ICDDR,B LIBRARY
DHAKA 1212

Attachment 1.
(FACE SHEET)

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date DMONO 12-5-97
06-1-98

Principal Investigator TASNIM AZIM Trainee Investigator (if any) _____

Application No. 97-020 (modified) Supporting Agency (if Non-ICDDR,B) _____

Title of Study EFFECT OF ZINC ON THE IMMUNE & INFLAMMATORY RESPONSES OF CHILDREN TO Shigella/Infection & CORRECTION WITH CLINICAL SEVERITY, PERSISTENCE & GROWTH

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

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

1. Source of Population:
   (a) Ill subjects (Yes) No
   (b) Non-ill subjects Yes (No)
   (c) Minors or persons under guardianship (Yes) No
2. Does the study involve:
   (a) Physical risks to the subjects Yes (No)
   (b) Social Risks Yes (No)
   (c) Psychological risks to subjects Yes (No)
   (d) Discomfort to subjects (Yes) No
   (e) Invasion of privacy (Yes) No
   (f) Disclosure of information damaging to subject or others Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) (Yes) No
   (b) Use of fetal tissue or abortus Yes (No)
   (c) Use of organs or body fluids (Yes) No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study (Yes) No
   (b) Procedures to be followed including alternatives used (Yes) No
   (c) Physical risks (Yes) No
   (d) Sensitive questions (Yes) No
   (e) Benefits to be derived (Yes) No
   (f) Right to refuse to participate or to withdraw from study (Yes) No
   (g) Confidential handling of data (Yes) No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure (Yes) No
5. Will signed consent form be required:
   (a) From subjects Yes (No)
   (b) From parent or guardian (if subjects are minors) (Yes) No
6. Will precautions be taken to protect anonymity of subjects (Yes) No
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ✓ Protocol (Required)
   ✓ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ✓ Informed consent form for parent or guardian
   ✓ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

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

Jasmin Azim
Principal Investigator

Trainee

27 NOV 2004

ICDDR,B LIBRARY
DHAKA 1212

APPLICATION FOR A PROJECT GRANT

TITLE:
Effect of zinc supplementation on the immune and inflammatory responses of children to *Shigella dysenteriae* type 1 infection, and correlation with clinical severity of illness and growth following recovery.

INVESTIGATORS FROM ICDDR,B:

Principal Investigators: Dr. Tasnim Azim, Laboratory Sciences Division
Dr. Swapan Kumar Roy, Clinical Sciences Division

Co-principal Investigator: Dr. George Fuchs, Clinical Sciences Division

Co-investigators: Dr. Rubhana Raqib, Laboratory Sciences Division
Dr. Dilara Islam, Laboratory Sciences Division
Dr. Nurul Huq Alam, Clinical Sciences Division
Mr. M. A. Wahed, Laboratory Sciences Division
Dr. Manuel John Albert, Laboratory Sciences Division

EXTERNAL INVESTIGATOR:
Prof. Anne Ferguson, Professor of Gastroenterology,
University of Edinburgh, Western General Hospital,
Edinburgh, UK.

TOTAL BUDGET: US$ 155,072

STARING DATE: As soon as possible

ENDING DATE: Two years from start

HEAD OF PROGRAMME: Director, LSD

ABSTRACT:
Shigellosis is a major cause of morbidity and mortality in young children in Bangladesh and other developing countries. Further, the increasing emergence of resistance to a wide range of antibiotics is of great concern. Another major public health problem in Bangladesh is malnutrition which is closely linked with shigellosis and with a high mortality. In shigellosis, a heavy nutritional burden is placed on children and vital micronutrients such as vitamin A is lost in the urine. We recently found that the immune response in *S. flexneri* infection was lower in children who were severely malnourished

27 NOV 2004

ICDDR,B LIBRARY
DHAKA 1212

(weight-for-age ≤65% as a percentage of the National Centre for Health Statistics median) when compared to children with weight-for-age from >65-75% . T cell responses were primarily affected with lowered CD4/CD8 ratios, lowered proliferative responses to T cell mitogens, Conconavalin A (ConA) and phytohaemagglutinin (PHA). However, proliferation of peripheral blood mononuclear cells (PBMs) was lowered only in the presence of autologous plasma suggesting that a factor(s) in plasma, probably nutritional, rather than a defect in cells themselves was responsible. In children with *S. dysenteriae* 1 infection, proliferative responses to PHA were similarly lowered in the presence of autologous plasma but inhibition correlated to lowered transferrin levels in plasma and not to the weight-for-age of the children. Also severely malnourished children with either *S. flexneri* or *S. dysenteriae* 1 infection were more severely ill. These findings show that immunity in malnourished children with *Shigella* infection is impaired which may lead to more severe illness. As zinc has profound effects on immunity as well as clinical outcome in diarrhoeal diseases, it is possible that zinc deficiency may be a factor in reducing immunity and increasing severity of acute illness in malnourished children with shigellosis. In this study, we will investigate the effect of zinc supplementation, in a double blind placebo controlled trial, on inflammatory responses, immune responses, outcome of acute illness and growth following recovery from acute illness with *S. dysenteriae* 1 infection. As *S. dysenteriae* 1 causes more severe illness and is associated with the highest mortality, this study will concentrate on children with *S. dysenteriae* 1 infection.

HYPOTHESES:

1. Zinc supplementation in malnourished children with *S. dysenteriae* 1 infection reduces intestinal inflammation.

2. Zinc supplementation in malnourished children with *S. dysenteriae* 1 infection improves key elements of the immune response.

3. Zinc supplementation in malnourished children with *S. dysenteriae* 1 infection decreases the severity of the acute illness and promotes subsequent growth.

4. Enhanced immune response secondary to zinc supplementation is associated with rapid recovery from acute shigellosis and improved growth after recovery.

OBJECTIVES:

1. To determine whether zinc supplementation improves the inflammatory response in malnourished children with *S. dysenteriae* 1 infection.

27 NOV 2004

2. To determine whether zinc supplementation improves the immunological response in malnourished children with *S. dysenteriae* 1 infection.

3. To determine whether zinc supplementation reduces the severity of the acute illness, and improves growth following recovery in malnourished children with *S. dysenteriae* 1 infection.

4. To correlate the changes in immunity, if any, with the severity of acute illness and growth following recovery from the acute illness.

BACKGROUND:

Significant morbidity and mortality is caused by shigellosis in developing countries such as Bangladesh and particularly in children below 5 years of age. The highest mortality with shigellosis is seen in children with malnutrition (Bennish and Wojtyniak, 1991). In shigellosis, a heavy nutritional burden is placed on children and vital micronutrients such as vitamin A is lost in the urine (Mitra et al, 1996). Therefore, nutritional supplementation during the acute illness and during convalescence from shigellosis appears attractive as a simple means of reducing mortality. This is confirmed by the findings that a protein rich diet given to children during convalescence from shigellosis improved subsequent growth in those children (Kabir et al, 1993) and more recent, unpublished data suggest that vitamin A supplementation improves the outcome in shigellosis (Shahadat et al, personal communication). We recently found that the immune response in *S. flexneri* infection was lower in children who were severely malnourished (weight-for-age $\leq$65% as a percentage of the National Centre for Health Statistics median) when compared to children with weight-for-age from >65-75% (Azim et al, 1996a). We also found that the immune response in children with *S. dysenteriae* 1 infection is lower in children with lower levels of transferrin in their plasma (our unpublished data). Thus nutritional status appears to be an important determinant of the immune response to *Shigella* infection which may then influence outcome of illness. Since amongst the shigellae, infection with *S. dysenteriae* 1 is associated with the most morbidity, we will study the effect of zinc supplementation on the inflammatory and immunological responses to *S. dysenteriae* 1 infection and correlate responses to severity of acute illness and growth following recovery from the acute illness.

The inflammatory response in shigellosis:
Shigellae are highly invasive and during the process of invasion they induce a strong inflammatory response. The inflammatory lesions primarily involve the distal colon (Speelman et al, 1984) and are characterised by oedema, patchy haemorrhage, goblet cell depletion, crypt hyperplasia, infiltration of polymorphonuclear cells (PMNs) and mononuclear cells, shedding of epithelial cells and focal haemorrhage (Mathan and Mathan, 1991). Shigellae invade gut epithelial cells via the M cells overlying Peyer's patches in the small intestine (Perdomo et al, 1994a) and via the basolateral surface of

epithelial cells (Mounier et al, 1992). Entry via the basolateral surface follows the opening of the paracellular pathway by PMNs (Perdomo et al, 1994b). PMNs may be attracted to the site of infection by bacteria themselves. Renesto et al, (1996) have shown that shigellae can themselves induce activation of PMNs via the Ipa invasins. Once shigellae invade, they infect the resident macrophages and induce apoptotic cell death (Zychlinsky et al, 1992). Results from rectal biopsies of adults during the acute phase of infection is characterised by increased cell turnover in the lamina propria and the epithelium, increased inducible nitric oxide synthase (iNOS) expression in the surface epithelium and apoptosis of the lamina propria macrophages (Islam et al, 1997). Following apoptosis, IL1 is released from the macrophages and inflammation is initiated (Zychlinsky et al, 1994). IL1 has been shown to be a major trigger in the inflammatory reaction induced by shigellae as treatment with IL1 receptor antagonist lowers the extent of inflammation, tissue destruction and bacterial invasion in animal models (Sansonetti et al, 1995). Also, in adults with shigellosis, significantly more IL1 producing cells are found in rectal biopsies of patients with more severe inflammation compared to those with milder inflammation (Raqib et al, 1995a). *S. flexneri* has also been shown to initiate PMN degranulation (Renesto et al, 1996) the products of which can lead to tissue destruction and one of the major injurious enzymes released by PMNs is elastase. In children with *S. dysenteriae* 1 infection, PMNs are very polarised (activated) and polarisation is more marked in children with systemic complications (Azim et al, 1995b). Inflammatory changes in the gut of adults with shigellosis have been found to persist even during convalescence (30 days after illness) (Raqib et al, 1995a). Most of these adults had protein deficiency so that it is possible that their nutritional status influenced the extent of inflammation. It can, therefore, be hypothesised that with malnutrition and micronutrient deficiencies, the balance between inflammation and protection is perturbed in shigellosis.

Thus, some of the key factors in the inflammatory response in shigellosis are activation of PMNs and release of elastase, apoptosis of macrophages and release of IL1. All these responses are influenced by malnutrition and micronutrient deficiencies such as zinc. PMNs from malnourished children are more polarised (activated) (Azim et al, 1995), more adherent and show diminished directed migration (Anderson et al, 1983). All these abnormalities in PMN function are reversible with nutritional recovery (Anderson et al, 1983). The relationship between PMN function and zinc concentrations is more complex as subnormal concentrations of zinc in the serum induces peak phagocytosis by PMNs (Lennard, 1980) while excessive zinc inhibits chemotaxis and phagocytosis (Chandra, 1984). Apoptosis of macrophages is inhibited by zinc (Meikle et al, 1996). However, the effect on IL1 secretion is less clear. In PEM, IL1 secretion is lowered (Bhaskaram and Shivakumar, 1986) but zinc deficiency does not affect IL1 levels (Falus et al, 1996) although the kinetics of IL1 secretion is altered (Flynn et al, 1984). Therefore, it is possible that supplementation with zinc may modulate the inflammatory response in shigellosis such that it is beneficial to the host.

The immune response in shigellosis:
Recently considerable work has been published on the immune response in shigellosis in adults (Raqib, 1995c; Islam, 1995a) and in children (Azim et al, 1995a; 1996a; 1996b; 1996c). To summarise, T lymphocytes appear to have a role in protection in shigellosis as they are activated during the acute infection in both the peripheral blood (Islam et al, 1995) and the gut (Raqib et al, 1994) and patients with AIDS have a more severe illness (Baskin et al, 1987). The humoral response shows higher antibody levels to all *Shigella* antigens in the mucosal versus the systemic compartments and the antibody levels directly correlate with disease severity so that they may be a marker of infection rather than protection (Islam et al, 1996). Of the cytokines, IL1β, tumour necrosis factor (TNF)α, IL6, IL8, are all elevated in stool during the acute illness (Raqib et al, 1995b; Azim et al, 1995a) and cytokine levels were found to be higher in patients with more severe illness. On the other hand, interferon (IFN) γ is lowered during acute infection and levels rise with recovery so that upregulation of IFNγ production correlates with immunity in shigellosis (Raqib et al, 1996). Data from these studies also show that the immune responses in patients with *S. dysenteriae* 1 and *S. flexneri* infection are quantitatively different so that patients with *S. dysenteriae* 1 infection who are more severely ill have a stronger response. Also, the immune responses in patients with *S. dysenteriae* 1 and *S. flexneri* infection are qualitatively different as the phenotype of PBMs is different in the two groups of patients (Islam et al, 1995b).

We have recently shown that in children with *S. flexneri* infection with severe malnutrition (≤65% weight-for-age of the National Centre for Health Statistics median) T cell responses are impaired when compared with better nourished children (Azim et al, 1996a). We found lowered CD4/CD8 ratios in the peripheral blood, lowered proliferative responses of peripheral blood mononuclear cells (PBMs) to T cell mitogens Conconavalin A (ConA) and phytohaemagglutinin (PHA). However, proliferation of PBMs was lowered only in the presence of autologous plasma suggesting that a factor in plasma, probably nutritional, rather than a defect in cells themselves was responsible. In children with *S. dysenteriae* 1 infection, PHA responses are similarly inhibited in the presence of autologous plasma (our unpublished data). However, inhibition in children with *S. dysenteriae* 1 infection was observed irrespective of weight-for-age rather it correlated with plasma transferrin levels. Therefore, it is possible that nutritional factors in the plasma may influence the immune response. It is known that zinc has strong effects on immunity (Myrvik, 1994). There are a large number of zinc dependent enzymes and these include enzymes required for transcription and translation. Cells of the immune system are continuously proliferating and differentiating and require numerous enzymes that use zinc as a cofactor. Therefore, zinc deficiency results in impairment of several aspects of the immune system, particularly T cell function. The effects include thymic atrophy, lowered delayed type hypersensitivity responses, reduced responses of lymphocytes to mitogens, impaired T cell maturation and impaired secondary antibody responses (Chandra, 1991; Sanderson and Walker, 1991). Zinc supplementation can enhance IFNγ production (Salas and Kirchner, 1987). It is therefore

possible that the immune response will be boosted by zinc supplementation during acute shigellosis.

Effect of nutrition on severity of acute illness and growth following shigellosis.
We found that malnourished children with *S. flexneri* (Azim et al, 1996a) and *S. dysenteriae* 1 infection (our unpublished data) are more severely ill and it is known that malnourished children with shigellosis have the highest mortality (Bennish and Wojtyniak, 1991). Also a decrease in linear growth is more common in children with a history of dysentery (Black et al, 1984; Henry et al, 1987) but this may be prevented by providing children with a high protein diet during convalescence (Kabir et al, 1993). Zinc supplementation has been shown to enhance linear growth in children with malnutrition and in children with acute watery diarrhoea (Behrens et al, 1990; Roy, 1995). Zinc supplementation also reduces the severity and duration of acute watery diarrhoea (Sazawal et al, 1995) as well as persistent diarrhoea (Tomkins et al, 1993). Additionally, zinc supplementation improves intestinal permeability in malnourished children with acute or persistent diarrhoea which could be a reflection of enhanced mucosal repair (Roy et al, 1992). Similarly, in shigellosis zinc supplementation was shown to improve intestinal permeability (Alam et al, 1994). These findings strongly suggest a positive role for zinc in the outcome of acute illness and growth following shigellosis.

In summary, malnutrition does compromise the immune response to *Shigella* infection and if this affects the clinical outcome of the disease, a similar effect may be encountered with vaccines that are currently under trial for shigellosis (Karnell et al, 1992; Lindberg et al, 1990). Therefore, micronutrient supplementation may not only enhance the immune response to natural infection but also to vaccines.

METHODS

Study design: A double blind randomised clinical trial with subsequent follow-up.

Study Population:
Children with dysentery (passage of mucus and/or blood in stools with abdominal pain) attending the CRSC will initially be enrolled. Of these, only those who fulfil the specified inclusion and exclusion criteria will be included.

Inclusion criteria:
- moderate malnutrition (weight-for-length 70-80% by Waterlow classification)
- 12-60 months of age
- duration of diarrhoea 0-3 days
- culture-confirmed *S. dysenteriae* 1 in stool on enrolment

Exclusion criteria:

- measles infection in the last six months
- presence of obvious systemic illnesses such as pneumonia, meningitis, septicaemia, leukemoid reaction, haemolytic uraemic syndrome
- residence beyond a distance requiring more than 2 hours journey from the CRSC
- received zinc supplementation

Children with culture-confirmed *S. dysenteriae* 1 infection will be randomised into two treatment groups using a random number table and permuted block lengths of 16. The two groups are:

Group I zinc and multivitamin
Group II multivitamin (control)

Double blinding will be done by having identical bottles of syrup labelled with numbers which will be allocated to the children in the two groups chronologically according to their serial number within each group. All children with *S. dysenteriae* 1 infection will receive pivmecillinum at 60 mg/kg body weight/day in four divided doses for 5 days along with one of the two syrups. Zinc will be given as 20 mg elemental zinc in two divided doses daily for two weeks and multivitamins will be given as twice the RDA. When children are discharged, they will be given the bottles of syrup to drink at home for up to two weeks from the beginning of supplementation. As the diet of children during acute shigellosis can influence growth during convalescence (Kabir et al, 1993), all children will receive a standardised diet of 100-125 kcal and 3-4 g of protein in mixed food per kg body weight per day during hospitalisation.

Study samples will be collected only from children with culture confirmed *S. dysenteriae* 1 infection, i.e. approximately 2 days after initial enrolment. Study samples include venous blood (7 ml), rectal biopsy material (three pinches each approximately 2 mm in size, using a rectosigmoidoscope), urine and stool and these will be collected before supplementation and 7 days after supplementation. Blood and stool will also be collected at convalescence (30 days after the onset of diarrhoea). Children will be followed monthly for up to 6 months after the onset of diarrhoea to assess growth.

Sample size calculation:

a) Based on linear growth:
Zinc supplementation in patients with acute diarrhoea was shown to increase growth by 11.8±5.5 (Behrens et al, 1990). Therefore, if a target difference of 30% is to be achieved, with 5% significance and 80% power, the number of patients to be studied in each group is 38.

b) Based on clinical recovery (duration of illness) with a 25% reduction, 5% significance and 80% power, the number of patients to be studied in each group is 30.

c) Based on immunological responses (lymphocyte proliferation) with a 20% reduction, 5% significance and 80% power, the number of patients to be studied in each group is 38.

Therefore, 76 (38 X 2) children with *S. dysenteriae* 1 infection will have to be enrolled. The CRSC surveillance data of 1995 (Fuchs and Faruque, 1995) show that of the children between 70-80% weight-for-length, 12-60 months of age and with 0-3 days history of dysentery, 75 had *S. dysenteriae* 1 infection. Given our strict inclusion criteria, competition with other ongoing studies and a 15% drop out rate, approximately 18 months will be required to enrol 76 children with *S. dysenteriae* 1 infection.

Investigations:

The following will be investigated:

Nutritional status:

1. Assessment of growth and growth velocity:
Measurements will include weight, height, triceps skinfold thickness (TSF), bioelectric impedance assessment (BIA) and knemometry. These will be done before supplementation, 7 days after supplementation, and every month thereafter for up to 6 months after the onset of diarrhoea. Weights will be obtained to the nearest 10 g using an electronic digital scale (Secca model 770, Sweden) standardised with a one kg standard weight prior to each weighing. Recumbent length will be measured in children less than two years of age using a slideboard infantometer (Harpenden, St. Albans, England). In older children, standing height will be determined with a locally constructed instrument in which a metal tape measure is extended between a footplate and head bar. The mean of two consecutive height measurements to the nearest 0.5 cm will be recorded as the observed value. Measurements will be compared to the standards according to the NCHS data and the nutritional status will be assessed by Z score (Hamil et al, 1979; Waterlow et al, 1977). Percent of median equivalents of standard deviation units will be determined and categorised according to the system of Waterlow (Waterlow, 1972). Body mass index (weight/height^2) and TSF will also be assessed. BMI results will be compared to the standardised curves of the National Health and Nutrition Examination Study while TSF will be compared to standards of Karlberg et al (1968). The mean of two consecutive measurements for all indices will be recorded as the observed value. Health workers will be extensively trained in the anthropometric measurements, and measurements will be repeated until minimum in-between variance is reached.

Knemometry
Height increments are such that measurements at least 8 weeks apart are required before height velocity can be accurately assessed (Wales et al, 1994). Portable knemometry by experienced operators permits far more accurate measurements of the heel-knee length, enabling meaningful length velocity assessment over shorter periods of time (Michaelson

et al, 1991). A hand-held electronic knemometer, resembling a pair of callipers will be used for knee heel measurement. The measuring system is based on a magnetic encoder, has a digital read-out and a resolution of 0.01 mm. The knee heel length will be recorded automatically when the pressure applied on the heel reaches a pre-set value. The measuring will be done by relaxing the child in a supine position. With 90° flexion at both knee and hip, the left arm of the callipers will be held against the child's knee. The knee will be supported with the left hand such that it is not too tight yet not allowing it to move while pressure is applied to the foot. The foot will be placed on a plate on the right arm of the callipers, so that the anterior end of the tibia will be parallel to the body of the callipers. The foot will then be supported by the fingers in the same way as the knee and increasing pressure will be applied on the right arm of the callipers. The readings will be recorded automatically when the pressure reaches a pre-set value.

BIA

Reactance and resistance will be measured with a RJL whole body plethysmograph model BIA-101. Total body water (TBW) will be calculated according to the formula of Fjeld et al, (1990) for use in infants in young children:

$$TBW\ (kg) = 0.48 + 0.68\ (height)2\ (cm)/Resistance\ (ohms)$$

(SEE = 0.36, r = 0.98).

2. Zinc will be measured in plasma using an atomic absorption photometer. This will be done prior to supplementation, 7 days after supplementation and at convalescence (30 days after the onset of diarrhoea).

Inflammatory responses:

1. Stool $\alpha 1$ anti-trypsin will be measured as described before (Alam et al, 1994). This will be done prior to supplementation, 7 days after supplementation and at convalescence.

2. C-reactive protein will be measured in plasma as described before (Khan et al, 1995) on all three sampling days.

3. Quantitation of luminal neutrophils will be done by measuring granulocyte elastase levels in stool (Handy et al, 1996). This will be done prior to supplementation, 7 days after supplementation and at convalescence.

4. Inflammatory responses in rectal pinch biopsy material will be ascertained by immunohistochemistry using a previously described pathological score (Raqib et al, 1995a). Rectal pinch biopsies will be obtained under direct visual guidance using limited flexible sigmoidoscopy. Quantitative microscopy of rectal biopsies and the use of computerised image analysis will be used to characterise epithelial and stromal constituents as further indices of tissue damage with the help of the Edinburgh group. This will be done prior to supplementation and 7 days after supplementation.

5. iNOS activity will be measured by a spectrophotometric assay using Greiss reaction for nitrite and nitrate in blood and urine (Kruppa et al, 1992). Immunohistochemical methods will be used to assess apoptotic cell death (Islam et al, 1997). The former will be done on three sampling days (prior to supplementation, 7 days after supplementation and at convalescence) and the latter, in the first two sampling days.

Immune responses:

1. Phenotype of circulating and gut lymphocytes (Islam et al 1995b; Raqib et al, 1994). 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. Additionally, expression of adherence antigens and activation antigens will be determined using appropriate monoclonal antibodies (from commercial sources). Phenotyping of PBMs will be analysed using a fluorescence activated cell sorter (FACS) in Edinburgh. Similar antigens will be determined in sections prepared from rectal biopsy material. The rectal biopsy sections will be stained by standard immunocytochemical techniques (peroxidase, alkaline phosphatase, fluorescence). Phenotyping of PBMs will be done on all three sampling days while that on rectal biopsy material will be done prior to supplementation and 7 days after supplementation.

2. Proliferation of blood mononuclear cells with *Shigella* antigens and T cell mitogens such as Conconavalin A and phytohaemagglutinin (PHA) in the presence of autologous plasma (Azim et al, 1996a). This will be done prior to supplementation, 7 days after supplementation and at convalescence.

3. Cytokines (IL1, IL8 and IFNγ) in plasma, stool and rectal biopsy material (Raqib et al, 1995b). Measurements in plasma and stool will be done by ELISA using commercially available kits (Endogen, USA and Genzyme, USA) and will be done on all three sampling days. Assays on rectal biopsy material will be done by immunohistochemistry with the help of the Edinburgh group. Assays on rectal biopsy material will be done before supplementation and 7 days after supplementation using commercially available reagents.

4. *Shigella*-specific antibodies (isotype and subclass) in plasma, stool and rectal biopsy material (Islam et al, 1996; Azim et al, 1996c). Measurements in plasma and stool will be done on all three sampling days but those on rectal biopsy material will be done before supplementation and 7 days after supplementation.

Severity of acute illness:

1. Clinical evaluation - duration of illness, frequency of stools, presence of blood in stool, tenesmus, vomiting, fever, appetite, dietary intake, weight changes.

2. Laboratory investigations- total and differential WBC counts in blood, serum electrolytes and creatinine concentrations. In stool, microscopy for semiquantitative estimation of leukocytes and erythrocytes will be done. These will be done on all three sampling days.

Dietary history following recovery:
Dietary intake assessment will be done weekly for six months following discharge. This will be carried out by dietary recall in the last 24 hrs. using a questionnaire. The following will be asked:

- amount of food consumed
- nature of food consumed
- frequency of intake
- assessment of fat, protein, energy and micronutrients from the history

Validation of the recall will be done by measuring spoons, bowls and serving pots before the recall. Individual items will be estimated from recall and compared with portions already estimated. Nutrient content will then be calculated from the reference food composition table of this area (Nutrient contents of Indian food. C. Gopalan, National Institute of Nutrition, Hyderabad, India).

Catch-up growth:
This will be determined by using anthropometric measures at monthly visits over six months following discharge by trained health assistants.

ANALYSIS PLAN:

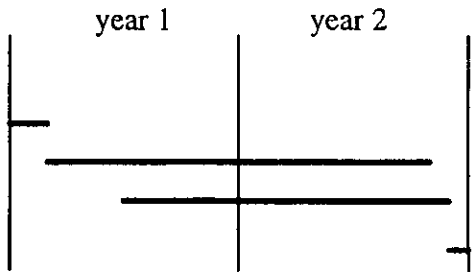
Children of the two study groups will be compared using the Mann Whitney U test (for non-parametric data). Categorical variables will be compared by the chi-square statistic. The Wilcoxon matched-pairs signed rank test will be used to analyse differences over time in the same group of children. Correlation between clinical and immunological parameters will be done using Spearman's correlation coefficient. The inflammatory and immunological parameters will be correlated with the severity of illness and growth following recovery in each of the two groups of children.

SIGNIFICANCE AND POTENTIAL IMPACT ON POLICY

This study will provide information regarding the effect of zinc supplementation on the immune and inflammatory responses and clinical outcome in shigellosis as well as catch-up growth and morbidity following shigellosis. The results from this study will have important direct implications in the management of shigellosis and in the prevention of morbidity following shigellosis. In addition, trials of candidate *Shigella* vaccines are now being planned at ICDDR,B. As in natural infection, a vaccine on its

own, without nutritional rehabilitation, may not elicit a strong immune response in malnourished children.

TIMEFRAME :



Initial set up
Patient enrolment with follow-up
Laboratory assays
Data analysis and write up

ETHICAL CONSIDERATIONS

The ethical implications of this study are outlined below:

1. The study will not, in any way, interfere with the management and treatment of the children. None of the procedures will be harmful and none will result in permanent physical damage or injury.

2. Seven ml (7.0 ml) of whole blood will be drawn before supplementation, 7 days after supplementation and at convalescence (30 days after the onset of diarrhoea) . Thus, not more than 14 ml will be drawn from any child within a 7-day period. Since all children will be 12-60 months old, who are expected to have a blood volume of over 400 ml, drawing of a maximum of 14 ml blood for the purpose of study will not be detrimental to the participating children.

3. Three pinches of rectal biopsies, approximately 2 mm each, will be taken using fibreoptic, paediatric rectosigmoidoscope before supplementation and 7 days after supplementation. This is a safe procedure when performed by an experienced physician. All patients will be hospitalised at the time of biopsy so that if there is excessive bleeding following biopsy, the patients can be appropriately and promptly managed. The hospital has its own blood bank and if necessary, immediate blood transfusion will be provided. The blood bank screens blood for major blood-borne pathogens including HIV and Hepatitis B.

BUDGET in US$

	Year 1	Year 2
T. Azim (30%)	4,067	4,271
S. K. Roy (20%)	4,200	4,410
N. H. Alam (5%)	784	823
Research Officer (100% X 2)	9,600	10,250
Health Assistant (100% X 2)	7,200	7,560
Medical Officer (100%)	8,133	8,654
Subtotal	**33,984**	**35,968**
Consumables (reagents and plastics)	20,000	21,000
Biopsy charges (US$ 20/endoscopy +US$15 for histopathological processing of each sample)	1,400	1,400
Clinical lab investigations (TC, DC, stool microscopy, culture, etc. US$19 per sample)	2,200	2,200
Patient hospitalisation (US$20/day for 5 days)	4,560	4,560
Rent, communication, utilities	3,000	3,000
Fax, email, communication, etc.	500	500
Print and publication	1,000	1,000
Travel		
Local (patient follow-up cost)	1,850	1,850
International (UK for~ 2 wk.)	3,500	3,500
Equipment		
Liquid nitrogen tank	800	
-20°C freezer	800	
Laser printer	1,500	
Knemometer	5,000	
Total (direct cost)	**80,094**	**74,978**

S. H. 11/9/97

Shamima Moin
Controller, Budget & Costing

JUSTIFICATION FOR BUDGET:

1. International travel - Prof. Anne Ferguson will provide help with the histopathology, immunohistochemistry and assays measuring neutrophil elastase. In addition,

ICDDR,B does not have fluorescence activated cell sorter (FACS) which will be required for the phenotyping of peripheral blood mononuclear cells. The FACS in Edinburgh will therefore be used in this study. Samples stored will be assayed at the end of the first year and again towards the end of the third year when patient enrolment is complete. At least two weeks in each trip will be needed for this purpose.

2. Local travel - approximately US$2 each way will be required for each visit. Therefore, for weekly visits over three months, i.e. 12 visits for 76 children, approximately US$3,650 will be needed.

3. Equipment - a liquid nitrogen tank will be needed to store the PBMs and rectal biopsy samples. A -20°C freezer will be required for storage of plasma and urine samples. A laser printer will be needed for the computer which is already present. A knemometer will be required to measure growth velocity.

REFERENCES

Alam AN, Sarker SA, Wahed MA, et al. Enteric protein loss and intestinal permeability changes in children during acute shigellosis and after recovery: effect of zinc supplementation. Gut. 1994. 35:1707-1711.

Anderson DC, Krishna GS, Hughes BJ, et al. Impaired polymorphonuclear leukocyte motility in malnourished infants: relationship to functional abnormalities of cell adherence. J Lab Clin Med. 1983. 101:881-895.

Azim T, Halder RC, Sarker MS, et al. Cytokines in the stools of children with complicated shigellosis. Clin Diagn Lab Immunol 1995a. 2:492-495.

Azim T, Islam LN, Halder RC, et al. Peripheral blood neutrophil responses in children with shigellosis. Clin Diagn Lab Immunol 1995b. 2:616-622.

Azim T, Sarker MS, Hamadani J, et al. Effect of nutritional status on lymphocyte responses in children with *Shigella flexneri* infection. Immunol Infect Dis. 1996a. 6:151-158.

Azim T, Sarker MS, Hamadani J, et al. Alterations in lymphocyte phenotype and function in children with shigellosis who develop complications. Clin Diagn Lab Immunol. 1996b. 3: 191-196.

Azim T, Qadri F, Ahmed S, et al. Lipopolysaccharide specific antibodies in plasma and stools of children with *Shigella*-associated leukemoid reaction and hemolytic uremic syndrome. Clin Diagn Lab Immunol. 1996c. 3:701-705.

Baskin DH, Lax JD, Barenberg D. *Shigella* bacteremia in patients with the Acquired Immune Deficiency Syndrome. Am J Gastroenterology. 1987. 82: 338-341.

Behrens RH, Tomkins AM, Roy SK. Zinc supplementation in diarrhoea:fortification against malnutrition. Lancet (letter). 1990. 336:8712.

Bennish ML, Wojtyniak B. Mortality due to shigellosis: community and hospital data. Rev Infect Dis. 1991. 13 (Suppl 4): S245-251.

Bhaskaram P, Sivakumar B. Interleukin-1 in malnutrition. Arch Dis Child. 1986. 61:182-185.
Black RE, Brown KH, Becker S. Effects of diarrhoea associated with specific enteropathogens on the growth of children in rural Bangladesh. Pediatr. 1984. 73:799-805.

Chandra RK. 1990 McCollum Award lecture. Nutrition and immunity: lessons form the past and new insights into the future. Am J Clin Nutr. 1991. 53:1087-1101.

Falus A, Beres J Jr. A trace element preparation containing zinc increases the production of interleukin 6 in human monocytes and glial cells. Biol Trace Elem Res. 1996. 51:293-301.

Fjeld CR, Freundt-Thurne J, Schoeller DA. Total body water measured by ^{18}O dilution and biolelectrical impedance in well and malnourished children. Pediatr Res. 1990. 27:98-102.

Flynn A, Loftus MA, Finke JH. Production of interleukin-1 and interleukin-2 in allogeneic mixed lymphocyte cultures under copper, magnesium and zinc deficient conditions. Nutr Res. 1984. 4:673-679.

Fuchs GJ, Faruque ASG. Hospital surveillance programme. In: Annual report of ICDDR,B. Dhaka: International Centre for Diarrhoeal Disease Research, Bangladesh. 1995: 23.

Hamil PV, Drizd TA, Johnson CL, et al. Physical growth: National Center for Health Statistics Percentages. Am J Clin Nutr. 1979. 32:607-629.

Handy LM, Ghosh S, Ferguson A. Investigation of neutrophils into the gut lumen by assay of granulocyte elastase in whole gut lavage fluid. Scand J Gastroenterol. 1996. 31:700-705.

Hammer LD, Kraemer HC, Wilson DM, et al. Standardized percentile curves of body mass index for children and adolescents. Am J Dis Child. 1991. 145:259-263.

Henry FJ, Alam N, Aziz KMS, et al. Dysentery, not watery diarrhoea, is associated with stunting in Bangladeshi children. Hum Nutr Clin Nutr. 1987. 41C:243-249.

Islam D. Cellular and humoral immune responses in shigellosis. Ph.D. thesis. Dept. of Immunology, Pathology and Infectious Diseases, Division of Clinical Bacteriology and Pathology, Karolinska Institute, Huddinge Hospital, Huddinge, Sweden. 1995a.

Islam D, Bardhan PK, Lindberg AA, et al. *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. 1995b. 63: 2941-2949.

Islam D, Wretlind B, Hammarstrom L, et al. Semiquantitative estimation of *Shigella* antigen-specific antibodies: correlation with disease severity during shigellosis. Acta Pathol Microbiol Immunol Scand. 1996. 104: 563-574.

Islam D, Veress B, Bardhan PK, et al. In situ characterization of inflammatory responses in the rectal mucosa of patients with shigellosis. Infect Immun. 1997. 65:739-749.

Kabir I, Malek MA, Mazumder RN, et al. Rapid catch-up growth of children fed a high-protein diet during convalescence from shigellosis. Am J Clin Nutr 1993. 57:441-445.

Karlberg P, Engstorm I, Lichtenstein H, et al. The development of children in a Swedish urban community: a prospective longitudinal study. III. Physical growth during the first three years of life. Acta Paediatr Scand (Suppl). 1968. 197:48.

Karnell A, Stocker BAD, Katakura S, et al. Live oral auxotrophic *Shigella flexneri* SFL124 vaccine with a deleted aro D gene: characterisation and monkey protection studies. Vaccine. 1992. 10: 389-394.

Khan WA, Salam MA, Bennish ML. C reactive protein and prealbumin as markers of disease activity in shigellosis. Gut. 1995; 37:402-405.

Kruppa G, Thoma B, Machleidt T, et al. Inhibition of tumour necrosis factor (TNF)-mediated NF-kB activation by selective blockade of the human 55kDa TNF receptor. J Immunol. 1992. 148:3152-3157.

Lindberg AA, Karnell A, Pal T, et al. Construction of an auxotrophic *Shigella flexneri* strain for use as a live vaccine. Microbial Pathogen. 1990. 8: 433-440.

Lennard ES. Implications in the burn neutrophil of serum and cellular zinc levels. J Surg Res 1980. 29:75-82.

Meikle I, Cummings J, Macpherson JS, et al. Induction of apoptosis in human cancer cell lines by the novel anthracenyl-amino acid topoisomerase 1 inhibitor NU/ICRF 505. Br J Cancer. 1996. 74:374-379.

Mitra A, Stephenson CB, Alvarez JO, et al. Depressed serum concentration and urinary excretion of retinol during acute shigellosis. FASEB J. 1996. 10: Abstr. A466.

Mounier J, Vasselon T, Hellio R, et al. *Shigella flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole. Infect Immun. 1992. 60:237-248.

Myrvik QN. Immunology and nutrition. From: Modern Nutrition in Health and Disease. Eds. ME Shils, JA Olsen, M Shike. Lea and Ferbiger. Philadelphia, Baltimore, Hong Kong, London, Munich, Sydney, Tokyo. 1994. pp623-662.

Perdomo OJJ, Gounon P, Sansonetti PJ. Polymorphonuclear leukocyte transmigration promotes invasion of colonic epithelial monolayer by *Shigella flexneri*. J Clin Invest. 1994a. 93:633-643.

Perdomo OJJ, Cavaillon JM, Huerre M, et al. Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis. J Exp Med. 1994b. 180:1307-1319.

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

Raqib R, Reinholt F, Bardhan PK, et al. Immunopathological patterns in the colonic mucosa of patients with shigellosis: expression of HLA-DR antigens and T lymphocyte subsets. Acta Pathol Microbiol Immunol Scand. 1994. 120: 13-24.

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

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

ICDDR,B LIBRARY
DHAKA 1212

Raqib R. Pathogenesis and immune responses in shigellosis. Ph.D. thesis. Dept. of Immunology, Pathology and Infectious Diseases, Division of Clinical Bacteriology and Pathology, Karolinska Institute, Huddinge Hospital, Huddinge, Sweden. 1995c.

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

Renesto P, Mounier J, Sansonetti PJ. Induction of adherence and degranulation of polymorphonuclear leukocytes: a new expression of the invasive phenotype of *Shigella flexneri*. Infect Immun. 1996. 64:719-723.

Roy SK, Behrens RH, Haider R, et al. Impact of zinc supplementation on intestinal permeability in Bangladeshi children with acute watery diarrhoea and persistent diarrhoea syndrome. J Pediatr Gastroenterol Nutr. 1992. 15:289-296.

Roy SK. Zinc supplementation in the treatment of childhood diarrhoea. Indian J Pediatr. 1995. 62:181-193.

Salas M, Kirchner H. Induction of interferon γ in human leukocyte cultures stimulated by Zn2+. 1987. 45:139-142.

Sansonetti PJ, Arondel J, Cavaillon J-M, et al. Role of interleukin 1 in the pathogenesis of experimental shigellosis. J Clin Invest. 1995. 96:884-892.

Sanderson IR, Walker WA. Nutrition and immunity. Current Opinion Gastroenterol. 1991. 7:463-470.

Sazawal S, Black RE, Bhan MK, et al. Zinc supplementation in young children with acute diarrhoea in India. N Engl J Med. 1995. 333:839-844.

Speelman P, Kabir I, Islam M. Distribution and spread of colonic lesions in shigellosis: a colonoscopic study. J Infect Dis. 1984. 150:899-903.

Mathan VI, Mathan MM. Morphology of the rectal mucosa of patients with shigellosis. Rev Infect Dis. 1991. 13 (Suppl 4):314-318.

Michaelson KF, Skov L, Badsberg JH, et al. Short term measurement of linear growth in preterm infants: validation of a hand-held knemometer. Pediatr Res. 1991. 30:464-468.

Tomkins A, Behrens R, Roy SK. The role of zinc and vitamin A deficiency in diarrhoeal syndromes in developing countries. Proceedings of the Nutrition Society. 1993. 52:131-142.

Wales JKH, Gibson AT. Short term growth: rhythms, chaos, or noise? Arch Dis Chld. 1994. 71:84-89.

Waterlow JC, et-al. The presentation and use of height and weight data for comparing the nutritional status of groups of children under the age of ten years. Bull World Health Organisation. 1977. 55:489-498.

Waterlow JC. Classification and definition of protein calorie malnutrition.. Br Med J. 1972. 3: 565-569.

Zychlinsky A, Prevost MC, Sansonetti PJ. *Shigella flexneri* induces apoptosis in infected macrophages. Nature. 1992. 358:167-169.

27 NOV 2004 A-040689

Zychlinsky A, Fitting C, Cavaillon J-M, et al. Interleukin 1 is released by murine macrophages during apoptosis induced by *Shigella flexneri*. J Clin Invest. 1994. 94:1328-1332.

BIOGRAPHICAL SKETCHES:

Tasnim Azim
Associate Scientist, Immunology, Laboratory Sciences Division, ICDDR,B.

Born: September 22, 1956.
Nationality: Bangladeshi

Education:

Degree	Institution	Year
MBBS	University of Dhaka	1983
Ph.D., Immunology	University of London	1989

Internship (Medical) Dhaka Medical College Hospital, 1983-1984.

Positions:

September 1995-present — Associate Scientist, Immunology, Laboratory Sciences Division, ICDDR,B

March 1989-September 1995 — Assistant Scientist, Immunology, Laboratory Sciences Division, ICDDR,B

Professional Societies:

British Society for Immunology
Society for Mucosal Immunology
Bangladesh Society for Immunology

Publications (1993 to present):

1. Qadri F, **Azim T**, Hossain A, Islam D, Mondol G, Faruque SM, Albert MJ. A monoclonal antibody to *Shigella dysenteriae* serotype 13 cross-reacting with Shiga toxin. FEMS Microbiol Lett 1993; 107:343-348.

2. Qadri F, Haque MA, Hossain A, **Azim T**, Alam K, Albert MJ. Role of *Shigella dysenteriae* type 1 slime polysaccharide in resistance to serum killing and phagocytosis. Microbial Pathogen 1993: 14;441-449.

APPENDIX I

COMPOSITION OF THE MULTIVITAMIN SYRUP

5ml syrup contains the following:

Vitamin A.	3000 IU
Vitamin D	600 IU
Vitamin B1	1.2 mg
Riboflavin	2.0 mg
Nicotinamide	6.0 mg
Calcium D Panthonate	6.0 mg

APPENDIX II

CONSENT FORM

EFFECT OF ZINC SUPPLEMENTATION IN MALNOURISHED CHILDREN WITH *SHIGELLA DYSENTERIAE* TYPE 1 INFECTION.

Your child is suffering from blood dysentery which is caused by infection due to a germ called *Shigella*. It has been shown that dysentery due to *Shigella* causes malnutrition and also decreases the immune response so that children become more susceptible to various infections. It is thought that improvement in children's nutritional status by nutritional supplements during shigellosis helps in improving immunity, promoting rapid recovery from the acute illness, better growth and health following recovery. One such nutritional supplementation could be zinc, a micronutrient, which has been shown to help growth of children following watery diarrhoea. In this study, we will study the effect of zinc which will be given for two weeks with multivitamins and conventional antibiotic therapy, i.e. pivmecillinum. For this purpose, children with shigellosis will be given either zinc plus multivitamin or multivitamin alone and the responses in the two groups of children will be compared. We would like to enrol your child in this study and if you agree to our proposal, you may expect the following:

1. Your child will be admitted in the research ward of this hospital until his/her condition permits discharge from the hospital, and during hospitalisation s/he will receive the usual good care of this hospital.

2. On the day of admission we will ask you questions related to the illness of your child including history and physical examination, and record all information. We will ask about medical problems and perform physical examination on your child on each day s/he remains in this hospital.

3. We will perform some tests which are routinely done at this hospital for such children which will include microscopic examination and culture of a stool sample.

4. Along with the routine treatment for shigellosis, your child will receive a syrup containing either zinc with multivitamin or multivitamin alone. This will have to be taken twice daily for two weeks. The syrup will provide additional nutrition to your child.

5. We will draw 7 ml (<1.5 teaspoonful) of venous blood from your child for special investigations. Blood will be drawn 2 days after enrolment but before starting the supplementation, 7 days after the first sample and 30 days after the onset of diarrhoea. Apart from the discomfort and mild pain involved in taking blood, drawing this amount of blood will not be harmful for your child. For the second and third samples of blood, we will ask you to bring your child back to this hospital.

6. We will collect urine from your child on the same days that we draw blood for special investigations.

7. We will collect three tiny pieces of mucosa, 2 mm each (about 1/4 the size of a grain of rice), from the rectum of your child for special investigation. This will be done after pacifying your child by administering diazepam injection in standard dose through introduction of a flexible paediatric endoscope by one of the investigators well experienced in performing the procedure. This will not be harmful to your child. This will be done twice, 2 days after enrolment of your child into the study and 7 days later. For the second biopsy procedure, we will ask you to bring your child back to hospital.

8. After your child has improved and been discharged from hospital we will visit your home regularly at weekly intervals to assess whether your child is well, to monitor growth and to ask you questions about the feeding habits of the child. This will be done for three months.

9. You are the one to decide participation of your child in this study. If your child does not participate in this study, or even if you withdraw your consent during the study, s/he will continue to receive the standard care and treatment of this hospital.

10. Information obtained from history and the laboratory investigations of your child will be kept strictly confidential and no one other than the investigators of this study and the Ethics Committee of this Centre will/can have access to the information.

11. If you want to know the results of any or all investigations we will happily provide those to you, subject to their availability. It may be mentioned that some of the tests will be done long after your child is discharged.

If you agree to our proposal for participation of your child in this study, please put your signature or left thumb impression at the specified space below:

Thank you for your co-operation.

_____	_____
Signature of the investigator	Date:
_____	_____
Signature/LTI of parent/guardian	Date:
_____	_____
Signature of the witness	Date: