THE INFLUENCE OF INNATE IMMUNE MECHANISMS ON T CELLS STIMULATION IN SHIGELLOSIS

DILARA RAHMAN
AND OTHERS

1998-031

Attachment 1

Date: 12.11.98.

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

Principal Investigator: Dilara Islam & Birger Christensson

Application No. 98-031

Title of Study: The influence of innate immune mechanisms on T cells stimulation in shigellosis.

Trainee Investigator (if any): X

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

Project Status:

[✓] 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 — Yes (No)
   - (e) Benefits to be derived — (Yes) No
   - (f) Right to refuse to participate or to withdraw from study — (Yes) No
   - (g) Confidential handling of data — (Yes) No
   - (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — Yes (No)

5. Will Signed Consent Form be Required:
   - (a) From subjects — (Yes) No
   - (b) From 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:
   - No — Umbrella proposal - Initially submit an with overview (all other requirements will be submitted with individual studies
   - Yes — Protocol (Required)
   - Yes — Abstract Summary (Required)
   - Yes — Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw) (Required
   - Yes — Informed consent form for subjects
   - No — Informed consent form for parent or guardian
   - Yes — Procedure for maintaining confidentiality
   - ___ — Questionnaire or interview schedule*

   * If the final instrument is not completed prior to review, the following information should be included in the abstract summary
   1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy
   2. 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.

Dilara Islam
Principal Investigator

Trainee

Principal Investigator: Last, first, middle: Islam, Dilara & Christensson, Birger

International Centre for Diarrhoeal Disease Research, Bangladesh

RESEARCH PROTOCOL

FOR OFFICE USE ONLY
Protocol No: 98 - 031 Date:
RRC Approval: Yes/ No Date:
ERC Approval: Yes/No Date:

1. Title of Project (Do not exceed 60 characters including spaces and punctuations)
STUDIES ON SHIGELLOSIS.
a) The influence of innate immune mechanisms on T cells stimulation in shigellosis.

2a. Name of the Principal Investigator(s) (Last, Middle, First). Qualifications	2b. Position / Title	2c.
Islam, Dilara (ICDDR,B)	Asst. Scientist	PhD
Christensson, Birger (Karolinska Institute (KI), Sweden)	Assoc. Prof.	MD, PhD

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

4. Contact Address of the Principal Investigator
4a. Office Location:
Immunology, LSD

4b. Fax No:
4c. E-mail: dilara@icddrb.org
4d. Phone / Ext: 2404

5. Use of Human Subjects: Yes [x] No [ ]

5a. Use of Live Animal: Yes [ ] No [x]

5b. If Yes, Specify Animal Species

6. Dates of Proposed Period of Support (Day, Month, Year - DD/MM/YY)
Three years (Jan'99-Dec'2001)

7. Cost Required for the Budget Period
7a. *1st Year ($):* 66,049 *2nd Year ($):* 71,090 *3rd Year ($:* 70,205
7b. Direct Cost ($) 185,947 Total Cost ($) 207,344

8. Approval of the Project by the Division Director of the Applicant

The above-mentioned project has been discussed and reviewed at the Division level as well by the external reviewers.
The protocol has been revised according to the reviewer's comments and is approved

Prof. V. I. Mathan, MD, PhD, FRCP
Name of the Division Director

Signature

24/10/98
Date of Approval

9. Certification by the Principal Investigator
I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

10. Signature of PI
Dilara Islam.

Date: 27.10.98.

Table of Contents

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: Dilara Islam (LSD, ICDDR,B)
Birger Christensson (KI, Sweden)

Project Name: **STUDIES ON SHIGELLOSIS.**
a) The influence of innate immune mechanisms on T cells stimulation in shigellosis.

Total Budget : 207,347 US$. Beginning Date: Jan'1999 Ending Date: Dec'2001

Shigellosis is one of the major causes of morbidity and mortality in many developing countries. Shigellosis is an invasive infection of the colonic epithelium characterized by the formation of abscess and ulcerations of the mucosa. Inflammation is initiated by invasion of epithelial cells by *Shigella*, and is characterized by strong mucosal infiltration by polymorphonuclear neutrophils preceding infection and destruction of epithelial cells. The current study will be undertaken to understand the interplay between immune effector cells and molecules of the innate and adaptive systems in shigellosis. Antibacterial peptides and proteins are an integral part of the epithelial defense barrier that makes up immediate protection against bacterial invasion. In humans, α-defensins, β-defensins and LL-37 are the primary antibacterial proteins and peptide. It has been suggested that human colon epithelial cells can express and secret several C-X-C and C-C chemokines that can chemoattract specific populations of leukocytes, including neutrophils, monocytes, cosinophils, and sub-sets of T lymphocytes. These existing findings indicate that both antibacterial proteins/peptides and chemokines may play an important role in host defense mechanisms in shigellosis. Besides the destruction of potential pathogens, components of the innate defense system have an instructive role for the highly specific lymphocytes of the adaptive immune system. It is well established that T cells play a central role in protection from infectious diseases. T cells use T cell receptor (TCR) heterodimers to specifically recognize antigens. The third hyper-variable region (CDR3) of TCR α and β chains is mainly involved in recognition of the antigen fragments associated with the MHC molecule. Thus, involvement of CDR3-β loop is expected in a conventional antigen-specific response. Therefore, in detail analysis of the involvement of TCR variable gene usage and CDR3-β loop region analysis will be carried out in this study as a part of adaptive immunity in shigellosis. Adult males a) with naturally acquired shigellosis (culture confirmed *S. dysentriae* 1 or *S. flexneri*), b) with naturally acquired acute watery (other than *Shigella*) diarrhea and age- and sex matched healthy subjects from the similar socio-economic background will be enrolled in this study. Blood and rectal biopsies will be collected from them.

It is envisaged that the findings of this study would help in better understanding of the role of innate immunity on adaptive immunity in shigellosis, and also may help to specify the "loop holes" of our defense mechanisms due to *Shigella* infection. Thus, the outcome of this study may indicate the strategies of prevention/efficient therapy against shigellosis.

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

Name	Professional Discipline/ Specialty	Role in the Project
1. Dilara islam, PhD	Clinical-Immunologist	Principle Investigator (LSD)
2. Birger Christensson, MD, PhD	Immuno-Pathologist	PI (KI, Sweden)
3. N. H. Alam, MBBS	Clinician/diarrheal diseases	Co-Investigator (CSD)
4. Minnie Mathan, MD, PhD	Histopathologist	Consultant (LSD)

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.

Our body defense- system comprises of two main compartment: innate and adaptive immunity. Although, innate immunity is not pathogen specific, its complex cascade of events may influence the adaptive immunity, which is primarily represented by T cells in diarrheal disease- shigellosis.

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).

Objective: To study the influence/effect of effector mechanisms of the innate immune system on adaptive immune system by analyzing the activity of antibacterial peptides and proteins on stimulation of T cells by *Shigella*-antigens (T cells are fundamental in initiating and maintaining an immune response), both *in vivo* and *in vitro*. As, antibacterial peptides and proteins are effector molecules in innate immunity and are the main mediators of the bacterial killing process. In addition, the role of chemokines (small proinflammatory proteins) will be assessed at the site of inflammation in shigellosis. Three group of subjects will be included in the study: adult patients with shigellosis, adult patients with acute watery diarrhea and adult healthy individuals (controls), to monitor the specificity of the mentioned parameters in patients with shigellosis.

Specific objectives:

- the functional-status of innate immunity will be assessed by: measuring the antimicrobial peptide- LL-37, α-defensins and β-defensins in rectal biopsies from: patients with shigellosis, with watery diarrhea and controls. Both the tissue distribution and the m-RNA levels of these peptide/proteins will be measured. The expression of C-X-C and C-C chemokine families will also be assessed by measuring the m-RNA levels of these chemokines in rectal tissues, and the quantity of excreted chemokines in feces by ELISA. In addition the expression of pro• inflammatory cytokine-TNF-α will be assessed by measuring the m-RNA level in rectal tissues, and the quantity of excreted TNF-α in feces by ELISA.
- Detailed analysis of *Shigella*- specific T cell responses in adults with shigellosis: by analyzing T cell receptor (TCR) variable (V) gene usage; complementary determining region 3 (CDR3) fragment analysis to estimate the degree of clonality, cloning and sequencing of TCR V gene products. For this purpose, cells from peripheral blood and rectal biopsies will be utilized from patients with shigellosis, with acute watery diarrhea and healthy individuals.
- *In vitro* activation and expansion of i) *Shigella* antigen specific T cells from patients and ii) *Shigella*-antigen stimulated T cells from healthy subjects

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:

Shigellosis is one of the major causes of morbidity and mortality in many developing countries. It is estimated that *Shigella* species infect over 200 million people yearly and the world-wide mortality rate from acute shigellosis is estimated to be 650,000 per year (1). In a study from rural Bangladesh, bloody diarrhea was the presenting complaint in 18% of all patients. In 65% of those patients *Shigella* species was isolated. The highest case-fatality rate was observed in *Shigella* patients (1.4%) (2). The main causes of death reported in shigellosis in endemic areas, are severe colitis complicated by septicemia and concomitant malnutrition

and pneumonia (3, 4, 5). Out of the four pathogenic *Shigella* species, *S. dysenteriae* type 1 and *S. flexneri* are of major importance for dysentery in developing countries.

Shigellosis is an invasive infection of the colonic epithelium characterized by the formation of abscess and ulcerations of the mucosa (6). Inflammation is initiated by invasion of epithelial cells by Shigella, and is characterized by strong mucosal infiltration by polymorphonuclear neutrophils (PMN) preceding infection and destruction of epithelial cells (7). PMN therefore destabilize epithelial cohesion and facilitate bacterial access to their invasion zone. Such a process differ from the classical scheme of PMN involvement during infections (8).

Innate immunity:

During the last decade, antibacterial peptides and proteins have gradually been accepted as important effectors in innate immunity (9). Several important defense properties have been assigned to these peptides and proteins, e.g. bactericidal, cytolytic, antiviral, antifungal, LPS binding, and chemotactic functions (10-11). These peptides are widespread in nature. Antibacterial peptides and proteins are an integral part of the epithelial defense barrier that makes up immediate protection against bacterial invasion. Besides the destruction of potential pathogens, components of the innate defense system have an instructive role for the highly specific lymphocytes of the adaptive immune system, thereby amplifying the local clearance and the defense potency. Thus, effective, specific and long-lasting immune response is dependent on the interplay between immune effector cells and molecules of the innate and adaptive systems (12-13). The outcome of the immune response is dependent on the stimulatory pathways that are activated (13). In humans, the α-defensins are mainly bactericidal efectors in circulating granulocytes, β-defensin-1 is synthesized in epithelial cells, and LL-37 is produced in granulocytes, and is also located in bronchial epithelial cells, and induced in skin epithelial cells during inflammation (11, 14-16). In addition to surface defenses, antibacterial components are synthesized and stored by granulocytes that are recruited to the site of inflammation or immediately activated upon contact with bacteria that enter the circulation.

Acute inflammatory response:

Acute inflammatory response is an important aspect of the host response to infection with pathogenic microbes and is characterized in the intestinal mucosa by a rapid influx and accumulation of neutrophils, monocytes, and other inflammatory cells that play a significant role in microbial destruction. Chemokines are small proinflammatory proteins that have a broad range of activities on the recruitment and function of specific populations of leukocytes at sites of inflammation and have an important role in the initiation and maintenance of the host inflammatory response (17-18). It has been suggested that, epithelial cells serve as an

early signaling system to host immune and inflammatory cells in the underlying mucosa following bacterial entry by secreting chemotactic mediators (19).

Antigen-specific T cells stimulation:

A T cell recognizes a foreign antigen via its T cell receptor (TCR). The variable (V) region and junctional region (CDR3) of TCR determine the specificity of a T cell for the antigenic peptide presented as a complex with a MHC class II molecule on antigen presenting cells (20-22). The CDR3 fragment length analysis can be used to disclose the size distribution of TCR products from T cells expressing a particular V gene segment and thus estimate the degree of clonality within a T cell population. T cells using a particular TCR CDR3 length may expand in response to a peptide (23-25). Polyclonality is usually seen in normal T cell populations, with in most cases 6-12 peaks distributed in a gaussian pattern, while dominant peaks showing T cells using a particular CDR3 length are found in oligoclonal or clonal T cell populations. As such, the TCR represents a key element of the cell-mediated arm of the immune response to pathogenic challenge. In general, a specific antigenic response results into the preferential or restricted usage of distinct TCR gene segments. Antigen-specific T cells orchestrate many reactions of the specific and non-specific systems. The breadth of protective cell-mediated responses in infection may depend upon how many different peptides derived from the key *Shigella* antigens and are presented by their MHC class II molecule. The study of TCR α/β chain and the detection of clonal kinetics, is a power tool to determine the responsible T cell clonotypes against immunodominant epitopes of that pathogen. It is generally believed that an immune response to an antigen is accomplished by clonal cooperation of lymphocytes. Since T cells are known to play an important role in such a cascade, it is essential to understand how T cell clones are activated in normal and pathological immune reactions. For successful vaccine design against shigellosis, both efficient antigen presentation and specific T cell activation would be required. Since effective T cell responses mediate both the humoral and cell-mediated immune responses to pathogen, it should be emphasize in designing peptide-based vaccine strategies. However, little is known about the field of T cell activation and the influence of modes of antigen presentation for T cell function and the kinetics of these T cell clonotypes in shigellosis. Thus, analysis of the TCR in shigellosis may help in understanding the nature of a presumed antigen responsible for eliciting T cell responses, subsequently creating T cell expansions, and may open the possibility to a highly selective immunotherapy by targeting shigellosis mediated T cells.

Our previous studies:

Previously and recently we have published a series of papers elucidating cellular, humoral and to some extent inflammatory responses in naturally acquired shigellosis in adults (26-

31). A two and a half year study on "Further studies of systemic and local immune responses in shigellosis in order to establish a protective vaccine" is on going and will be closed at the end of 1998. Data and results are being in the process of analysis, and a number of manuscripts will be prepared

from those data, which will illustrate preliminary involvement PMN in shigellosis, in detail analysis of involvement of nitric oxide in inflammatory process in shigellosis, and preliminary analysis of TCR V gene usage in shigellosis.

The proposed study for the coming three year period will focus on interplay between innate and adaptive immunity in shigellosis. To a large extent this study is natural extension of the work of the on going project.

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).**

Study population:

Inclusion criteria:

1) Adult male of age 20 to 45 years (n=30 in each group).
2) H/O bloody mucoid diarrhea of <4 day duration and stool culture +ve for *S. dysentriae* 1 or *S. flexneri.*
3) H/O watery diarrhea of <4 day duration (diarrheal control group)
4) Apparantly healthy male of similar age.
5) Both patients and controls from the population of similar socio-economic background.

Exclusion criteria:

1) If the patients and controls have experienced diarrhea in the 6 months prior to inclusion in the study.

2) Patients with concomitant gastrointestinal infections (other than shigellosis) or other infections.

Case management:
All patients with shigellosis will be treated with Pivmecilinum (400 mg 6 hourly) for atleast 5 days. Patients with watery diarrhea will be treated with fluid therapy (ORS or IV) and antimicrobial if indicated. Before recruiting healthy individuals as controls, single fecal sample will be collected from each healthy individual on first routine visit for microscopic examination and routine culture, if the microscopic examination of fecal sample would be positive for helminthes infection, they will be dewormed immediately. Within one week after deworming, and if the routine culture of fecal sample would be negative for common enteric pathogens, healthy individuals will be enrolled in the study.

Study materials: Colonic biopsies, peripheral blood and feces. Multiple (10-12) colonic biopsies will be obtained at colonoscopy and peripheral blood will be drawn by the median anticubital venipuncture in EDTA-vacuum tube (Becton Dickinson (BD), San Jose, CA, USA), and feces will be collected on days 0, 11 and 60 after disease onset, from the patient groups. Blood, feces and rectal biopsy will be obtained from healthy subjects at one time point. On follow-up visit, fecal sample from each patient at each time point will be examined microscopically and routine culture will be carried out. Routine blood analysis (TC, DC and Hb%) will be carried out for each individual at each time point.
Fresh fecal samples will be extracted as described earlier (28, 31) for chemokines assays.

Methodology:

1. **Peripheral blood mononuclear cells (PBMC) & $CD4^+$, $CD8^+$ T cells separation from peripheral blood:** PBMC will be isolated from heparinized peripheral blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and will be frozen in RPMI containing 20% FBS and 10% dimethylsulfoxide and stored at -196°C for immunophenotypic characterization at Karoliska Institute, Sweden (laetr on). For analysis of clonality, $CD4^+$ and $CD8^+$ T cells will be separated with magnetic beads (Dynabeads, Norway) according to the manufacturers description. Briefly, 2.5 ml blood will be incubated end over end for 30 min at 4°C with the beads coated with human anti-CD4 antibody, and then beads with $CD4^+$ cells will be removed with a magnetic separator and will be washed three times. Similarly, $CD8^+$ T cells will be separated with beads coated with human anti-CD8 antibody. This procedures results in >95% enriched $CD4^+$ or $CD8^+$ T cell populations.

2. **RNA extraction and RT-PCR amplification:** Total RNA will be extracted from enriched $CD4^+$, $CD8^+$ T cells or rectal biopsies using RNAzol B(Cinna/Biotek Laboratories Inc., Tx, USA) according to the manufacturers recommendation. First strand cDNA will be generated using random hexanucleotides (Pharmacia, Sweden) with reverse transcriptase (Gibco BRL Life Technology Inc., Maryland, USA).
   cDNA from biopsies will be used as template DNA for a number of PCR amplification with 5' and 3' primers encoding for: LL-37, α-defensin, β-defensin gene segments; for 11 chemokines (GRO- α, GRO- γ, ENA-78, IP-10, MCP-1, MCP-3, MIP-1 α, MIP-1 β, RANTES, I-039 and Lymphotactin), and for TNF-α (19). Each would be prepared cDNA will be amplified with primers for "house keeping gene" β-$_2$ microglobulin to monitor the cDNA synthesis.
   An aliquot of each amplification reaction will be loaded in ethidium bromide-stained 2% agarose gel to confirm the expected size of the amplified fragment.
   cDNA from enriched $CD4^+$, $CD8^+$ T cells and biopsy samples will be amplified with 5'V specific primers (for 28 TCR Vβ specific gene fragments) and 3' constant TCR β primers. An aliquot of each amplification reaction will be loaded in ethidium bromide-stained 2% agarose gel to confirm the expected size of the amplified fragment.

3. **CDR3 fragment analysis:** The size distribution of different TCR fragments will be studied by utilizing a CDR3 length analysis. Briefly, TCR Vβ amplified products will be amplified again in a PCR reaction with a 5' Vβ specific primer in conjunction with a 3' constant β primer labeled with FITC. A fraction of the PCR amplified product will subsequently be denatured and loaded on a 6.75% acrylamide gel (Readymix, Pharmacia) and will run for 400 min. at 42°C on an automated DNA sequencer, ALF (Pharmacia). The CDR3 length distribution will be analyzed with ALF Fragment Manager software version 1.1 (Pharmacia).

4. **Cloning and sequencing:** PCR amplified TCR V gene products will be cloned and sequenced according to standard procedures. Briefly, the PCR product will be purified on a spin column (qiagen, Dusseldorf, Gemany). After phosphorylation with T4 polynucleotide kinase (Pharmacia) the product will be ligated to a *Smal* cleaved pUC18 vector that will subsequently be used to transform competent bacteria. Single colonies will be used as templates for solid phase sequencing (32) and will be analyzed with the ALF (Pharmacia). Alternatively, PCR will be performed with a biotinylated C β primer, and the PCR product will be used for "direct" solid phase sequencing with a non-labeled βV specific primer and Fluoro-dATP (Pharmacia) according to the recommendation of the manufacturer. The CDR3 region will be defined and CDR3 lengths will be calculated using the formula by Rock et. al. (33), i.e. the number of amino acids between the

conserved GXG triplet in the J region and the nearest preceding C in the V region, minus 4 amino acids.

5. **Assessment of chemokines in fecal extract:** Chemokines in fecal extract will assayed by ELISA using ELISA-Kit from R&D Systems. These ELISAs are sensitive to ≅20 pg/ml.

6. **Lymphocyte separation from Biopsies:** The aseptically taken rectal biopsies will be cut into smaller pieces and will be digested using an enzyme mixture consisting collagenase, DNase and hyaluranidase. After washing, lymphocytes will be separated by percoll density gradient centrifugation. From patients, purified lymphocytes will be phenotyped, and will be put in culture with antibiotics, human AB+ sera and rIL-2. The cells will be grown for 10-20 days before TCR V gene analysis (34). From healthy controls, purified lymphocytes will be put in culture with antigen presenting cells (APC) pulsed with *Shigella* antigens. *Shigella* antigen-specific clone T cells will be phenotyped and will be grown for 10-20 days before TCR V gene analysis. Dendritic cells will be used as APC.

7. **Immunophenotypic characterization of PBMC and separated lymphocytes from biopsies:** Flow cytometric immuno-phenotyping has become a standard method for characterizing lymphoid cell populations in clinical as well as experimental medicine. By multicolor analysis, the expression of individual cell-surface molecules can be defined on sub-population of lymphocytes. Immunophenotyping of PBL may reflect the character and magnitude of the cellular immune response in disease conditions. The regulated expression of cell surface molecules plays a crucial role in lymphocyte activation, tissue homing and cell to cell interactions. Not only the presence, but also the magnitude of expression of a particular molecule may determine the functional competence of a cell.

7-a) Cell preparation, staining and Flow cytometric analysis. Cell preparation and staining will be followed exactly the previously described procedure (23, 24). Frozen PBMC will be thawed and viability will be checked by trypan blue exclusion. Thawed PBMC ($1x10^6$ cells in 100 ml of phosphate buffered saline pH 7.4 (PBS) containing 2% heat inactivated pooled human serum (AB sera)) will be dispensed into tubes containing saturating concentrations of triple combinations (FITC, PE and PerCP conjugated) of monoclonal antibodies against T cell subsets (CD4 and CD8) and TCR Vβ's for 30-min at 4°C. After washing the cells will be resuspended in PBS containing 1%

paraformaldehyde and will be analyzed within 4 h. Negative controls will be included in each experiment. Three color fluorescent analysis will be performed according to the previously described procedures on a FACSort flow cytometer (BD). Data acquisition will be made on Lysys II software (BD). For each sample 3,000 lymphocytes will be acquired using log amplified fluorescence- and linearly amplified side and forward scatter signals. The data will be analyzed with the Paint-A-Gate^{+} software (BD). All sampleswill be analyzed by setting appropriate FSC/SSC gates around the lymphocyte population using back gating on $CD45^{+}CD14^{-}$ cells. Multiple samples from individual patients will always be stained and run at a time.

8. ***In vitro* differentiation of dendritic cells (DC) from PBMC:** DC are considered to be the most potent APC and to play a critical role in initiation of primary immune responses (35). DC are generated from PBMC as earlier described (36-37). In brief, PBMC from controls will be isolated from EDTA-blood by density centrifugation on Ficoll Hypaque density gradient, resuspended in culture media, and will be then allowed to adhere for 1.5 h at 37°C. The cell culture flusk will be washed, and the adherent cells will be cultured in media containing human recombinant IL-4 and GM-CSF. On day 5 TNF-α will be added to the culture media for maturation of DC. Immature DC will be pulsed with *Shigella*-specific antigens for efficient antigen processing and presentation on cell surface.

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 equipment 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).**

The laboratories at LSD, ICDDR,B have the required expertise to perform assays for this study. However, due to lack of some of the delicate equipment, a portion of the research work will be carried out at KI, Stockholm, Sweden under the guidance of Birger Christensson.

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).**

Data analysis: This is a descriptive type of study, using mathematical formula sample size calculation is not appropriate, as the primary outcome is not quantifiable. There will be three different group of individuals (with shigellosis, with watery diarrhea and healthy controls). From each patient, sample will be collected on admission day and day-11 and day-60. Specific immune response in patients will reach pick level on day-11, and whether it will decrease or remain elevated that can be assessed from day-60 samples. There, within the patients, individual immune response can be monitored. In addition, immune responses in patients with shigellosis will be compared to watery diarrheal patient group and healthy individuals.

Based on the sample size for our previous and on going studies in shigellosis, the sample size in each group is chosen. In our previous study, with sample size 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.

The CDR3 profiles will be analyzed with ALF fragment manager Software 1.1 and peak areas will be determined according to the formula:

$$\frac{\text{X peak area} \times 100}{\text{Total peak area}} = \% \text{ of total peak area}$$

The size of obtained fragments will be compared to a FITC-labeled 50-5000 bp size marker.

All data will be analyzed by: i) 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., 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 proposed studies involved:

(i) Management of patients with shigellosis and acute watery diarrheal diseases.

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

For inclusion of patients and healthy subjects (controls), informed consents will be required according to guide lines from the local ethical committee at ICDDR,B. Patients will be recruited among those seeking medical care at the outpatient section at Clinical Research Service Center (CRSC) of ICDDR,B. Included patients will receive clinical care and therapy free of charge and of the same type that they would have received had they not been enrolled in the study. Age and sex matched controls will be recruited from the same socioeconomic status as well as the same endemic areas. Patients may discontinue their participation in the study at any time point. This decision would not have any influence on the clinical management or therapy of the patients. Repeated sampling of blood, stool and rectal mucosa will be performed from patients. All samples from controls will be collected once (rectal biopsies only from adults), to monitor the constancy of the research parameters in the healthy adult population. Approximately 10 ml of venous blood (from median cubital vein) will be taken from adults (3 times). With an colonoscope 6-8 tiny pieces of biopsies (about the size of rice grains, 4 mm across) will be obtained from the rectosigmoid area of adults. This instrument has a tube which will be passed through anus upto 20-25 cm. All sampling procedures will be performed by clinically fully trained experts in the procedures concerned.

Patients and controls management, including repeated biopsy sampling has been done previously at ICDDR,B in conjunction with studies of immune responses in shigellosis. As has been seen in previous occasions we consider that the potential scientific benefit obtained by the proposed studies well warrants the inconveniences and local pain associated with sampling. No serious side effects have been associated with sampling with previous studies and are not expected to occur in the proposed study. Apart from known complications of blood drawing which are generally minor including temporary discomfort/pain, potential for bruising at the site of vein puncture, etc. participation in the study poses no substantial likely risks to the subjects. The subject will have the access to the information and retain the right to refuse participation in this study, and also to withdraw from the study at any time. Confidentiality of data will be maintained.

Use of Animals

Describe in the space provided the type and species of animal that will be used in the study. Justify with reasons the use of particular animal species in the experiment and the compliance of the animal ethical guidelines for conducting the proposed procedure

Animal will not be used in this study

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 programme.

Findings of this study may help in better understanding the influence of innate immunity on adaptive immunity in shigellosis. As a result this may help to understand host response in shigellosis, and the extent of involvement of innate immune mechanisms in host response, therefore, a better therapeutic and/or prevention strategy may be formulated. All the finding will be published in internationally well renowened scientific journals.

Collaborative Arrangements:

This will be a collaborative study between LSD, ICDDR,B and Division of Pathology, Huddinge Hospital, Karolinska Institute (KI), Sweden.
Flow Chart for sequence of tasks (ICDDR,B+KI) within time frame (January 1999 to December 2001):

Year	Parts of the project to be performed at: ICDDR,B	KI
1999	Procurement of supplies and method standardisation Recruitment of patients and controls Routine analyses Processing and storing of samples In vitro growth and cloning of Shigella specific T cells.	Procurement of supplies and method standardisation Establishment of in situ analysis methods for antibacterial peptides In vitro analysis of the regulation of antibacterial peptide expression in mucosal cells Flow cytometric analysis of T cell subset TCR v-beta repertoir expression
2000	Procurement of supplies and method standardisation Recruitment of patients and controls Routine analyses Processing and storing of samples cDNA preparation and RT-PCR analysis of samples Chemokine analyses In vitro analysis of Shigella derived peptides on the antigen presentation to Shigella specific T cell clones	Flow cytometric analysis of T cell subset TCR v-beta repertoir expression CDR3 length analysis of TCR heterogeneity by molecular run-off methodology In vitro analysis of the influence of Shigellae spp on the regulation of antibacterial peptide expression in epithelial and dendritic cells
2001	In vitro analysis of Shigella derived peptides on the antigen presentation to Shigella specific T cell clones Competion of analyses, data analysis and reporting	CDR3 length analysis of TCR heterogeneity by molecular run-off methodology Molecular analysis of immunogenic Shigella derived peptides Molecular TCR v-beta gene sequencing Detailed analysis of the signalling path ways regulating antibacterial peptide expression Competion of analyses, data analysis and reporting

Itemized specific tasks for investigator (ICDDR,B):

- Dilara Islam (50%)
  Overall supervision, data analyses, compilation and reporting.
- Minnie Mathan (5%) Consultant, LSD
  Guidence of the histopathological work
- N. H. Alam (5%) -CSD
  Overall in-charge patient management and of colonoscopy
- Debasish Saha* (Medical officer), (25%) -LSD
  Patient enrollment and clinical management and follow-up, and colonoscopy
- Research officer* (50%) -LSD
  Carry out tests specified for the protocol involving both microbiological and immunological techniques
- Lab. attendant* (50%) -LSD
  Patient recruitment and others.
  *Will also be involved in other project.

Biography of the Principal Investigator

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

Name	**Position**	**Date of Birth**
Dilara Islam	Asst. Scientist, LSD, ICDDR,B,; Dhaka, Bangladesh	30th January, 1961

Academic Qualifications (Begin with baccalaureate or other initial professional education)

Institution and Location	**Degree**	**Year**	**Field of Study**
Karolinska Institute, Stockholm, Sweden	PhD	1995	Immuno-Pathology in shigellosis
Department of Biochemistry, University of Dhaka	MsC	1987	Biochemistry
Department of Biochemistry, University of DhakaDhaka	BsC	1983	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. Different aspects of immune responses and immmune mechanisms in shigelosis. 1996-1998
2. PhD thesis work . 1989-1995
3. Research officer at Biochemistry and Nutrition Lab. 1987-1989

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6. **Islam, D.**, Wretlind, B., Lindberg, A. A. and Christensson, B. Changes in the peripheral blood T cell receptor Vβ repertoire *in vivo* and *in vitro* during shigellosis. Infect Immun. 64:1391-1399, 1996.
7. **Islam, D.**, Bardhan, P. K., Lindberg, A. A. and Christensson, B. *Shigella* infection induces cellular activation of T and B cells, and distinct species-related changes in peripheral blood lymphocyte subsets during the course of the disease. Infect Immun. 63:2941-2949, 1995.

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Detailed Budget for a period of three year:

Project Title: STUDIES ON SHIGELLOSIS.
a) The influence of innate immune mechanisms on T cells stimulation in shigellosis.

Name of PI: Dilara Islam (LSD, ICDDR,B); Birger Christensson (KI, Sweden)

Protocol Number: Name of Division: LSD

Funding Source: SIDA/SAREC: **Total: $207,347** for 3 years

Starting Date: 01-01-1999 Closing Date: 31-12-2001

PART A: ICDDR,B

EXCHANGE RATE **$1** = 8

			Yearly budget					
			Ist year	Ist year	2nd year	2nd year	3rd year	3rd year
1. Personnel:	% of salary	of tim	**US$**	**SKR**	**US$**	**SKR**	**US$**	**SKR**
Principal Investigator (PI)	30	50	3,900	31,200	4,095	32,760	4,300	34,398
Medical Officer	25	25	1,700	13,600	1,785	14,280	1,875	15,000
Medical Officer	0	5	800	6,400	840	6,720	880	7,040
Research Officer	50	50	2,100	16,800	2,205	17,640	2,300	18,400
Laboratory Attendant	50	50	1,000	8,000	1,050	8,400	1,100	8,800
Research Histopathologist	0	5	0	0	0	0	0	0
SUBTOTAL			9,500	76,000	9,975	79,800	10,455	83,638
OVERHEAD PERSONELL	10%		950	7,600	998	7,980	1,045	8,364
TOTAL PERSONNEL COST			**10,450**	83,600	**10,973**	87,780	**11,500**	92,002
Local travel			100	800	100	800	100	800
International travel	Trip to KI		1500	12,000	1,700	13,600	1,900	15,200
OVERHEAD			Nil		Nil		Nil	
TOTAL TRAVEL COST			**1,600**	12,800	**1,800**	14,400	**2,000**	16,000
Supplies and Materials:								
a) Laboratory investigations:								
Immunological assays:			2,200	17,600	3,600	28,800	2,700	21,600
RT-PCR & TCR Vb usage analysis			1,000	8,000	2,500	20,000	2,500	20,000
Chemokine ELISA by Kit								
b) Laboratory supplies:			550	4,400	300	2,400	300	2,400
Plastic ware, glassware and office supplies			3,750	30,000	6,400	51,200	5,500	44,000
SUBTOTAL			375	3,000	640	5,120	550	4,400
OVERHEAD LAB COST	10%		**4,125**	33,000	**7,040**	56,320	**6,050**	48,400
TOTAL LABORATORY COST								

	Yearly budget					
	1st year US$	1st year SKR	2nd year US$	2nd year SKR	3rd year US$	3rd year SKR
Other services:						
Repair and Maintenance	0	0	0	0	0	0
Rent, Communications, Utilities						
Training Workshop, Seminars						
Printing and Publication	100	800	100	800	1,500	12,000
Staff Development						
SUBTOTAL	100	800	100	800	1,500	12,000
OVERHEAD OTHER SEVI 10%	10	80	10	80	150	1,200
TOTAL OTHER SEVICES	**110**	880	**110**	880	**1,650**	13,200
Interdepartmental Services:						
Computor Charges	100	800	100	800	100	800
Pathological Tests	500	4,000	800	6,400	800	6,400
Microbiological Tests	500	4,000	1,000	8,000	925	7,400
Biochemistry Tests	200	1,600	450	3,600	400	3,200
X-Rays						
Patients Study (Study ward cost &wage loss)	2,000	16,000	3,400	27,200	3,500	28,000
Research Animals						
Biochemistry & Nutrition						
Transport						
Xerox,Mimeographs etc.	120	960	100	800	100	800
SUBTOTAL	3,420	27,360	5,850	46,800	5,825	46,600
OVERHEAD OTHER SEVI 10%	342	2,736	585	4,680	583	4,660
TOTAL OTHER SEVICES	**3,762**	30,096	**6,435**	51,480	**6,408**	51,260
Other Operational Costs						
Capital Expenditure (Small equipment)	2,000	16,000		0		0
SUBTOTAL OTHER OPERATIONAL COSTS	2,000	16,000	0	0	0	0
OVERHEAD 10%	200	1,600	0	0	0	0
TOTAL OTHER OPERATIONAL COSTS	**2,200**	17,600	0	0	0	0
TOTAL (ICDDR,B)	**22,247**	177,976	**26,358**	210,860	**27,608**	220,862

= **$76,212**
548980(SKR)

PART B:

Detailed budget for studies to be performed at Karolinska Institute:

Swedish project leader: Birger Christensson
Dept. of Pathology, Huddinge Univ. Hosp.
Karolinska Institute, Sweden

EXCHANGE RATE **$1 = 8**

		Yearly budget					
		Ist year	Ist year	2nd year	2nd year	3rd year	3rd year
1. Personnel:	% of time Positic	**US$**	**SKR**	**US$**	**SKR**	**US$**	**SKR**
Biomed assistant	30%	9,770	78,160	9,770	78,160	9,770	78,160
Laboratory Attendant	10%	3,123	24,984	3,123	24,984	3,123	24,984
SUBTOTAL PERSONNEL COST		**12,893**	103,144	**12,893**	103,144	**12,893**	103,144
Over Head (KI) 13.64%		**1,759**	14,069	**1,759**	14,069	**1,759**	14,069
TOTAL PERSONNEL COST		**14,652**	117,213	**14,652**	117,213	**14,652**	117,213
Accomodation & subsistence gra	2 months	1,500	12,000	1,500	12,000	1,500	12,000
For Banglad. guest, res.				2,000	16,000		
International travel							
TOTAL TRAVEL COST		**1,500**	12,000	**3,500**	28,000	**1,500**	12,000
Supplies and Materials:							
a) Laboratory investigations:							
Immunological reagents		5,000	40,000	5,000	40,000	5,000	40,000
(Flow cytometry, Confocal microscopy)							
Molecular biological reagents		10,000	80,000	10,000	80,000	10,000	80,000
(Sequencing, In situ hybridisation)							
b) Laboratory supplies:							
Plastic ware, glassware,office supplies,		1,332	10,656	1,332	10,656	1,332	10,656
and image-print material							
C) Laboratory reagents		5,000	40,000	5,000	40,000	5,000	40,000
bought in Sweden for ICDDR,B							
SUBTOTAL LABORATORY COST		**21,332**	170,656	**21,332**	170,656	**21,332**	170,656
Over Head (KI) 13.64%		**2,910**	23,277	**2,910**	23,277	**2,910**	23,277
TOTAL LABORATORY COST		**24,242**	193,933	**24,242**	193,933	**24,242**	193,933
Other services:							
Repair and Maintenance, Service of equipment		500	4,000	500	4,000	500	4,000
Communications/transport		500	4,000	500	4,000	500	4,000
(Telecommunication/ parcel service)							
Capital Expenditure (Small equipment)		2,000	16,000	1,000	8,000	1,000	8,000
SUBTOTAL OTHER OPERATIONAL COSTS:		**3,000**	24,000	**2,000**	16,000	**2,000**	16,000
Over Head (KI) 13.64%		**409**	3,274	**273**	2,182	**273**	2,182
TOTAL OTHER OPERATIONAL COSTS:		**3,409**	27,274	**2,273**	18,182	**2,273**	18,182
TOTAL (KI)		**43,802**	350,420	**44,666**	357,329	**42,666**	341,329
GRAND TOTAL (ICDDR,B+KI)		**66,049**	528,396	**71,024**	568,189	**70,274**	562,191

= **$207,347**
1,656,061 SKR

APPENDIX

International Centre for Diarrhoeal Disease Research, Bangladesh

Voluntary Consent Form

Title of the Research Project: The influence of innate immune mechanisms on T cells stimulation in shigellosis.

Principal Investigator: Dilara Islam & Birger Christensson

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.

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

"SHIGELLA"

CONSENT FORM FOR ADULT PATIENTS

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 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, we will need to collect blood, stool and rectal biopsies from you. For your treatment and for the convenience of the study, you will have to stay in the hospital for 4-5 days. You will be requested to come for follow-up visits on days 11 and 60 after discharge. About 10 ml of blood (two tea-spoon full) will be collected from your median cubital vein on the day of admission and 11 and 60 days after that (3 times). This will not be harmful to you in any way. You will be examined by a colonoscope which has a tube that will be passed through your anus (20-25 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 not feel any discomfort or pain during this examination. Six/seven tiny pieces of biopsies (about the size of rice grains, 4 mm across)

will be obtained from your rectum. Although the procedure is not harmful, you will be kept under observation for 3-4 hours

after the examination to ensure it. This procedure will not cause you any pain or harm afterwards. Biopsies will be obtained from you three times, on the day of admission, 11 and 60 days later.

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 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. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit.

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

_____ _____

Signature of a witness

Date

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

"CONTROL"

CONSENT FORM FOR ADULTS

Bloody diarrhea due to pathogen ***"SHIGELLA"*** usually causes 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 lives 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 blood, stool and rectal biopsies only one time. Approximately 10 ml (two tea-spoon full) of venous blood will be taken from your from median cubital vein. You will be examined by an instrument called flexible sigmoidoscope. This instrument has a tube which will be passed through your anus (20-25 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 not feel any discomfort or pain during this examination. Six/seven tiny pieces of biopsies (about the size of rice grains, 4 mm across) will be obtained from your rectum. Although the procedure is not harmful, you will be kept under observation for 3-4 hours after the examination to ensure it. This procedure will not cause you any pain or harm afterwards.

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

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

"WATERY DIARRHEAL CONTROL"

CONSENT FORM FOR ADULT PATIENTS

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. However, individuals who are suffering from watery diarrhea caused by pathogens other than *Shigella* usually do not face these complications. For this purpose, adult who are suffering from watery diarrhea are needed to be examined in order to compare the findings in this group 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 request you to participate in this study. Your participation in this study may help to save lives 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, we will need to collect blood, stool and rectal biopsies from you. For your treatment and for the convenience of the study, you will have to stay in the hospital for 4-5 days. You will be requested to come for follow-up visits on days 11 and 60 after discharge. About 10 ml of blood (two tea-spoon full) will be collected from your median cubital vein on the day of admission and 11 and 60 days after that (3 times). This will not be harmful to you in any way. You will be examined by a colonoscope which has a tube that will be passed through your anus (20-25 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 not feel any discomfort or pain during this examination. Six/seven tiny pieces of biopsies (about the size of rice grains, 4 mm across) will be obtained from your rectum. Although the procedure is not harmful, you will be kept under observation for 3-4 hours after the examination to ensure it. This procedure will not cause you any pain or harm afterwards. Biopsies will be obtained from you three times, on the day of admission, 11 and 60 days later.

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 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. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit.

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
_____	
_____	
Signature of a witness	Date

আন্তর্জাতিক উদারাময় গবেষণা কেন্দ্র
মহাখালী, ঢাকা-১২১২
"শিগেলা"

সম্মতি পত্র (প্রাপ্তবয়স্ক)

আপনি "শিগেলা" নামক এক প্রকার জীবানুর কারণে রক্ত আমাশয়ে ভুগছেন, যা কখনো খুব মারাত্মক এবং জটিল অবস্থার সৃষ্টি করতে পারে। শিগেলা মানুষের শরীরে কিভাবে জটিলতার সৃষ্টি করে তা এখন পর্যন্ত সম্পূর্ন রূপে জানা যায়নি। এই রোগ সম্পর্কে আরো ভালোভাবে জানার জন্য এবং কিভাবে এর রোগ প্রতিরোধ ক্ষমতা বাড়ানো যায় তার জন্য আমরা একটা গবেষণা করছি যা ভবিষ্যতে এ রোগের প্রতিরোধ এবং চিকিৎসায় আমাদের সাহায্য করবে। আমরা আশা করছি যে, এই গবেষণার ফল সাফল্যজনক হলে আমাদের এবং আমাদের দেশের মত উন্নয়নশীল দেশের লক্ষ লক্ষ রোগী এই মারাত্মক রোগ হতে রক্ষা পাবে। সেই কারনে, এই গবেষণায় আপনাকে অংশগ্রহন করার জন্য আমরা আপনার কাছে অনুমতি চাচ্ছি।

গবেষণায় ভর্তির সময় এবং পরবর্তী প্রতিদিন আপনার অবস্থা সম্পর্কে জিজ্ঞাসাবাদ ও শারীরিক পরীক্ষা করা হবে এবং আপনাকে হাসপাতালের প্রয়োজনীয় চিকিৎসা দেওয়া হবে। গবেষণা চলাকালীন সময় আপনাকে সাত দিন হাসপাতালে ভর্তি থাকতে হবে। ভর্তির দিন এবং ভর্তির ১১, এবং ৬০ দিন পরে (৩ বার) আপনার হাতের শিরা থেকে দুই চা-চামচ মত রক্ত (১০ মিঃ লিঃ) এবং পায়খানা নেওয়া হবে। ভর্তির দিন এবং ভর্তির ১১ এবং ৬০ দিন পরে আপনার মল দ্বারে একটা নলের মত যন্ত্র (flexible sigmoidoscope) ঢুকিয়ে (মল দ্বার থেকে ২০-২৫ সে: মি: ভিতরে) ৬-৭ টুকরা ঝিল্লি (ছোট চালের দানার মত) সংগ্রহ করা হবে। এই প্রক্রিয়ায় আপনার মলদ্বারে এক প্রকার জেলি লাগান হবে, যাতে আপনার মলদ্বার পিচ্ছিল হয় এবং আপনি কোন ব্যাথা অনুভব না করেন। এই ব্যবস্থা গুলো সম্পুর্ন নিরাপদ কিন্তু তবুও যদি কোন অসুবিধা ঘটে আমরা তার জন্য প্রয়োজনীয় ব্যবস্থা নেব। ছুটির পর, ভর্তির ১১, ও ৬০ দিন পরে পুনরায় হাসপাতালে আসতে হবে পুর্বে উল্লেখিত নমুনাগুলো আমাদের কাছে দেওয়ার জন্য। আপনি এই গবেষণায় অন্তর্ভূক্ত হউন আর নাই হউন এই চিকিৎসা কেন্দ্রের যে সকল সেবা ও চিকিৎসা দেওয়া হয় তা আপনি নিয়ম মতই পাবেন। আপনি চাইলে যে কোন সময় এ গবেষণা থেকে আপনাকে প্রত্যাহার করতে পারেন এবং সে কারণে আপনি এ হাসপাতলের প্রচলিত সূচিকিৎসার সুযোগ থেকে বঞ্চিত হবেন না। এই গবেষণার সকল তথ্য গবেষণার স্বার্থে গোপন রাখা হবে। তবে আপনি জানতে আগ্রহী হলে এবং আমাদের জানা থাকলে তা আপনাকে জানানো হবে। ছুটির পর প্রতিবার আপনার আসার জন্য আপনাকে আপনার সেই দিনের মজুরী এবং যাতায়াত খরচ আমরা দেব।

আপনি যদি এই গবেষণায় অংশগ্রহণ করতে রাজি হন, তবে দয়া করে নিচে আপনার সই বা বাম হাতের বৃদ্ধাঙ্গুলির টিপসই দিন।।

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রোগির স্বাক্ষর/টিপ সই তারিখ

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গবেষণাকারীর স্বাক্ষর তারিখ

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সাক্ষীর স্বাক্ষর তারিখ

আন্তর্জাতিক উদারাময় গবেষণা কেন্দ্র
মহাখালী, ঢাকা-১২১২
"পাতলা পায়খানা কন্ট্রোল"

সম্মতি পত্র (প্রাপ্তবয়স্ক)

"শিগেলা" নামক এক প্রকার জীবানুর কারণে যে সমস্ত রোগী রক্ত আমাশয়ে ভুগছে তারা বিভিন্ন ধরনের মারাত্মক জটিলতার সম্মুখিন হয়। শিগেলা মানুষের শরীরে কিভাবে জটিলতার সৃষ্টি করে তা এখন পর্যন্ত সম্পূর্ন রূপে জানা যায়নি। এই রোগ সম্পর্কে আরো ভালোভাবে জানার জন্য এবং কিভাবে এর রোগ প্রতিরোধ ক্ষমতা বাড়ানো যায় তার জন্য আমরা একটা গবেষণা করছি যা ভবিষ্যতে এ রোগের প্রতিরোধ এবং চিকিৎসায় আমাদের সাহায্য করবে। শিগেলা ছাড়া অন্য প্রকার জীবানুর কারনে যে সমস্ত রোগী পাতলা পায়খানা জনিত রোগে ভুগছে তাদের এ ধরণের জটিলতা কেন হয় না তা জানার জন্য তাদেরকেও এই গবেষণায় অর্ন্তভূক্ত করার প্রয়োজন আছে। আমরা আশা করছি যে, এই গবেষণার ফল সাফল্যজনক হলে আমাদের এবং আমাদের দেশের মত উন্নয়নশীল দেশের লক্ষ লক্ষ রোগী এই মারাত্বক রোগ-রক্ত আমাশয়ে হতে রক্ষা পাবে। সেই কারনে, এই গবেষণায় আপনার শিশুকে অংশগ্রহন করতে দেওয়ার জন্য আমরা আপনার কাছে অনুমতি চাচ্ছি ।

গবেষণায় ভর্তির সময় এবং পরবর্তী প্রতিদিন আপনার অবস্থা সম্পর্কে জিজ্ঞাসাবাদ ও শারীরিক পরীক্ষা করা হবে এবং আপনাকে হাসপাতালের প্রয়োজনীয় চিকিৎসা দেওয়া হবে। গবেষণা চলাকালীন সময় আপনাকে সাত দিন হাসপাতালে ভর্তি থাকতে হবে। ভর্তির দিন এবং ভর্তির ১১, এবং ৬০ দিন পরে (৩ বার) আপনার হাতের শিরা থেকে দুই চা-চামচ মত রক্ত (১০ মিঃ লিঃ) এবং পায়খানা নেওয়া হবে। ভর্তির দিন এবং ভর্তির ১১ এবং ৬০ দিন পরে আপনার মল দ্বারে একটা নলের মত যন্ত্র (flexible sigmoidoscope) ঢুকিয়ে (মল দ্বার থেকে ২০-২৫ সে: মি: ভিতরে) ৬-৭ টুকরা ঝিল্লি (ছোট চালের দানার মত) সংগ্রহ করা হবে। এই প্রক্রিয়ায় আপনার মলদ্বারে এক প্রকার জেলি লাগান হবে, যাতে আপনার মলদ্বার পিচ্ছিল হয় এবং আপনি কোন ব্যাথা অনুভব না করেন। এই ব্যবস্থা গুলো সম্পুর্ন নিরাপদ কিন্তু তবুও যদি কোন অসুবিধা ঘটে আমরা তার জন্য প্রয়োজনীয় ব্যবস্থা নেব। ছুটির পর, ভর্তির ১১, ও ৬০ দিন পরে পুনরায় হাসপাতালে আসতে হবে পুর্বে উল্লেখিত নমুনাগুলো আমাদের কাছে দেওয়ার জন্য। আপনি এই গবেষণায় অন্তর্ভূক্ত হউন আর নাই হউন এই চিকিৎসা কেন্দ্রের যে সকল সেবা ও চিকিৎসা দেওয়া হয় তা আপনি নিয়ম মতই পাবেন। আপনি চাইলে যে কোন সময় এ গবেষণা থেকে আপনাকে প্রত্যাহার করতে পারেন এবং সে কারণে আপনি এ হাসপাতলের প্রচলিত সূচিকিৎসার সুযোগ থেকে বঞ্চিত হবেন না। এই গবেষণার সকল তথ্য গবেষণার স্বার্থে গোপন রাখা হবে। তবে আপনি জানতে আগ্রহী হলে এবং আমাদের জানা থাকলে তা আপনাকে জানানো হবে। ছুটির পর প্রতিবার আপনার আসার জন্য আপনাকে আপনার সেই দিনের মজুরী এবং যাতায়াত খরচ আমরা দেব।

আপনি যদি এই গবেষণায় অংশগ্রহণ করতে রাজি হন, তবে দয়া করে নিচে আপনার সই বা বাম হাতের বৃদ্ধাঙ্গুলির টিপসই দিন।।

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রোগির স্বাক্ষর/টিপ সই তারিখ

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গবেষণাকারীর স্বাক্ষর তারিখ

....

সাক্ষীর স্বাক্ষর তারিখ

আন্তর্জাতিক উদারাময় গবেষণা কেন্দ্র
মহাখালী, ঢাকা-১২১২
"কন্ট্রোল"

সম্মতি পত্র (প্রাপ্তবয়স্ক)

"শিগেলা" নামক এক প্রকার জীবানুর কারণে যে সমস্ত রোগী রক্ত আমাশয়ে ভুগছে তারা বিভিন্ন ধরনের মারাত্মক জটিলতার সম্মুখিন হয়। শিগেলা মানুষের শরীরে কিভাবে জটিলতার সৃষ্টি করে তা এখন পর্যন্ত সম্পূর্ন রূপে জানা যায়নি। এই রোগ সম্পর্কে আরো ভালোভাবে জানার জন্য এবং কিভাবে এর রোগ প্রতিরোধ ক্ষমতা বাড়ানো যায় তার জন্য আমরা একটা গবেষণা করছি, যা ভবিষ্যতে এ রোগের প্রতিরোধ এবং চিকিৎসায় আমাদের সাহায্য করবে। এই রোগে আক্রান্ত হওয়ার ফলে রোগীর অবস্থা সুস্থ ব্যক্তির সাথে তুলনা করার জন্য, এই গবেষণায় সুস্থ ব্যাক্তির অংশগ্রহন বাঞ্ছনিয়। আমরা আশা করছি যে, এই গবেষণার ফল সাফল্যজনক হলে আমাদের এবং আমাদের দেশের মত উন্নয়নশীল দেশের লক্ষ লক্ষ রোগী এই মারাত্মক রোগ হতে রক্ষা পাবে। সেই কারনে, এই গবেষণায় আপনার অংশগ্রহনের জন্য আমরা আপনার কাছে অনুমতি চাচ্ছি।

আমরা মাত্র একবার আপনার হাতের শিরা থেকে দুই চা-চামচ মত রক্ত (১০ মিঃ লিঃ), পায়খানা, লালা, এবং মূত্র নেব। সেই সাথে আপনার মল দ্বারে একটা নলের মত যন্ত্র (flexible sigmoidoscope) ঢুকিয়ে (মল দ্বার থেকে ২০-২৫ সে: মি: ভিতরে) ৬-৭ টুকরা ঝিল্লি (ছোট চালের দানার মত) সংগ্রহ করা হবে। এই প্রক্রিয়ায় আপনার মলদ্বারে এক প্রকার জেলি লাগান হবে, যাতে আপনার মলদ্বার পিচ্ছিল হয় এবং আপনি কোন ব্যাথা অনুভব না করেন। এই ব্যবস্থা গুলো সম্পুর্ন নিরাপদ কিন্তু তবুও যদি কোন অসুবিধা ঘটে আমরা তার জন্য প্রয়োজনীয় ব্যবস্থা নেব। আপনার আসার জন্য আপনাকে আপনার সেই দিনের মজুরী এবং যাতায়াত খরচ আমরা দেব।

আপনি যদি এই গবেষণায় অংশগ্রহণ করতে রাজি হন, তবে দয়া করে নিচে আপনার সই বা বাম হাতের বৃদ্ধাঙ্গুলির টিপসই দিন।

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অংশগ্রহণকারীর স্বাক্ষর/টিপ সই তারিখ

....

গবেষণাকারীর স্বাক্ষর তারিখ

....

সাক্ষীর স্বাক্ষর তারিখ

Title: Studies on shigellosis

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

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

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification ☒

- on technical grounds ☒
- on level of financial support ☒

I do not support the application ☐

Name of Referee: [signature]

Signature: ..... Date: Sept 11 1998

Position: Professor
Chairman MD PhD Hon FRCPCH (UK)

Institution:
Dept Clinical Immunology
Göteborg University
Göteborg
Sweden

Detailed Comments

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

(Use additional pages if necessary)

Title: Studies on shigellosis

PI:

Reviewer: L A Hanson

Lars Å Hanson

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In the background description of this project the applicants correctly bring out the recent understanding of native immunity for the immediate response to infections. They most correctly mention the role, now being better appreciated, of components like defensins. They also mention the very important role of chemokines and cytokines. The latter, as they originate from many of the cells of the innate immune system presumably truly play a major role, especially by activating the adaptive immune system, especially by stimulating the dendritic cells in their central role as the main antigen presenting cell.

Based on this the investigators want to determine in patients with shigellosis the appearance of chemokines with the T cell reactivity of healthy controls and patients with watery diarrhoea which is not caused by Shigella.

This is a well planned study of an interesting topic with excellent methodology. The kind of co-operation which is included in the study is positive and the possibility for an obviously well trained main applicant to apply her knowledge should be encouraged.

The plans made are already extensive and it might be difficult to add further aspects to the study. However, when such a nice clinical material is collected it takes quite an effort and I would be specifically curious to know how much TNF-α (mRNA and/or product) is produced by the innate immune cells. This is because TNF-α produced such seems to play a major role in activating the dendritic cells to optimal antigen presentation (Nature this year).

Also the applicant has, to judge from the bibliography, quite some experience of work with Shigella antibodies. Would no further information be obtained from such analyses in parallel? Various studies and a new vaccine show good protection against Shigella via antibodies. It seems therefore surprising not to study this protective parameter.

As a detail I would like to know which Shigella or, Shigella antigens, will be used in the cultures of the patient's lymphocytes preparation from the patients' own strains?

The budget seems adequate although the travel to Sweden seems to be very expensive (Business class?) and the >100% increase in the applicant's salary during the three years seems striking. However, I do not know enough about the local situation to comment on that.

(As a minor detail, irrelevant for my evaluation, but perhaps important if the application is sent to outside agencies: there are a number of small unnecessary linguistic errors.)

Date: Thu, 3 Sep 1998 15:07:37 +0000
From: Anne Ferguson <af@srv0.med.ed.ac.uk>
To: mathan@icddrb.org
Subject: research proposal

I have been over this carefully. Of course, I am familiar with the previous work and the success in collaboration with the Karolinska, including safe transfer of certain samples to allow use of techniques only available in Sweden.
I have absolutely no criticisms of the project and plans, and score as high on all items - thus supported without qualification.

Please do not pay me a fee for this review - either don't send anything, or transfer $50 to the ICDDRB development fund (as I think it is called).
Best wishes to all of you
Anne Ferguson

Title of the proposal: **The influence of innate immune mechanisms on T cells stimulation in shigellosis.**

PI: **Dilara Islam & Birger Christensson.**

Subject: **Response to the Reviewers' Comments**

Reviewer I:

A. We have incorporated the suggestion of the reviewer in the protocol, and will perform assays to determine TNF-α mRNA levels and secretion of TNF-α in fecal samples (page 5 & 9).

B. A number of studies have already been performed to measure the Shigella-specific antibody response, which are: 1) **Islam, D.**, Veress, B., Bardhan, P. K., Lindberg, A. A. and Christensson, B. Quantitative assessment of IgG and IgA producing cells in rectal mucosa during shigellosis. J Clin Pathol 50:513-520, 1997.

2) **Islam, D.**, Wretlind, B., Bardhan, P. K., Hammarstrom, L., Christensson, B., and Lindberg, A. A. Semi-quantitative estimation of *Shigella* antigen specific antibodies: Correlation to disease severity during shigellosis. APMIS. 104:563-574, 1996. 3) **Islam, D.**, Wretlind, B., Ryd, M., Lindberg, A. A. and Christensson, B. Immunoglobulin subclass distribution and dynamics of *Shigella*-specific antibody responses in serum and stool samples in shigellosis. Infect Immun. 63:2054-2061, 1995.

In addition, recently a number of articles have published about the *Shigella*-specific antibodies, their functions etc. Therefore, in this study we didn't include that part.

C. Heat killed *Shigella flexneri* and *S. dysenteriae* 1 and invasion plasmid associated antigens and Shiga toxin will be used as antigens for cell culture.

D. About the budget part: The budget that was submitted to the reviewer is reduced from 269,000 US$ to 169,000. Now the budget fro travel/year is 1500 US$, which is not a business class ticket cost for Dhaka-Stockholm-Dhaka. There was a miss calculation in the PI's salary part, which has been corrected already.

Reviewer II:

There was no comment from the reviewer.