chment 1. Date April 04, 1994

E SHEET) ETHICAL REVIEW COMMITTEE, ICDDR,B.

cipal Investigator Dr.Tasnim Azim Trainee Investigator (if any) _____

ication No. 94-007 Supporting Agency (if Non-ICDDR,B) _____

e of Study Cellular immunity and munohistopathology of the gut of children th shigellosis with and without complitions.

Project status:
(✓) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

cle the appropriate answer to each of the following (If Not Applicable write NA).

Source of Population:
(a) Ill subjects (Yes) No
(b) Non-ill subjects (Yes) No
(c) Minors or persons under guardianship (Yes) No

Does the study involve:
(a) Physical risks to the subjects (Yes) No
(b) Social Risks Yes (No)
(c) Psychological risks to subjects Yes (No)
(d) Discomfort to subjects (Yes) No
(e) Invasion of privacy Yes (No)
(f) Disclosure of information damaging to subject or others Yes (No)

Does the study involve:
(a) Use of records, (hospital, medical, death, birth or other) (Yes) No
(b) Use of fetal tissue or abortus Yes (No)
(c) Use of organs or body fluids (Yes) No

Are subjects clearly informed about:
(a) Nature and purposes of study (Yes) No
(b) Procedures to be followed including alternatives used (Yes) No
(c) Physical risks (Yes) No
(d) Sensitive questions Yes No NA
(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 NA

5. Will signed consent form be required:
(a) From subjects Yes (No)
(b) From parent or guardian (if subjects are minors) (Yes) No
6. Will precautions be taken to protect anonymity of subjects (Yes) No
7. Check documents being submitted herewith to Committee:
___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
✓ Protocol (Required)
✓ Abstract Summary (Required)
___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
___ Informed consent form for subjects
✓ Informed consent form for parent or guardian
___ Procedure for maintaining confidentiality
___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

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

Tasnim Azim
Principal Investigator

Trainee

A-031973

APPLICATION FOR PROJECT GRANT

1.	PRINCIPAL INVESTIGATOR	:	Tasnim Azim
	COINVESTIGATORS	:	Firdausi Qadri M.M. Islam M. John Albert Pradip K. Bardhan
	CONSULTANTS	:	Dr. J. Alero Thomas* Prof. D.H. Crawford*

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London School of Hygiene and Tropical Medicine.
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2.	TITLE OF PROJECT	:	Cellular immunity and immunohistopathology of the gut of children with shigellosis with and without complications
3.	STARTING DATE	:	When funds are available
4.	COMPLETION DATE	:	3 years from start
5.	TOTAL BUDGET REQUESTED	:	US$ 220.272
6.	FUNDING SOURCE	:	
7.	HEAD OF PROGRAMME	:	Dr. R. Bradley Sack Associate Director Laboratory Sciences Division

R. Bradley Sack

8. AIMS OF PROJECT

a) General aim

To ascertain whether changes in different aspects of granulocyte function and immunohistology of the gut are associated with complications from shigellosis.

b) Hypotheses and Specific aims

1) *Shigella dysenteriae* type 1 induces changes in different aspects of granulocyte function which is more severe in children with complications and therefore a crucial factor in determining disease outcome. Thus, the study will compare the generation of superoxide and bacterial killing by polymorphonuclear cells from children suffering from shigellosis (with and without complications) with that of healthy children.

2) Leukemoid reaction and haemolytic uremic syndrome from *S. dysenteriae* type 1 is accompanied by alterations in lymphocyte phenotype in the circulation which may be secondary to phenotypic changes in the gut. To test this hypothesis the study will compare the distribution of different subsets of lymphocytes and the expression of various lymphocyte antigens in the circulation and gut of children suffering from shigellosis (with and without complications) with those of children suffering from watery diarrhoea.

3) Children with complications from shigellosis may have altered expression of cell adhesion molecules (CAMs) in the gut. In order to ascertain whether this is so, the study will compare the expression of CAMs in the gut of children suffering from shigellosis (with and without complications) with that of children with watery diarrhoea.

c) Rationale

Complications from shigellosis may be severe and sometimes fatal. Some of these complications, such as leukemoid reaction and haemolytic uremic syndrome (HUS) are almost exclusively seen in children, particularly children from 1-5 years of age infected with *S. dysenteriae* type 1. The cause(s) for these complications are unknown, however, they may be precipitated by an abnormal or inappropriate immune response. Early results of an ongoing study being conducted in our laboratory show changes in the phenotype of peripheral blood mononuclear cells (PBMs) of children with leukemoid reaction and HUS from shigellosis. These systemic changes are only an indication of the actual events occurring in the gut. A thorough study of the phenotype of gut lymphocytes and the expression of CAMs is essential not only to the understanding of the immunopathogenesis of the disease but also to relate systemic with local changes.

Results from the same ongoing study also show that in children with shigellosis, granulocytes polarise less in response to chemoattractants than in healthy children. In addition, microbial killing (of *S. dysenteriae* 1) by granulocytes was examined in 8 children with complications from shigellosis. No killing was observed in any of these experiments while 100% killing was always observed when granulocytes from healthy adults were used. Therefore, defects in various aspects of granulocyte function may be responsible for determining the disease outcome in shigellosis. It is therefore relevant to examine the different functional properties of granulocytes from children with shigellosis (with and without complications).

d) Significance

The study will define cellular and immunohistological changes in shigellosis which have not been investigated previously in paediatric patients. This will provide further understanding of disease pathogenesis, identify specific immunopathological changes of prognostic significance, indicate areas of treatment and new strategies for clinical management.

9. ETHICAL IMPLICATIONS

Children between 1-5 years will be enrolled in the study. Children enrolled will include those who come to the Clinical Research Centre (CRC) of the ICDDR,B with a history of passage of bloody, mucoid stools; children with a history of acute watery diarrhoea matched for age and nutrition and healthy children (i.e. with no history of illness in the last month) also matched for age and nutrition.

The ethical implications of this study are outlined below:

a) The study will not interfere with the management and treatment of the children and none of the procedures will be harmful.

b) Seven ml blood will be drawn at initial enrollment from all children. In children with confirmed *S. dysenteriae* 1 infection, blood will be taken again 3-5 days later and 14 days after discharge (follow-up). Thus, not more than 14 ml blood will be drawn from any child in one week. Since most children will be 1-5 years old, their blood volume will be over 400 ml. Therefore, drawing of 14 ml blood will not be detrimental to the patient.

c) From children with confirmed *S. dysenteriae* 1 infection and from children with watery diarrhoea, rectal biopsies (3 punches each 5-6 mm in size) will be taken using a rectosigmoidoscope on the day following enrollment. If required children will be sedated using Diazepam (0.1-0.2 mg/kg) intravenously. This is a safe procedure when performed by an experienced physician.

d) The nature and purpose of the study including the procedures involved will be explained to the guardian of the children and a written consent will be obtained.

10. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) BACKGROUND

The immune response in shigellosis has not been clearly elucidated. Studies carried out so far have concentrated on specific antibodies to various antigens of *Shigella*. Thus, antibodies to the lipopolysaccharide (LPS), invasion plasmid antigens and Shiga toxin have been found in serum (Keusch *et al.*, 1976; Lindberg *et al.*, 1984; Oaks *et al.*, 1986; Chen *et al.*, 1991; Oberhelman *et al.*, 1991), saliva (Oberhelman *et al.*, 1991; Schultz *et al.*, 1992), stool (Winsor *et al.*, 1988; Oberhelman *et al.*, 1991), breast milk (Cleary *et al.*, 1989) and duodenal aspirates (Oberhelman *et al.*, 1991). Very few studies have addressed the question of cellular immunity in shigellosis. Natural killer cells have been shown to exert cytotoxic effects against *Shigella flexneri* infected cells (Klimpel *et al.*, 1986, 1988) and a CD4+ T cell clone directed against *S. flexneri* has been generated from an infected individual (Zwillich *et al.*, 1989). Furthermore, antibody secreting cells to LPS of *S. dysenteriae* type 1, *S. flexneri* (Raqib *et al.*, 1993) and *S. sonnei*

(Orr *et al.*, 1992) have been demonstrated in infected individuals. Locally, there is an increase in the percentage of CD4+ and CD8+ T lymphocytes in the lamina propria and in intraepithelial lymphocytes (IELs) as well as in the expression of major histocompatibility complex II (MHCII) antigens by IELs (Raqib *et al.*, in press).

Studies on the immune response in complications from shigellosis are even fewer. Many patients, particularly children, infected with *Shigella* may develop severe complications and a chronic illness associated with malnutrition, shigellaemia, leukemoid reaction or HUS. Leukemoid reaction is associated with a WBC count of ≥40,000/cumm, granulocytosis and an increase in immature granulocytes (Rahaman *et al.*, 1975; Butler *et al.*, 1984). HUS consists of a triad of haemolytic anaemia, thrombocytopenia and acute renal failure which may be related to the deposition of immune complexes (Koster *et al.*, 1978). The cause(s) for these severe complications is unknown but it is possible that they are precipitated by an inappropriate immune response.

An ongoing study in our laboratory is investigating whether there are dysfunctional granulocytes and immature lymphocytes in leukemoid reaction and HUS in shigellosis. Preliminary results of this study show that granulocytes from children with shigellosis polarise (change shape) less in response to the chemoattractant *N*-formyl-methionyl-laucyl-phenylalanine (FMLP) than granulocytes from healthy children (a 2 log higher concentration of FMLP is required to obtain peak polarisation). However, the same granulocytes are able to ingest yeast particles efficiently. For optimal anti-bacterial activity, granulocytes must polarise in response to chemoattractants, leave the circulation, by first adhering to endothelial cells and then migrating through the blood vessels (diapedesis), to the site of infection (chemotaxis)

where they engulf (phagocytosis) and finally degranulate and kill the organism. It appears that in shigellosis, the initial polarisation step is somewhat hampered. This defect in itself may be enough to allow bacterial accumulation. On the other hand, as phagocytosis appears to be intact it is possible that granulocytes that are able to polarise are sufficient in number to effectively reduce bacterial load. However, it is not known whether these granulocytes, after phagocytosis, can degranulate and kill microorganisms. Experiments on microbial killing conducted in our laboratory suggest that there could be a defect in this aspect of granulocyte function. Using granulocytes from 8 children with complications from *S. dysenteriae* 1 infection, no killing of *S. dysenteriae* 1 was observed in any patient while 100% killing occurred with granulocytes from healthy adults. However, comparisons with healthy children and children with shigellosis but without complications could not be carried out. These early results suggest that granulocyte defect contributes to the development of complications in shigellosis. However, to be certain, various aspects of granulocyte function need to be investigated systematically. For this purpose, this study will compare the ability of granulocytes from children with shigellosis, with and without complications, to generate superoxide in response to stimulants and to kill *Shigella*, with that of granulocytes from control children.

Our findings also show that lymphocytes in peripheral blood have an altered phenotype in children with shigellosis. Children who recover without developing complications have a reduced percentage of CD3+ (pan T) lymphocytes and an increased percentage of CD8+ (suppressor/cytotoxic) lymphocytes when compared to healthy children (P=0.002 and P=0.02, respectively). These findings suggest that defense against *Shigella* may be partly mediated via

cytotoxic CD8+ T cells. Raqib *et al.* (in press) also showed an increased percentage of CD4+ and CD8+ T lymphocytes in the gut of adults suffering from shigellosis. In children who develop complications there are reduced percentages of CD3+ (P=0.005) and CD4+ (helper/inducer) (P=0.001) lymphocytes and no change in the percentage of CD8+ T lymphocytes when compared to healthy children or children with shigellosis but without complications. It therefore appears that in children who develop complications not only is there a reduced percentage of CD4+ T lymphocytes, which are usually pivotal in specific immune responses, but also no increase in the percentage of CD8+ T lymphocytes which may be required for defense against *Shigella*. The cause for the decrease in circulating CD3+ T lymphocytes and their major subsets cannot be gauged from this study. Either there is decreased production of mature T lymphocytes as suggested by the findings of Jackson *et al.*, (1979a,b) or reduced migration of T lymphocytes to local sites of infection i.e. the gut as suggested by the findings of Raqib *et al.* (in press). However, neither study systematically compared cellular changes in the gut with those in the periphery. This study will attempt to clarify this point by comparing lymphocyte phenotype in the gut with that of peripheral blood lymphocytes in the blood of children with shigellosis (with and without complications) and compare the findings with that of children with watery diarrhoea.

In addition to looking at the distribution of lymphocyte subsets and their state of activation in the gut it is important to consider the expression of cell adhesion molecules (CAMs) on leucocytes and endothelial cells. CAMs are critical to the generation of inflammatory and immune responses and regulate cell-cell and cell-matrix interaction (table 1) (for reviews see Pardi *et al.*, 1992; Zimmerman *et al.*, 1992; Shimizu *et al.*, 1992). Thus, a critical event in inflammatory and immune responses is the migration of leucocytes (both

granulocytes and lymphocytes) from the circulation to tissue. This process involves a coordinated sequence of events using multiple receptor-ligand interactions, the so called "adhesion cascade". There are four steps in this cascade: first is tethering or loose binding of leucocytes to endothelium via one of the selectin family of molecules, second is triggering involving integrin molecules which are converted from an inactive to an active form in the leucocytes, third is strong adhesion of leucocytes to the endothelium via the activated integrin molecules and fourth is migration to the endothelial cell junction and transmigration of leucocytes through vascular endothelium. This requires reduction in adhesion possibly occurring due to shedding of selectin from the cell surface and the transient expression of activated integrins. The regulatory mechanisms of CAMs are complex and not clearly understood: circulating leucocytes become adherent in response to changes in the microenvironment such as the presence of thrombin, histamine and cytokines including IL-1 and TNFα (Bevilacqua *et al.*, 1989). In addition, LPS from Gram negative bacteria increases leucocyte adhesiveness (Bevilacqua *et al.*, 1989) while pertussis toxin inhibits integrin-related adhesion (Mackay and Imhof, 1993). Thus, bacterial antigens as well as inflammatory cytokines affect the expression of CAMs. Therefore, it can be postulated that in shigellosis two opposing factors influence CAM expression: LPS and bacterial induced cytokines enhancing expression and Shiga toxin inhibiting expression as it is known to inhibit protein synthesis (Brown *et al.*, 1980). Perhaps the latter effect predominates in children who develop complications from shigellosis due to *S. dysenteriae* 1. A comparison of the expression of CAMs in children who recover from shigellosis versus those who develop complications will reveal differences, if any, between the two.

b) RESEARCH PLAN

The following groups of children will be studied:

Disease condition	No. of patients	Age of patients (yrs)	Samples	Source of patients
S. dysenteriae 1 infection	30	1-5	Peripheral blood (7 ml) Rectal biopsy	ICDDR,B
S. dysenteriae 1 infection with complications	30	1-5	Peripheral blood (7 ml) Rectal biopsy	ICDDR,B
Watery diarrhoea (as controls)*	30	1-5	Peripheral blood (7 ml) Rectal biopsy	ICDDR,B
Healthy controls**	30	1-5	Peripheral blood (7 ml)	ICDDR,B

*Watery diarrhoea controls will be matched for age and nutrition

**Healthy controls will be matched for age and nutrition and will include children with no history of fever, runny nose, cough or diarrhoea for the last one month. Siblings of patients at ICDDR,B or children of volunteers in the urban volunteer programme of ICDDR,B will serve as healthy controls.

The nutritional status will be assessed by calculating weight for age and will be classified as follows:

Weight (percentage of standard*)	Oedema	
	Present	Absent
80-60	Kwashiorkor	Under weight
<60	Marasmic Kwashiorkor	Marasmus

Standard* : 50th Centile of NCHS

The required sample, n, for estimating different immunological parameters for each of the groups has been obtained using the following equation:

$$n = \frac{\alpha^2 \delta^2}{\epsilon^2}$$

Where α is the value of normal variate for which the estimated value will be within $\pm\epsilon$ of the population with a probability of $(1-2\alpha)$. We have considered the variances of different immunological parameters and found that a sample size of 30 is sufficient to limit the error within 20% of the population parameter with 95% confidence limit.

Seven ml blood will be obtained by venepuncture from all children on enrollment. After confirmation of *S. dysenteriae* type 1 infection, 7 ml blood and stool will again be taken 3-5 days later and at follow-up which will be 14 days after discharge. From children with confirmed *S. dysenteriae* type 1 infection and from children with watery diarrhoea, rectal biopsies (3 punches each 5-6 mm in size) will be taken using a rectosigmoidoscope on the day following enrollment. The reason for enrolling only children with *S. dysenteriae* type 1 infection is that most children with complications are infected with *S. dysenteriae* type 1. Our ongoing study (quoted earlier in the text) shows that out of 30 children with complications only 29 were infected with *S. dysenteriae* type 1. Thus, enrolling only children with *S. dysenteriae* type 1 infection will preclude any differences in immune responses due to differences in the infecting organism.

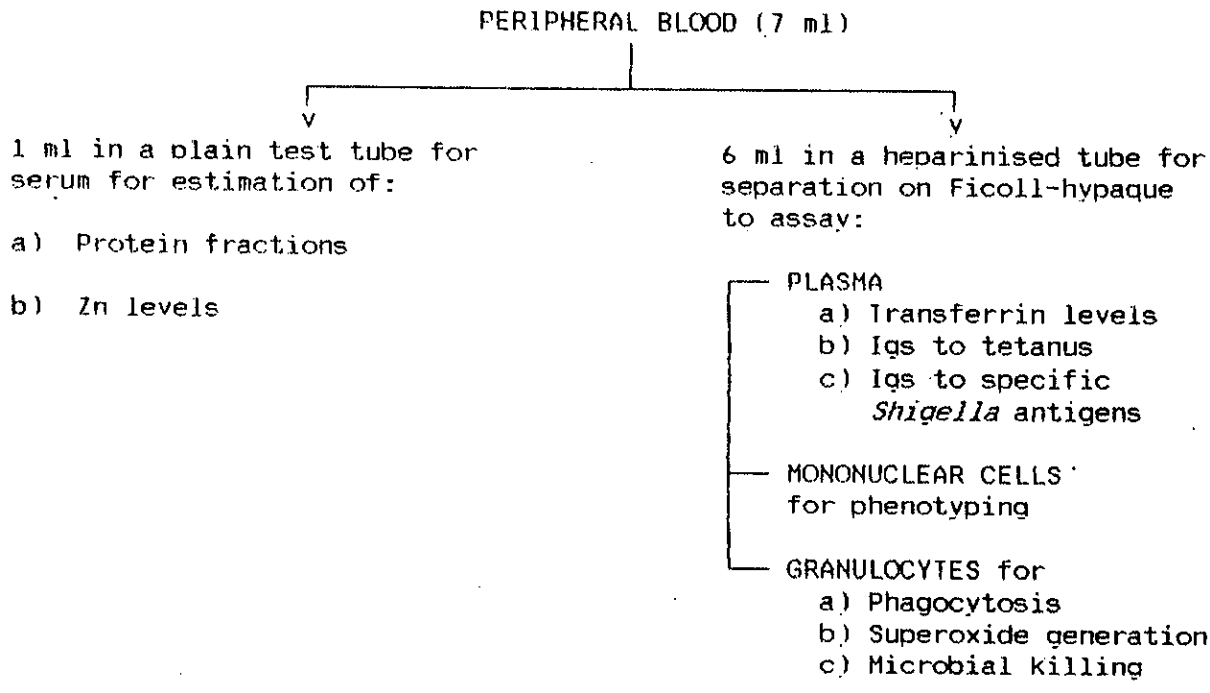
All children will be clinically evaluated by a physical examination and measurements of weight, temperature, heart and respiratory rates will be

recorded as per the procedures of the CRC of ICDDR,B. Stools from all children will be examined microscopically for cellular elements and will also be cultured.

Tests will be carried out on: (i) peripheral blood, and (ii) rectal biopsies.

Peripheral Blood: Peripheral blood will be used for experiments on serum, plasma, lymphocytes (mononuclear cells) and granulocytes. Seven ml blood will be required from children and this will be obtained by venepuncture. The haematological tests to be carried out include measurements of haematocrit and WBC counts (total and differential). The immunological and nutritional parameters to be studied are described below and shown in the following flow chart:

Nutritional and immunological assays on peripheral blood



1. Protein fractions will be estimated by electrophoresis.

2. Zinc levels will be measured as zinc has profound effects on immunity and may be lowered in malnutrition. Zinc will be measured by an atomic absorption spectrophotometer.

b) Plasma

1. Transferrin levels will be measured by turbidimetry using COBAS BIO. Like zinc it has profound effects on immunity and can be lowered in malnutrition.

2. Antibodies to tetanus will be estimated by ELISA only if children have been immunised against tetanus. This will be measure of the overall immune status of children.

3. Antibodies to LPS, Shiga toxin and outer membrane porteins of different *Shigella* serotypes will be estimated by ELISA.

c) Mononuclear cells

Phenotyping by indirect immunofluorescence will be carried out to determine proportions of peripheral blood T lymphocytes, B lymphocytes and T lymphocyte subsets using monoclonal antibodies against CD3 (pan T lymphocytes), CD4 (helper/inducer subset of T lymphocytes), CD8 (suppressor/cytotoxic subset of T lymphocytes) and CD20 (B lymphocytes) antigens.

d) Granulocytes

1. Phagocytosis: Granulocytes will be incubated with Baker's yeast suspension and pooled human plasma from 6 healthy individuals for

60 minutes. Cells will then be centrifuged and resuspended in a drop of medium (HBSS). A cell smear will be made on a glass slide and stained with Wright's stain. Ingested yeast particles in 50 neutrophils will be counted under a light microscope.

2. Measurement of superoxide generation: Measurements will be based on cytochrome c reduction by superoxide (O^-_2) ions released from granulocytes. Briefly, granulocytes will be incubated with *S. dysenteriae* type 1 at 37°C for 90 min in the presence of cytochromec in phenol-red free Earle's balanced salt solution. The mixture will be centrifuged and the cell-free supernatant will be used to measure reduced cytochrome c in a spectrophotometer at 550 nm (Goldhar *et al.*, 1991).

3. Microbial killing: Granulocytes will be incubated with *S. dysenteriae* 1 and pooled human sera for 2 hr at 37°C. Granulocytes will then be lysed and the mixture plated and incubated overnight at 37°C when viable bacteria will be counted (Qadri *et al.*, 1993).

Rectal biopsies: Rectal biopsies will be obtained using a rectosigmoidoscope when 3 punches, each 5-6 mm in size will be taken. These will be collected in separate containers: 1 in 10% buffered neutral formal saline and 2 in HISTOCON (tissue transport medium). Samples fixed in formal saline will be embedded in paraffin and 5 µ thick serial sections will be made. The sections will be stained by haematoxylin and eosin before examination. HISTOCON samples will be snap frozen immediately and stored at -70°C till use. Frozen samples will be cut into 6 µ sections using a cryostat, the sections will be mounted on

glass slides, dried at room temperature and stained by standard immunocytochemical techniques (peroxidase, alkaline phosphatase, fluorescence) using monoclonal antibodies to lymphocyte antigens and CAMs. The antigens that will be examined include:

LYMPHOCYTE ANTIGENS

CD1	expressed on epithelial dendritic cells, e.g. Langerhans cells involved in antigen presentation, and on cortical thymocytes
CD3	pan T lymphocytes
CD4	T helper/inducer subset
CD8	T suppressor/cytotoxic subset
CD5	T and B lymphocyte subset
CD19	pan B lymphocyte
CD20	pan B lymphocyte
CD23	B lymphocyte subset
CD38	plasma cells

Immunoglobulins (Ig) M, A, G, D, E, k, l (to define lamina propria B lymphocyte and plasma cell populations).

CELL ADHESION MOLECULES

CD22	B lymphocyte adhesion molecule
CD44	lymphocyte homing molecule
HML1	human mucosal lymphocyte antigen-1
ELAM-1	endothelial-leucocyte adhesion molecule
ICAM-1,-2	intercellular adhesion molecules expressed on haemopoeitic and epithelial cells and inducible by cytokines
VCAM-1	vascular adhesion molecule-1
LFA-1 (CD11a/18)	lymphocyte function antigen (-1) acts as ligand for ICAMs-1,-2

MAC-1 (CD11/CD18)	macrophage associated ligand for ICAMs-1,-2
VLA-4 (CD49)	very late antigen expressed on B and T lymphocytes and ligand for VCAM-1

Data analysis

Statistical differences in the various immunological parameters will be determined using the Wilcoxon's rank sum test. Comparisons will be made between the patient groups i.e. children with shigellosis with and without complications and between the patient groups and the control groups i.e. healthy children and children with acute watery diarrhoea.

c) BIBLIOGRAPHY

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11. PUBLICATIONS OF PRINCIPAL INVESTIGATOR (last five years)

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12. FLOW CHART

- Children with dysentery will be enrolled whenever available.

- Nutritionally matched children with acute watery diarrhoea and healthy controls will be enrolled subsequently. This will require approximately 3 years, however that will depend on the number of patients available, which can be very variable.

- From all these children, samples of serum and plasma will be stored at -20°C while fresh granulocytes and lymphocytes will be used in all experiments. Biopsy samples will be processed and appropriately stored.

- When all samples have been collected they will be assayed for the different parameters. Assays on serum and plasma will take approximately 6 month. Immunohistochemistry of biopsy samples for lymphocyte antigens and CAMs will be done during the same 6 months.

- Data analysis will be carried out in the last 6 months.

13. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATOR

Dr. Tasnim Azim (75%)

1. Lymphocyte phenotyping
2. Immunohistochemistry for lymphocyte antigens and CAMs
3. Supervise work in the laboratory and coordinate with physicians regarding patient enrollment and sample collection
4. Analyse data and write reports.

Dr. Firdausi Qadri (10%)

- Granulocyte studies

Dr. M.M. Islam

- Provide help with the immunohistochemical techniques

Dr. J. Alero Thomas

- Provide scientific and academic feedback

Dr. P. Kumar Bardhan

- Perform biopsies and ensure patient care

Research Trainee (Clinical)

- Enroll patients, provide patient-care and collect blood

Dr. M. John Albert

- Ensure smooth running of protocol

14. BUDGET (US$)

		Year-1	Year-2	Year-3	Total
a)	Personnel costs:				
	Tasnim Azim (NOB. S4) (75% 1st year, 100% for 2nd and 3rd year)	8,463	12,412	13,652	34,527
	Research Officer (GS-IV, S1) (2) (100%)	10,004	11,004	12,104	33,112
	Laboratory Attendant (GSII, S1) (100%)	2,412	2,653	2,918	7,983
	Research Trainee	1,800	1,800	1,800	5,400
	Subtotal:	22,679	27,869	30,474	81,022
b)	Supplies and materials:				
	Plasticwares	12,000	13,200	15,000	40,200
	Reagents (antibodies, etc.)	12,000	13,000	15,000	40,000
	Media, sera, etc.	3,000	3,300	3,630	9,930
c)	Patient cost:				
	Hospitalization	3,000	3,000	3,000	9,000
	Follow-up transport cost	500	500	500	1,500
d)	Other costs:				
	Clinical laboratory cost (for TC, DC, stool microscopy, etc.)	1,000	1,100	1,210	3,310
	Clinical biochemistry	0	0	2,000	2,000
	Interdepartmental services (bioengineering/maintenance/ medical illustration)	1,000	1,100	1,210	3,310
	Subtotal:	32,500	35,200	41,550	109,250
e)	Capital equipment - Cryostat	30,000			
	TOTAL (US$)	85,179	63,069	72,024	220,272

TA:mh/T1A:CELIM2.PRT

JUSTIFICATION FOR CAITAL BUDGET

Processing of frozen tissue sections require a good quality cryostat. The cryostat in the Histopathology Laboraory does not work properly as the sections we get from it are more than 6 μ in thickness. Such thick sections are not suitable for immunohistochemistry.

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM

We are conducting a study on children with bloody dysentery caused by a bacteria called *Shigella* to see why some children develop severe complications. For this purpose, healthy children will also have to be studied. The study will help us to better understand the disease and thereby prevent complications in future. We would like to enroll your child in the study with your permission. For this, we will take 7.0 ml (1½ teaspoon) of venous blood and stool once from your child. These are safe procedures, but if any untoward effect does occur, we will provide the necessary treatment. We will not mind even if you do not agree to let your child take part in the study. All the information obtained during the study will be confidential and if you wish to know the results, they will be provided to you on request as they become available. If you agree, please sign or put your left-thumb print impression below.

_____ _____

Signature or left-thumb print impression of guardian

Date

_____ _____

Signature of witness

Date

_____ _____

Signature of investigator

Date

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র, বাংলাদেশ

সম্মতি পত্র

"শিগেলা" নামক জীবাণুর কারণে যে সমস্ত শিশু রক্ত আমাশয়ে ভুগছে তাদের মধ্যে- কিছু রোগী বিভিন্ন ঔষধের প্রতি প্রতিরোধ গড়ে তুলছে যার কারণ জানার মধ্যে- আমরা একটা গবেষণা পরিচালনা করছি। এই মধ্যে- কিছু শিশুকে পরীক্ষা করতে হবে। এই গবেষণায় 'শিগেলা' জনিত রক্ত আমাশয় সম্পর্কে ভালভাবে জানতে এবং ভবিষ্যতে এই রোগের ঔষধ প্রতিরোধ করতে সহায়তা করবে। আপনার অনুমতি নিয়ে আমরা আপনার শিশুকে এই গবেষণায় নিতে চাই। এই জন্য আমরা আপনার শিশুর থেকে মাত্র এক বার থেকে চা চামচ রক্ত (৭ মি.লি.) ও পায়খানা নিয়ে পরীক্ষা করবো। এইটা সম্পূর্ন নিরাপদ ব্যবস্থা, কিন্তু তবুও যদি কোন অসুবিধা ঘটে আমরা তার জন্য প্রয়োজনীয় ব্যবস্থা নিব। আপনি যদি এই গবেষণার অনুমতি না দেন তাহলেও আমরা দুঃখিত হবো না।

আপনি যদি আপনার শিশুকে এই গবেষণায় অংশ গ্রহন করতে দিতে রাজী হন তবে দয়া করে নীচে আপনার নাম বা দিলসই (বাম হাতের বৃদ্ধাঙ্গুলীর ছাপ) দিন।

অভিভাবকের স্বাক্ষর / দিপ-সই — তারিখ

গবেষণাকারীর স্বাক্ষর — তারিখ

সাক্ষীর স্বাক্ষর — তারিখ

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM

We are conducting a study on children with bloody dysentery caused by a bacteria called *Shigella* to see why some children develop severe complications. We do not know how *Shigella* causes complications and in order to understand more about this illness, we are conducting a study which, in future, may help us prevent or treat it. For this purpose children with watery diarrhoea will also have to be studied. We would like your permission to enroll your child in this study.

During the study, your child will be hospitalized for 2 days. Stool and 7 ml of venous blood (approximately 1½ teaspoon) will be collected on the day of admission. On the day after admission, we will introduce a small tube in your child's rectum and collect three small samples of rectal tissue. If necessary, we will give some medicine to sedate your child during the procedure. All these procedures are safe. However, we will provide treatment for any untoward effect if it were to occur.

Your child will receive the same care and treatment that is normally provided whether he/she is enrolled in the study or not. If at any time you wish to withdraw your child from the study, you are free to do so. All the information obtained during the study will be confidential and if you wish to know the results, they will be provided to you on request as they become available.

If you agree to let your child participate in the study, please sign or put your left-thumb impression below.

_____	_____
Signature or left-thumb print impression of guardian	Date
_____	_____
Signature of witness	Date
_____	_____
Signature of investigator	Date

আন্তর্জাতিক উদরাময় গবেষণা কেন্দ্র, বাংলাদেশ।

অনুমতি-পত্র

কিশোরগঞ্জ" নামক জীবাণুর কারণে যে আমাশয় কিশোর-কিশোরীর শরীরে ঢুকছে তাদের অন্ত্রে কিছু রোগী বিশিষ্ট রকম জটিলতার সৃষ্টি হয়, যার কারণ জানার জন্য আমরা একটা গবেষণা পরিচালনা করছি। কিশোরগঞ্জ জীবাণুর আঘাতে জটিলতার সৃষ্টি করে তা এখন পর্যন্ত জানা যায়নি, তাই এই গবেষণা ভবিষ্যতে এ জীবাণুর প্রতিরোধ এবং চিকিৎসায় আমাদের সাহায্য করবে। এই জন্য যে আমাশয় রোগী পাওয়া যায় যাদের জীবাণু ঢুকছে তাদের কোন গবেষণা করার প্রয়োজন আছে। সেই কারণে এই গবেষণায় আপনার কিশোরের রক্ত গ্রহণ করতে দেওয়ার জন্য আমরা আপনার কাছ থেকে অনুমতি চাচ্ছি।

এই গবেষণার জন্য আপনার কিশোরকে দুইদিন হাসপাতালে থাকতে হবে। ভর্তির দিন আপনার কিশোর থেকে দেড় চা-চামচ (৭ মিলি) রক্ত এবং পায়খানা নেওয়া হবে, ভর্তির পরের দিন আমরা আপনার কিশোর-কিশোরীর হাতের নল ঢুকিয়ে তিনটা খাবার-মিশ্রণ প্রবেশ করব। যদি আপনার কিশোরকে আজীবন এই ক্যানুলা রাখার জন্য দেওয়া হবে। এই ঠাণ্ডাগুলো একদম নিরাপদ, তবুও যদি কোন অসুবিধা হলে আমরা তা

আপনি আপনার রোগীকে এই গবেষণায় অন্তর্ভুক্ত করুন আর নাই করুন এই চিকিৎসা কেন্দ্রে যে সকল রোগীকে চিকিৎসা দেওয়া হয় তা সে নিয়মমতই পাবে। আপনি চাইলে যে কোন সময় আপনার রোগীকে এ গবেষণা থেকে নিয়ে যেতে পারেন। এই গবেষণার সকল তথ্য গোপন রাখা হবে এবং যদি আপনি ফলাফল জানতে চান তবে তা লিখিত কাগজ পত্র আপনাকে জানানো হবে।

আপনি যদি আপনার রোগীকে এই গবেষণায় অংশ গ্রহণ করতে দিতে রাজী হন তবে নিম্ন করে নীচে আপনার নাম ও সিগনেচার (বাম হাতের বৃদ্ধাঙ্গুলির ছাপ) দিন।

চিকিৎসকের স্বাক্ষর _____ তারিখ _____

গবেষণা কর্মীর স্বাক্ষর _____ তারিখ _____

সাক্ষীর স্বাক্ষর _____ তারিখ _____

INTERNATIONAL CENTRE FOR DIARRHOEAL DISEASE RESEARCH, BANGLADESH

CONSENT FORM

Your child has bloody diarrhoea caused by a germ called *Shigella* which can be very serious and can lead to severe complications. We do not know how *Shigella* causes complications and in order to understand more about this illness, we are conducting a study which, in future, may help us prevent or treat it. We would like your permission to enroll your child in this study.

During the study, your child will be hospitalized for 5 days. Stool and 7 ml of venous blood (approximately 1½ teaspoon) will be collected on the day of admission and again 3-5 days later. On the day after admission, we will introduce a small tube in your child's rectum and collect three small samples of rectal tissue. If necessary, we will give some medicine to sedate your child during the procedure. All these procedures are safe. However, we will provide treatment for any untoward effect if it were to occur.

We will ask you to bring your child back 14 days after discharge when we will again collect samples of blood and stool.

Your child will receive the same care and treatment that is normally provided whether he/she is enrolled in the study or not. If at any time you wish to withdraw your child from the study, you are free to do so. All the information obtained during the study will be confidential and if you wish to know the results, they will be provided to you on request as they become available.

If you agree to let your child participate in the study, please sign or put your left-thumb impression below.

_____ _____

Signature or left-thumb print impression of guardian

Date

_____ _____

Signature of witness

Date

_____ _____

Signature of investigator

Date

আন্তর্জাতিক উদরাময় গবেষনা কেন্দ্র, বাংলাদেশ।

সম্মতি পত্র

আপনার শিশু শিগেলা নামক এক প্রকার জীবাণুর কারণে রক্ত আমাশায় ভুগছে যা সব সময় মারাত্মক রোগ জটিলা বিষক্রিয়া সৃষ্টি করতে পারে। শিগেলা কিভাবে মানুষের শরীরে এই ভাবে সৃষ্টি করে তা এখন পর্যন্ত জানা যায়নি। এই রোগ সম্পর্কে আরো ভালোভাবে জানার জন্য আমরা একটা গবেষনা করছি যা ভবিষ্যতে এ রোগের প্রতিরোধে এবং চিকিৎসায় আমাদের সাহায্য করবে। এই কারণে এই গবেষনায় আপনার শিশুকে অংশগ্রহন করতে দেওয়ার জন্য আমরা আপনার কাছ থেকে অনুমতি চাচ্ছি।

এই গবেষনার জন্য আপনার শিশুকে পাঁচ দিন হাসপাতালে ভর্তি থাকতে হবে। ভর্তির দিন এবং ডিসচার্জের ৩-৫ দিন পর আপনার শিশুর থেকে (দেড় চা চামচ) রক্ত (৭ মিলি) এবং পায়খানা সংগ্রহ করা হবে। ভর্তির পরের দিন আমরা আপনার শিশুর মলদ্বারে একটা ছোট নল ঢুকিয়ে তিনটা ছোট সিরিঞ্জ রক্ত গ্রহন করবো। প্রয়োজনে আপনার শিশুকে স্বাভাবিক এবং শান্ত রাখার ব্যবস্থা করবো। এই ব্যবস্থাগুলো সম্পূর্ন নিরাপদ। কিন্তু তবুও যদি কোন অসুবিধা হয় আমরা তার জন্য প্রয়োজনীয় ব্যবস্থা নিব।

বাড়ীতে ফেরার ২৪ দিন পরে আপনার শিশুকে আবার রক্ত এবং পায়খানা পরিক্ষার জন্য আমাদের কাছে নিয়ে আসতে হবে, তাপনি আপনার শিশুকে সেই

গবেষনায় - যুক্ত করুন আর নাই করুন সেই ব্যক্তি
কোন যে সকল প্রকার তথ্য দিতে হবে তা
যে নিয়ম গতিতে পাবে। আপনি চাইলে যে কোন
সময় আপনার শিশুকে এ গবেষনা থেকে নিয়ে যেতে
পারেন। এই গবেষনায় সকল তথ্য গোপন রাখা হবে
এবং যদি আপনি ফলাফল জানতে চান তবে তা দিতে
করার পর আপনাকে জানানো হবে।

আপনি যদি আপনার শিশুকে এই গবেষনায়
অংশ গ্রহন করতে দিতে রাজি হন তবে দয়া করে
নীচে আপনার সই বা টিপসই দিন।

অভিভাবকের স্বাক্ষর

তারিখ

গবেষনা কারীর স্বাক্ষর

তারিখ

সাক্ষীর স্বাক্ষর

তারিখ

SHIGELLA

ABSTRACT SUMMARY

Complications from shigellosis may be severe and sometimes fatal. The cause(s) for some of these complications, such as leukemoid reaction and haemolytic uremic syndrome (HUS) is not known but they may be precipitated by an abnormal or inappropriate immune response. Preliminary data from a recent study conducted in our laboratory (entitled "Study of the immune response to*S. dysenteriae* 1 in an effort to identify abnormalities leading to the development of leukemoid reaction", protocol no. 89-014) suggest alterations in some aspects of granulocyte function as well as changes in the phenotype of peripheral blood lymphocytes of children with leukemoid reaction and HUS from shigellosis. The defects observed in granulocyte function do not preclude the ability of those granulocytes to kill bacteria and therefore the role of granulocytes in precipitating complications in shigellosis remains unanswered. Changes in peripheral blood lymphocyte phenotype may be an indication of the actual events occuring in the gut, however, this cannot be said with certainity as cellular immunological changes in the gut of children with shigellosis have never been described. This study will, therefore, examine the ability of granulocytes to kill bacteria from children with shigellosis, with and without complications, and compare with that of children with watery diarrhoea and healthy controls. A thorough study of the phenotype of gut and peripheral blood lymphocytes will be carried out to relate systemic with local changes. Finally, the expression of cell adherence molecules in the gut, which will lead to a better understanding of the cellular dynamics at local sites in shigellosis, will also be carried out.

1. Leukemoid reaction and HUS are life threatening complications in shigellosis and are almost exclusively seen in children, particularly children between 1-5 yrs of age and rarely in adults. This study will therefore enroll children between 1-5 yrs. and the groups of children that will be included are: 30 children with *S. dysenteriae* 1infection without complications, 30 children with *S. dysenteriae*1 infection with complications, 30 children with watery diarrhoea and 30 healthy control children . Normal parameters are not available for Bangladeshi children for which reason healthy children need to be enrolled. However, only blood will

be taken from these children. Healthy children will be obtained from the Nutrition Follow Up Unit of ICDDR,B. Our recent study entitled "Study of the immune response to*S. dysenteriae* 1 in an effort to identify abnormalities leading to the development of leukemoid reaction" (protocol no. 89-014) has used the same source for healthy control children.

2. The risks of the study will include the pain associated with drawing blood and the potential for bruising which sometimes occurs, and secondly the small risk from the rectal biopsy. The biopsy risk will be minimised since this will be performed by an experienced gastroenterologist who wil obtain the specimens from the rectum and not higher (i.e. not from the intraperitoneal portion of the colon). For children with dysentery, this is a frequently used diagnostic procedure in all clinical settings including that of ICDDR,B.

3. The major inconvenience for the patient will be the proctoscopy and the biopsy procedures. These procedures are being performed currently in ICDDR,B for clinical reasons, and in experienced hands the risks are negligible. All standard precautions will be taken before these procedures.

4. Anonymity will be maintained by using only identification numbers during analyis.

5. a) A signed consent form will be obtained. The Bengali consent form will be read out to the guardian of the subject when the subject is brought to ICDDR,B.

b) Information will not be witheld.

c) This has been mentioned in the consent form.

6. Clinical interview will be carried out for clinical history and clinical examination which will take approximately 20 mins.

7. The subject will get the best possible treatment that is available in ICDDR,B. This study will provide further understanding of disease pathogenesis, identify specific immunopathological changes of prognostic significance, indicate areas of treatment and new strategies for clinical management.

8. The study will use hospital records, blood and rectal biopsies.