and 30 days later. Bleeding, though seldom and usually minor, may occur from the site of biopsy and there is a rare possibility of perforation during endoscopy. The chances of rectal perforation is very small since the procedure will be performed by a trained and well-experienced clinician, one of the investigators of this study. To prevent such complications, every possible precaution will be taken, and the child will be kept under observation for 3-4 hours after the sigmoidoscopic

examination for any untoward effect if it were to occur. Necessary treatment will be provided. Appropriate measures will be taken to avoid introduction of any infection during the procedure.

There will be no direct benefit to the child as a result of participation in this study. However, the results of this study will help us to understand better about this disease and will thus benefit the society. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit.

It is completely your decision whether your child should be enrolled in this study or not. After initial participation in the study, you have the right to withdraw your child from the study at any time point at your will. Your child will receive the standard good care and treatment of this hospital whether he/she is enrolled in the study or not. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit. All information/data of this study will be kept confidential and will be provided to you upon your request. If you agree to let your child participate in this study, please sign or put your left thumb imprint at the specified space below.

Thank you for your co-operation.

_____	_____
Signature / left thumb impression of the guardian	Date
_____	_____
Signature of the investigator	Date
_____	_____
Signature of a witness	Date

CONSENT FORM FOR CHILDREN "HEALTHY CONTROL"

Children suffering from bloody diarrhea/dysentery caused by the pathogen called "*Shigella*" often have severe complications. Very little is known about the immunopathogenic mechanisms of this disease. In order to understand more about this illness and how to increase immunity against this disease, we are conducting a study. Healthy subjects are needed to be examined in order to compare the findings in health to that in *Shigella* infection. Results obtained from this study may help to understand protective immunity and provide information on better therapeutic interventions and management of this disease. For this purpose, we would like your permission to enroll your child in this study.

For this purpose, your child will be examined by a qualified physician for a routine check up. For our study, we will collect single samples of blood, urine and stool from your child. Approximately 5 ml (one tea-spoon full) of blood will be taken from a vein on your child's forearm (median cubital vein). Other than momentary pain and a very small chance of bruising at the site of insertion of the needles,

drawing blood will not cause any harm to your child. We will take aseptic precautions and use disposable, sterile syringes and needles for drawing blood to minimize the chance of infection.

Your child will be examined by a narrow, flexible tube-like instrument called flexible sigmoidoscope which will be introduced into the rectum (10-12 cm from anus) to examine the rectosigmoid area. Your child will be sedated with a medicine Promethazine orally so as not to feel any pain during this examination. Only three tiny pieces of biopsies (about the size of a mustard seed, 2 mm across) will be obtained from the rectum. Biopsies will be obtained from the child once. Bleeding, though seldom and usually minor, may occur from the site of biopsy and there is a rare possibility of perforation during endoscopy. The chances of rectal perforation is very small since the procedure will be performed by a trained and well-experienced clinician, one of the investigators of this study. To prevent such complications, every possible precaution will be taken, and the child will be kept under observation for 3-4 hours after the sigmoidoscopic examination for any untoward effect if it were to occur. Necessary treatment will be provided. Appropriate measures will be taken to avoid introduction of any infection during the procedure.

Your child will not be directly benefited by participating in this study, however, the results of this study will help us to better understand the normal immune status of children living in areas endemic for diarrheal diseases and will thus benefit the society. We will compensate for any wage loss and travel costs that you may incur while your child participates in this study.

It is your decision to let your child participate in this study. All information/data of this study will be kept confidential and will be provided to you upon your request. If you agree, please sign or put your left thumb imprint at the specified space below. Thank you for your co-operation.

_____	_____
Signature / left thumb impression of the guardian	Date
_____	_____
Signature of the investigator	Date
_____	_____
Signature of the witness	Date

CONSENT FORM FOR ADULT PATIENTS "**SHIGELLA**"

You have bloody diarrhoea which is caused by a pathogen called *Shigella.* Very little is known about the immunopathogenic mechanisms of this disease. In order to understand more about this illness and how to increase immunity against this disease, we are conducting a study. Results obtained from this study may help to understand protective immunity and provide information on better therapeutic interventions and management of this disease. For this purpose, we request you to participate in this study. Your participation in this study may help to save lives in future due to this bloody-diarrhea.

During the study period, you will be examined thoroughly and you will receive necessary treatment of this hospital. For this study, you will be required to stay in the hospital for 3 days and will be discharged on the 4th day. You will be requested to come for follow-up visits on days 7, 11, 30 and 60 after discharge. About 10 ml of blood (two tea-spoon full) will be collected from a vein on your forearm (median cubital vein) on the day of admission and 4, 7, 11, 30 and 60 days after that (6 times). Other than momentary pain and a very small chance of bruising at the site of insertion of the needles, drawing blood will not cause any harm to you. To minimize the chance of infection, we will take aseptic precautions and use disposable, sterile syringes and needles for drawing blood. Stool and urine samples will also be collected from you on these above mentioned days.

You will be examined by a narrow, flexible tube-like instrument called flexible sigmoidoscope which will be passed through your anus (10-12 cm from anus) to examine the rectosigmoid area of the large intestine. A lubricating jelly (bacteriostatic) will be applied locally at the anal orifice so that you will only feel a slight discomfort during this examination. Seven tiny pieces of biopsies (about the size of a mustard seed, 2 mm across) will be obtained from your rectum. Biopsies will be obtained from you three times, on the day of admission, 11 and 30 days later. Bleeding, though seldom and usually minor, may occur from the site of biopsy and there is a rare possibility of perforation during endoscopy. The chances of rectal perforation is very small since the procedure will be performed by a trained and well-experienced clinician, one of the investigators of this study. To prevent such complications, every possible precaution will be taken, and you will be kept under observation for 3-4 hours after the sigmoidoscopic examination for any untoward effect if it were to occur. Necessary treatment will be provided. Appropriate measures will be taken to avoid introduction of any infection during the procedure.

There will be no direct benefit to you as a result of participation in this study. However, the results of this study will help us to understand better about this disease and will thus benefit the society. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit.

It is your decision to participate in this study. Even after initial participation in the study, you have the right to withdraw yourself at any time at your will. Even if you do not agree to participate or want to withdraw from the study, you will receive the standard treatment of this hospital. All information/data of this study will be kept confidential and will be provided to you upon your request. If you agree to participate in this study, please sign or put your left thumb imprint at the specified space below.

Thank you for your co-operation.

_____ _____

Signature / left thumb impression of the patient — Date

_____ _____

Signature of the investigator — Date

_____ _____

CONSENT FORM FOR ADULTS
"HEALTHY CONTROL"

Bloody diarrhea due to pathogen "*Shigella*" may cause severe complications. Very little is known about the immunopathogenic mechanisms of this disease. In order to understand more about this illness and development of immune responses of the host against it, we are conducting a study. Results obtained from this study may help to understand protective immunity and provide information on better therapeutic interventions and management of this disease. Healthy subjects are needed to be examined in order to compare the findings in health to that in *Shigella* infection. For this purpose we request you to participate in this study. Your participation in this study may help to save many lives in future due to this bloody-diarrhea.

For this purpose, you will be examined by a qualified physician for a routine check up. For our study, we will collect single samples of blood, stool, urine and rectal biopsies from you. Approximately 10 ml (two tea-spoon full) of venous blood will be taken from a vein on your forearm (median cubital vein). You will be examined by a narrow, flexible tube-like instrument called flexible sigmoidoscope which will be introduced into the rectum (10-12 cm from anus) to examine the rectosigmoid area of the large intestine. A lubricating jelly (bacteriostatic) will be applied locally at the anal orifice so that you will feel only little discomfort during this examination. Seven tiny pieces of biopsies (about the size of mustard seed, 2 mm across) will be obtained from your rectum. Bleeding, though seldom and usually minor, may occur from the site of biopsy and there is a rare possibility of perforation during endoscopy. The chances of rectal perforation is very small since the procedure will be performed by a trained and well-experienced clinician, one of the investigators of this study. To prevent such complications, every possible precaution will be taken, and you will be kept under observation for 3-4 hours after the sigmoidoscopic examination for any untoward effect if it were to occur. Necessary treatment will be provided. Appropriate measures will be taken to avoid introduction of any infection during the procedure. This procedure will not benefit you in any way but it will help us to better understand the normal immune status of an adult living in areas endemic for diarrheal diseases and will help others in future.

It is your decision to participate in this study. All information/data of this study will be kept confidential and will be provided to you upon your request. We will compensate for any wage loss and travel costs that you may incur while participating in this study. If you agree to participate in this study, please sign or put your left thumb imprint at the specified space below.

Thank you for your co-operation.

Signature/left thumb impression of the control

Date

Signature of the investigator

Date

Signature of the witness

Date