ument 1.

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date 24.8.89 29/8/89

Principal Investigator Azim et al

Trainee Investigator (if any) _____

Application No. 89-008

Supporting Agency (if Non-ICDDR,B) _____

Title of Study DEVELOPMENT OF AN IMMUNOLOGICAL LABORATORY AND APPLICATION OF IMMUNE FUNCTION IN PERSISTENT DIARRHOEA, RECURRENT DIARRHOEA, DYSENTERY AND MALNUTRITION

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 NA
   (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 NA
   (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.

SEP 5 - 1989

(PTO)

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

Yasmin Ayim
Principal Investigator

_____
Trainee

89-006
29/8/89

APPLICATION FOR PROJECT GRANT

1. INVESTIGATORS : Dr. Tasnim Azim
Dr. Pradip Kumar Bardhan
Dr. Laila Noor Islam
Mr. M. A. Wahed

2. TITLE OF PROJECT : Development of an immunology laboratory and application of immune function tests to persistent diarrhoea, recurrent diarrhoea, shigellosis and malnutrition

3. STARTING DATE : As soon as possible

4. COMPLETION DATE : 3 years from starting date

5. TOTAL BUDGET REQUESTED : US$ 190,000

6. FUNDING SOURCE :

7. PROGRAMME HEAD : Dr. S. Tzipori S. Tzipori
Associate Director
Laboratory Sciences Division

8. AIMS OF PROJECT :

a) GENERAL AIM

To set up a clinical immunology laboratory in order to carry out a systematic investigation of the immune status of patients with persistent diarrhoea, recurrent diarrhoea, shigellosis and malnutrition.

b) SPECIFIC AIMS

1) A comparative immunological investigation between patients with acute and persistent diarrhoeas, in order to identify immunological abnormalities in patients with persistent diarrhoea.

A-033783

2) Application of similar immune function tests to identify parameters which might help identify abnormalities in patients who develop complications after shigellosis compared to those who recover from the acute illness.

3) Application of immune function tests to identify an immunological basis in patients with recurrent diarrhoeal episodes.

4) Assessment of the immune status of children with malnutrition, with and without diarrhoea by carrying out similar immunological investigations.

c) SIGNIFICANCE

A systematic study of the immune status of a large number of patients with diarrhoea and malnutrition may provide immunological markers that predict complications as well as provide insights into the immunopathogenesis of these conditions. In addition, a body of information will be obtained which may be useful in future studies including vaccine efficacy.

Ideally in order to identify immunological abnormalities to which a disease status may be attributed, knowledge of his or her immune status before the onset of illness is essential. In the study patients will be examined during the course of their illness which will make the association between any

immunological abnormality that may be detected at that time with the current illness, without the preexisting information, tenuous. However, should one or more immunological abnormalities be identified in relation to a particular disease, specific follow-up studies can then be designed to address or confirm these observations as cause or effect.

9. ETHICAL IMPLICATION

a) ACUTE AND PERSISTENT DIARRHOEA

Patients from 0-2 years suffering from diarrhoea not due to Shigella will be included and 3 ml of venous blood will be drawn from such patients. If the diarrhoea persists, further 3 mls of blood will be drawn at days 10 and 17 after onset of diarrhoea. Saliva and stool samples will be collected at the same time and delayed type hypersensitivity (DTH) will also be studied. Such volumes of blood will not affect the patient.

b) RECURRENT DIARRHOEA

Patients (3 months-2 years) with acute diarrhoea and with a history of 4 or more separate episodes of diarrhoea in the preceding 3 months will be included in this study. 3-5 mls of blood will be drawn during the acute stage of illness and a further bleed will be obtained 3 weeks after discharge, at convalescence.

Stool and saliva will be collected at the same time. DTH will also be studied.

c) SHIGELLOSIS

Samples will be obtained from patients with shigellosis in collaboration with Dr. Bennish, when ~7 ml blood will be drawn on admission, on days 3, 5 after admission and 30 days after discharge. Saliva and stool will be collected and DTH tested.

d) MALNUTRITION

Children will be assessed clinically for malnutrition & those with severe malnutrition will not be included in the study. Venous blood will be drawn (3-5 ml) once from the patients. Stool and saliva samples will be collected and DTH tested.

e) CONTROLS

3-5 mls of venous blood will be taken once from control subjects admitted to Shishu Hospital and Pongu Hospital for children without infection. Stool and saliva will be collected and DTH tested. Their nutritional status will be assessed and only children without malnutrition will be included. Controls will be age-matched for which purpose they will be divided into 2 groups of age; 0-2 yrs and 2-5 yrs.

All these children will be assessed for malnutrition by anthropometry.

The study will not interfere with the management and treatment of the patients and none of these procedures will be harmful to the children. A written consent will, however, be obtained from the guardian.

Gut biopsies will be carried out on patients when the expertise is available to the Centre. For this purpose a separate ethical consideration will be sought.

10. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

The immune system constitutes a major host defense mechanism along with the non-specific defense mechanisms which include the skin, mucous membrane, the complement system, phagocytes and soluble mediators such as interferon. A malfunction, whether a decrease or an alteration, results in disease as is seen in certain hereditory conditions, e.g. chronic granulomatous disease and certain acquired conditions, e.g. in infectons, autoimmune disease, acquired immune deficiency syndrome (AIDS), lymphomas, etc. The importance of the role of host defense mechanisms cannot be overstated which emphasises the need for the assessment of the immune status of patients especially those who are suffering from chronic illness.

The Clinical Research Centre of ICDDR,B receives 150-300 patients daily and around 70,000 annually (Alam, 1986). The high incidence of diarrhoeal diseases in the community is

often associated with malnutrition, especially in children. Persistent diarrhoea and shigellosis are common causes of morbidity and mortality in Bangladesh. Although the centre is involved in carrying out microbiological and nutritional studies related to these conditions, it is not at present equipped for carrying out immunological studies.

Persistent diarrrhoea and recurrent diarrhoea

Studies in a rural and urban site in Bangladesh of children under 4 years with diarrhoea, reveal that 4% of these are persistent (Henry, 1987). And Matlab studies show that 28% of all deaths in children less than 5 years are associated with persistent diarrhoea and malnutrition (Fauveau, 1986).

One of the definitions of persistent diarrhoea is the passage of an increased volume of watery stool for a period greater than 2 weeks (Walker-Smith, 1988). The causes leading to the development of persistent diarrhoea are not known. It is common in infants and children with poor environmental hygiene (Snyder and Merson, 1982) and malnutrition suggesting an underlying impairment of immunocompetence. However, very little is known about the immune status of patients with persistent diarrhoea. Children with persistent diarrhoea may have lowered immunity. Although, this could be secondary to malnutrition there may be primary causes for this suppresion such as release of inhibitory factors or induction of cellular suppressor mechanisms. Possible identification of a common

immune defect would shed light on the pathogenesis of persistent diarrhoea.

Moreover, many children in Bangladesh suffer from recurrent attacks of diarrhoea where diarrhoea is defined as the passage of watery stool more than 4 times per day for at least 1 day (Black et al., 1982). These attacks decrease with age and the majority are due to infections by enteropathogenic organisms. It is not known why some children are more susceptible to repeated diarrhoeal infections. However, Koster et al. (1987) have shown that a decrease in cell-mediated immunity, as measured by skin hypersensitivity tests, is associated with an increase in morbidity due to diarrhoea which in turn leads to malnutrition and further infections. A more extensive examination of various aspects of the immune system may shed light on the nature of immune defect.

Shigellosis

Reports from Matlab reveal that the death rate from shigellosis (5.6%) is twice that of diarrhoea from other causes (2.8%) (Huda and Harris, 1986). Of the four species of Shigella, Shigella dysenteriae type 1 can cause severe epidemics and is rapidly becoming resistant to the most available antibiotics.

The immune mechanisms against shigellosis are not well understood. There is evidence to suggest that both cellular

and humoral defense mechanism are involved in recovery from shigellosis. Thus, serum Immunoglobulins (Ig) of the A, G and M isotype to Shigella LPS O antigen (Cohen et al., 1989) and to Shiga toxin have been detected. In addition, secretory IgA (sIgA) to Shigella antigens are present in faeces (Winser et al., 1988), saliva (Schultsz et al., ongoing work at the Centre), intestinal fluid (Schultsz et al.; Keren et al., 1978) and breast milk (Cleary et al., 1989). However, it is not certain how these antibodies afford protection. One of the possible mechanisms of protection may be the generation of antibody-dependent-cellular-cytotoxicity (ADCC). ADCC has been demonstrated using sera from infected individuals by peripheral blood mononuclear cells (Lowell et al., 1980; Morgan et al., 1984) and by gut lymphocytes in mice (Tagliabue et al., 1983, 1984). Although, ADCC may explain defense mechanisms once the bacteria has penetrated the gut mucosa, it does not explain how immune individuals, who still have detectable levels of Shigella specific sIgA are protected against reinfection. Protection may also be provided by natural killer cells as these cells exert cytotoxicity against S. flexneri infected cells (Klimpel et al., 1986, 1988). However, the cellular defense mechanisms in shigellosis have not been fully elucidated. Initial studies on a T cell clone responsive to Sh. flexneri (Zwillich et al., 1989) confirm that Shigella specific helper T cells are generated but it is not known whether the B cell response is entirely

T cell dependent or whether they can be polyclonally activated by Shigella. Furthermore, little is known about antigen presentation and the role of phagocytes in shigellosis.

Infection from Shigella can lead to a wide variety of complications. Thus, patients may develop a chronic illness with associated malnutrition, shigellaemia, leukemoid reaction or haemolytic-uremic syndrome. The development of chronic illness from acute shigellosis is often seen in children with malnutrition (Keusch, 1982) and in infants with malnutrition there is an increased risk of death due to Shigella (Struelens et al., 1985). Rahaman et al. (1974) reported leukemoid reactions in 15% of patients suffering from S. dysenteriae type 1 infection in Bangladesh. The cause of these complications is not known.

It has been postulated that cytokines may be involved in these conditions. The research centre for the ICDDR,B has an on-going study on the role of cytokines such as ILI alpha and beta, tumour necrosis factor (TNF) in the pathogenesis of shigellosis. However, other immunological parameters are not being assessed in these patients.

Malnutrition

Malnutrition is often associated with diarrhoeal diseases, either as a cause or effect of the diarrhoea. Malnutrition

affects several components of the immune system (Table 1). However, malnutrition is a complex syndrome involving deficiencies of different food components. The effects of deficiencies of trace elements have been reviewed by Chandra (1985) and are shown in Table 2. These studies show how profoundly malnutrition affects immunity.

Thus, in persistent diarrhoea, shigellosis and complications of shigellosis, the underlying cause is not known. There is often accompanying malnutrition which secondarily impairs immunocompetence. In addition, an impairment of immunocompetence is strongly indicated in each of these conditions. Although there have been isolated studies on some aspects of immunity in persistent diarrhoea and shigellosis, no systematic and properly controlled study involving large numbers of patients and covering a wide range of immunological tests have been carried out. The Clinical Research Centre of ICDDR,B treats large numbers of patients with persistent diarrhoea, shigellosis with and without complications, and has a rehabilitation unit for children with malnutrition. It has ongoing studies on persistent diarrhoea and shigellosis leading to complication. It, therefore, provides an ideal opportunity for carrying out such a study. The study will be useful for several reasons:

1. The immune status may provide important prognostic information predicting complications and mortality.

2. The study may provide insights into the immunopathology of these disease conditions. Due to the limitations of the study i.e. lack of information regarding the immune status of patients prior to illness, any relevant findings will require further investigation. This study therefore is a preliminary one which will hopefully lead to a more intensive investigation of the aspects that are highlighted here. These further studies will, in turn, ultimately shed light into the immunopathology of these conditions.

3. The study will provide useful information about the immune status of a large number of people which may direct future immunisation programmes.

Patients to be studied include:

Disease condition	No. of patients	Age of patients (yrs)	Samples	Source of patients
Acute diarrhoea	50	0-2	P.blood* Stool Saliva	ICDDR,B
Persistent diarrhoea	50	0-2	P.blood Stool Saliva	ICDDR,B
Recurrent diarrhoea	50	1/4-2	P.blood Stool Saliva	ICDDR,B
Acute shigellosis	50	1-5	P.blood Stool Saliva	ICDDR,B in collaboration with Dr. Bennish
Complications of shigellosis	50	1-5	P.blood Stool Saliva	ICDDR,B in collaboration with Dr. Bennish
Malnutrition	50	0-5	P.blood Stool Saliva	Possibly from Save the Children Fund, Shishu Hospital
Controls	50 50	0-2 2-5	P.blood Stool Saliva	Possibly from Pongu Hospital and Shishu Hospital

*P.blood = peripheral blood

The sample number for each group has been determined statistically after taking into consideration the variance of different immunological parameters.

Materials to be studied include:

I. Peripheral blood
II. Saliva and stool
III. Gut biopsy (from patients only) - when the technique is available at the Centre
IV. Delayed type hypersensitivity will also be tested

I. Peripheral blood will be used for experiments on serum, plasma, lymphocytes (mononuclear cells) and granulocytes. Upto 5 mls of periperal blood will be required from children and this will be obtained by venepuncture. In those cases where less blood is obtained, some tests will not be carried out. The plan is shown below:

*PERIPHERAL BLOOD (3-5 ml maximum)

---> 0.5 ml in glass tubes for SERUM to be used for:
1. C_3, C_4 levels
2. Complement activation
3. Autologous serum for use in neutrophil iodination
4. Protein fraction estimation

4 ml in heparin containing tube separated on Ficoll-Hypaque

-> PLASMA will be collected from above the band of mononuclear cells and used for:
1. IgG, IgM and IgA levels
2. CRP, iron, transferrin or ferritin, zinc
3. IgE levels
4. Autologous plasma for PWM and PHA stimulation and MLC
5. Ig to causative organism and its antigens
6. Ig to diphtheria and tetanus

<---- GRANULOCYTES will be obtained from the pellet after removal of RBC by hypotonic lysis and Dextran sedimentation. Cells will be used for:

1. Iodination --> 6×10^6 cells
2. Phagocytosis --> 1×10^6 cells
3. Chemotaxis --> 1×10^6 cells
4. Polarisation --> 2×10^6 cells

MONONUCLEAR CELLS will be obtained by collecting the band at the interface and used for:
1. PHA stimulation --> 2.5×10^5 cells
2. PWM stimulation --> 3.0×10^5 cells
3. Resting DNA synthesis --> 2.5×10^5 cells
4. MLC --> 1.5×10^6 cells
5. Phenotyping by immunofluorescence --> $1\text{-}2 \times 10^6$ cells

*Methods are described on next page and the significance of each test is shown in Table 3

METHODS

A. SERUM

1. C3, C4 levels will be measured by a discrete analyser (COBAS B10). Anti-C3 and anti-C4 antibodies will be obtained commercially.

2. Complement activation will be assessed by two-dimensional immunoelectrophoresis.

3. Protein fractions will be estimted by electrophoresis.

B PLASMA

1. IgG, IgA, IgM levels will be measured by COBAS BIO and by ELISA.

2. C reative protein (CRP), iron and transferrin/ferritin levels will also be measured by COBAS BIO.

3. IgE levels will be estimated using an ELISA kit.

4. Igs to the causative organism and its antigens will be estimated by ELISA (e.g. Shigella).

5. Igs to diphtheria and tetanus will be measured by ELISA.

6. Plasma zinc levels will be estimated by an atomic absorption spectrophotometer (AAS).

C. MONONUCLEAR CELLS

1. Phytohaemagglutinin (PHA) stimulation will be measured by culturing cells with various concentrations of PHA in the presence of autologous plasma, heterologous plasma or calf serum for 72 hours and assessing proliferation by ^{3}HTDR incorporation.

2. Pokeweed mitogen (PWM) stimulation will be measured by culturing cells with various concentrations of PWM in the presence of autologous plasma, heterologous plasma or calf serum for 138 hours (5 1/2 days) and assessing proliferation by ^{3}HTDR incorporation. The supernatant will be collected prior to addition of isotope and used for measuring non-specific Ig levels by ELISA or by COBAS BIO as well as specific Ig to the causative organism and its antigens.

3. Resting DNA synthesis will be measured by incubating mononuclear cells with ^{3}HTDR for 3 hours to assess proliferation.

4. Mixed lymphocyte cultue (MLC) - patient's mononuclear cells will be cultured with irradiated, pooled mononuclear cells from 6 patients in the presence of autologous or homologous plasma. Homologous plasma will be collected from 6 healthy individuals and pooled. Control cultures of irradiated cells with homologus plasma will also be set up. This will be

incubated for 138 hours (5x days) when proliferation will be assessed by the incorporation of ^{3}HTDR.

5. Phenotyping by indirect immunofluorescence will be carried out for determining proprotions of T cells, B cells and T cell subsets (T helper cells or CD4 and T suppressor/cytotoxic cells or CD8) using monoclonal antibodies UCHT1 (a kind gift of Prof. P.C.L. Beverley), B1 (CD19) (commercial souce), anti-CD4 (a kind gift of Dr. Q. Sattentau) and UCHT4 (from Prof. P.C.L. Beverley) respectively.

D. GRANULOCYTES

1. Neutrophil iodination - patient's granulocytes will be incubated with Staph. aureus or Candida albicans or bakers yeast in the presence of autologous serum, pooled human serum from 6 healthy controls or heat-inactivated patient's serum and $Na^{125}I$ for 60 minutes. Iodination will then be assessed in a -counter.

2. Phagocytic index - neutrophils will be incubated with baker's yeast suspension and pooled human serum from 6 healthy controls for 60 minutes. Cells will then be centrifuged and resuspended in a drop and a smear made on a glass slide and stained wih Wright's stain. Ingested yeast in 50 neutrophils will be counted under a microscope.

3. Neutrophil chemotaxis will be measured by using a Boyden chamber. Neutrophils will be placed in the upper chamber, the chemotactic peptide N-formyl-Met-Leu-Phe (FMLP) or bacterial antigens will be layered in the lower chamber and PBS (phosphate buffer saline) added to both. After incubation for 30 minutes, the filters will be removed, fixed in methanol and stained in haematoxylin and then counted under a miscroscope.

4. Morphological polarisation of neutrophils-neutrophils will be incubated with FMLP or bacterial antigens for 30 mins at 37^{o}C. The cells will then be fixed with glutaraldehyde, washed and scored for the proportion of neutrophils deviating from spherical morphology.

II. Saliva samples will be collected by spitting into a beaker or, in the case of younger children, by collecting with a pipette. The samples will then be heat-inactivated and centrifuged. ELISAs will be carried out on the clear supernatant to measure total Ig, non-specific IgA and specific IgA to the causative organism and its antigens and to poliovirus.

Stool samples will be homogenized by mixing thoroughly in phosphate buffered saline (PBS). The sample will then be centrifuged and the supernatant collected and frozen till use for measuring Ig as for saliva.

III. Gut biopsy will be obtained from patients in the future when the technique is established at the Centre. The fixed biopsy material will be used for determining numbers of B cells, T cells and T cell subsets by immunoperoxidase staining methods. This part of the project will be submitted later as a supplement pending the return of a post-graduate student after training at the Karolinska Institute in Sweden in collaboration with Prof. Lindberg's laboratory.

IV. Delayed type hypersensitivity (DTH). DTH will be tested using a Multitest CMI kit whereby 7 antigens and a control will be introduced intradermally into the forearm using a multiple puncture device. An induration of 2mm or more diameter after 48 hrs will be counted as a positive reaction. The antigens that will be tested include:

1. Tetanus antigen 550,000 Merieux units/ml
2. Diphtheria antigen 1100,000 Merieux units/ml
3. Streptococcus antigen (group C) 2,000 Merieux units/ml
4. Tuperculin antigen 300,000 IU/ml
5. Glycerin control : solution of glycerin to 70% weight/volume
6. Candida antigen (albicans) 2,000 Merieux units/ml
7. Trichophyton antigen (mentagrophytes) 150 Merieux units/ml
8. Proteus antigen (mirabilis) 150 Merieux units/ml

The t-test will be used to calculate the statistical significance of each measure, comparing the treatment group with the control group.

TABLE 1

Effects of malnutrition on immunity

	Reference
A. CELL-MEDIATED IMMUNITY	
↓ total lymphocyte count	Smythe et al., 1971
↓ T cell numbers especially T helper cells (Th)	Chandra, 1974
Ability of Th cells to provide help to B cells in antibody synthesis	Chandra, 1983
↓ PHA response of T cells especially in the presence of autologous plasma	Beatty & Dowdle, 1978
↓ skin reactions (delayed type hypersensitivity, DTH)	Chandra, 1972
B. COMPLEMENT	
↓ C3, C1q, C1s and other components but not C4	Sirisinha et al., 1973
↓ opsonic function of plasma	Chandra, 1983
C. LYMPHOID TISSUES	
Atrophy of thymus, lymphnodes and spleen in experimental animals and post-mortem examinations of humans	Smythe et al., 1971
↓ tonsillar size	Smythe et al., 1971
D. IMMUNOGLOBULIN SECRETION	
↓ sIgA levels and ↓ mucosal antibody response to viral vaccines	Sirisinha et al., 1975

E.	NEUTROPHILS	
	↓ chemotaxis	Chandra, 1983[b]
	Impaired intracellular bacterial killing although bacterial ingestion is normal	Chandra, 1983[b]
F.	LYMPHOKINES	
	↓ ILI secretion from macrophages in severe malnutrition	Bhaskaram & Sivakumar, 1986
	↓ Interferon production	Chandra, 1983[b]

TABLE 2

Effects of trace elements on immunity

	TRACE ELEMENT	EFFECT ON THE IMMUNE SYSTEM
1.	Zinc	↓ DTH
		↓ lymphocyte proliferation
		↓ T helper cells (Th)
		↓ ability of Th cells to provide help to B cells
		↓ T suppressor activity
		↓ neutrophil chemotaxis
2.	Iron	↓ intracellular digestion of bacteria by neutrophil
		↓ T cell numbers
		↓ DTH
		↓ lymphocyte proliferation to mitogens and antigens
3.	Copper (in rodents)	↓ thymus size
		↓ thymic hormone activity
		↓ number of antibody forming cells in the spleen
		↓ lymphocyte proliferation in response to conconvalin A (Con A)
4.	Iodine	↓ microbididal activity of neutrophils
5.	Selenium (particularly when coexisting with Vit. E deficiency)	↓ thymic hormone activity
		↓ antibody responses to heterologous red blood cells (RBC)

TABLE 3

SIGNIFICANCE OF PROPOSED TESTS

TEST	SIGNIFICANCE
A. SERUM	
1. Complement C3, C4 levels	C3 ↓ malnutrition, C4 is normal.
2. Complement activation	Complement is activated in cow's milk protein allergy.
3. Protein fraction estimation	↓albumin occurs in protein energy malnutrition and protein losing enteropathies
B. PLASMA	
1. Ig levels IgA, IgG, IgM	These may be ↓ in malnutrition or unaffected, ↑ in infections.
2. IgE levels	↑ cow's milk protein allergy
3. CRP	↑ in bacterial infections although not in viral infections.
4. Igs to causative organism and its antigens	Absent in non-immune individuals, ↑ during infection
C. MONONUCLEAR CELLS	
1. Mononuclear cell PHA stimulation	T cell proliferatrion in response to PHA may be reduced in malnutrition, especially in the presence of autologous plasma.
2. Mononulear cell PWM stimulation	B cells predominantly respond to PWM. Deficiency in B cell function will produce a ↓ in this response.
3. Resting cell DNA synthesis	Unstimulated mononuclear cells may be activated and show ↑ proliferaton as in viral infections.
4. Mixed lymphocyte culture (MLC)	When cells from various donors are mixed in culture, they are activated to proliferate and release soluble factors

	in response to major histocompatibility antigens (MHC I and II). A ↓ in response may be seen with ↓ immunity.
5. Phenotyping of mononuclear cells	T cells are reduced in malnutrition, especially T helper (CD4) cells. The T helper and T cytotoxic/suppressor ratio is also reduced. This can also occur in infections.

D. GRANULOCYTES

1. Neutrophil iodination	This is a measure of both opsonisation and bacterial killing by neutrophils. ↓ yeast opsonisation is seen in protracted diarrhoea.
2. Phagocytic index	This is a measure of bacterial killing by neutrophils.
3. Neutrophil chemotaxis	A decrease in chemotaxis occurs in children with recurrent infections.
4. Neutrophil polarization	This is a measure of the proportion of neutrophils responding to chemoattractant factors.

REFERENCES

Alam, A.N. (1986) Surveillance programme, Dhaka Treatment Centre. Annual Report of ICDDR,B, p.13

Beatty, D.W. and Dowdle, E.B. (1978) The effects of kwashiorkor on lymphocyte transformation in vitro. Clin. Exp. Immunol., 32:134.

Bhaskaram, P. and Sivakumar, B. (1986) Interleukin-1 in malnutrition. Arch. Dis. Child., 61:182.

Black, R.E., Brown, K.H., Becker, S., Alim, A.R.M.A. and Huq, I. (1982) Longitudinal studies of infectious diseases and physical growth of children in rural Bangladesh. II. Incidence of diarrhoea and association with known pathogens. Am. J. Epid., 115:315-324.

Butler, H.L., Byrne, W.J., Marmer, D.J., Euler, A.R. and Steele, R.W. (1981) Depressed neutrophil chemotaxis in infants with with cow's milk and/or soy protein intolerance. Pediatrics., 67:264.

Challacombe, D.N., Wheeler, E.E. and Campbell, P.E. (1986) Morphometric studies and eosinophil cell counts in the duodenal mucosa of children with chronic nonspecific diarrhoea and cow's milk allergy. J. Pediatr. Gastroenterol. Nutr., 5:887.

Chandra, R.K. (1985) Trace element regulation of immunity and infection. J. Am. College Nutr., 4:5.

Chandra, R.K. (1983a) Numerical and functional deficiency in T helper cells in protein energy malnutrition. Clin. Exp. Immunol., 51:126.

Chandra, R.K. (1983b) Nutrition, immunity and infection: present knowledge and future directions. Lancet, i, 688.

Chandra, R.K. (1974) Rosette-forming T lymphocytes and cell-mediated immunity in malnutrition. Brit. Med. J., 3:608.

Chandra, R.K. (1972) Immunocompetence in undernutrtion. Trop. Pediat., 81:1194.

Cohen, D., Block, C., Green, M.S., Lowell, G. and Opek, I. (1989) Immunoglobulin M, A and G antibody response to lipopolysaccharide O antigen in symptomatic and asymptomatic Shigella infections. J. Clin. Microbiol., 27:162.

Henry, F.J. (1987) Persistent diarrhoea and malnutrition in urban and rural Bangladesh. Annual Report of ICDDR,B, p.13.

Huda, N. and Harris, J. (1986) Deaths after shigellosis in Matlab. Annual Report of ICDDR,B, p.25.

Fauveau, V. (1986) Deaths of children younger than 5 years in Matlab. Annual Report of ICDDR,B, p.12.

Keren, D.F., Holt, P.S., Collins, H.H., Gemski, P. and Formal, S.B. (1978) The role of Peyer's pathces in the local immune response of rabbit ileum to live bacteria. J. Immunol., 120:1892-1896.

Keusch, G.T. (1982) Shigellosis. From: Bacterial infections of humans. Epidemiology and Control. Eds. A.S. Evans and H.A. Feldman, Plenum Med. Book Co., NY, London, p.487.

Klimpel, G.R., Niesel, D.W., Asuncion, M. and Klimpel, K.D. (1988) Natural killer cell activation and interferon production by peripheral blood lymphocytes after exposure to bacteria. Infect. Immun., 56:1436-1441.

Klimpel, G.R., Niesel, D.W. and Klimpel, K.D. (1986) Natural cytotoxic effector cell activity against Shigella flexneri infected HeLa cells. J. Immunol., 136:1081-1086.

Koster, F.T., Palmer, D.L., Chakraborty, J., Jackson, T. and Curlin, G.C. (1987) Cellular immune competence and diarrhoeal morbidity in malnourished Bangladeshi children: a prospective field study. Am. J. Clin. Nutr., 46:115-120.

Lindberg, A.A., Karnell, A., Stocker, B.A.D., Katakura, S., Sweiha, H. and Reinholt, F.P. (1988) Development of an auxotrophic oral live Shigella flexneri vaccine. Vaccine, 6:146.

Lowell, G.H., MacDermott, R.P., Summers, P.L., Reeder, A.A., Bertovich, M.J. and Formal, S.B. (1980) Antibody-dependent cell-mediated antibacterial activity: K lymphocytes, monocytes and granulocytes are effective against Shigella. J. Immunol., 125:2778.

Matthews, T.S. and Soothill, J.F. (1970) Complement activation after milk feeding in children with cow's milk allergy. Lancet, ii, 893.

Morgan, D.R., DuPont, H.L., Gonik, B. and Kohl, S. (1984) Cytotoxicity of human peripheral blood and colostral leukocytes against Shigella species. Infect. Immun., 46:25.

Rahaman, A.M., Alam, A.K.M.J., Islam, M.R. and Greenough, III, W.B. (1975) Shiga bacillus dysentery associated with marked leukocytosis and erythrocyte fragmentation. Johns Hopkins Med. J., 136:65.

Sirisinha, S., Suskind, R., Edelman, R, Asvapaka, C. and Olson, R.E. (1975) Secretory and serum IgA in children with protein-caloric malnutrition. Pediatrics, 55:166.

Sirisinha, S., Suskind, R., Edelman, R., Charupatana, C. and Olson, R.E. (1973) Complement and C3-proactivator levels in children with protein-caloric malnutrition and effect of dietary treatment. Lancet, i, 1016.

Smythe, P.M., Schonland, M., Brereton-Stiles, G.G., Coovadia, H.M., Grace, H.J., Loening, W.E.K., Mafoyane, A., Parent, M.A. and Vos, G.H. (1971) Thymolymphatic deficiency and depression of cell-mediated immunity in protein-caloric malnutrition. Lancet, ii, 939.

Struelens, M.J., Patte, D., Kabir, I., Salam, A., Nath, S.K. and Butler, T. (1985) Shigella septicaemia: prevalence, presentation, risk factors and outcome. J. Infect. Dis., 152:784.

Synder, J.D. and Merson, M.H. (1982) The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data. Bull WHO, 60:605.

Tagliabue, A., Borashi, D., Villa, L., Karen, D.F., Lowell, G.H., Rappuoli, R. and Nencioni, L. (1984) IgA-dependent cell-mediated activity against enteropathogenic bacteria: distribution, specificity and characterisation of the effector cells. J. Immunol., 133:988.

Tagliabue, A., Nencioni, L., Villa, L., Kere, D.F., Lowell, G.H. and Boraschi, D. (1983) Antibody-dependent cell-mediated antibacterial activity of intestinal lymphocytes with secretory IgA. Nature, 306:184.

Walker-Smith, J. (1988) Intractable diarrhoea from: Diseases of the small intestine in childhood. Butterworths London, Boston, Singapore, Sydney, Toronto, Wellington, p.390.

Winsor, D.K., Jr., Mathewson, J.J. and DuPont, H.L. (1988) Comparison of serum and fecal antibody responses of patients with naturally acquired Shigella sonnei infection. J. Infect. Dis., 158:1108-1112.

Zwillich, S.H., Duby, A.D. and Lipsky, P.E. (1989) T-lymphocyte clones responsive to Shigella flexneri. J. Clin. Microbiol., 27:417-421.

11. PUBLICATIONS OF INVESTIGATORS

a) Dr. Tasnim Azim

1. **Azim, T.** and Crawford, D.H. (1988) Lymphocytes activated by the Epstein-Barr virus to produce immunoglobulin do not express CD23 or become immortalised. Int. J. Cancer., 42:23-28.

2. Crawford, D.H., **Azim, T.**, Daniels, G.L. and Huehns, E.R. Monoclonal antibodies to the Rh D antigen. In: Progress in Transfusion Medicine III. Ed. J. Cash. Churchill Livingstone Press, pp.175-197.

3. **Azim, T.**, Golay, J., Lam, K. and Crawford, D.H. (1987) Polyclonal activation of B lymphocytes after EB virus infection. In: The Proceedings of the 2nd International Workshop on EB Virus and Related Malignant Diseases. pp.331.

4. Crawford, D.H. and **Azim, T.** (1987) The use of EB virus for the production of human monoclonal antibody secreting cell lines. In the Proceedings of the 1st IRI International Symposium on Biotechnology: Monoclonal antibodies in the treatment of human disease. Ed. J. Brown, pp.1-6.

5. **Azim, T.**, Crawford, D.H. and Beverley, P.C.L. (1987) The role of activation antigens on the surface of B cells. In: Leucocyte Typing III, Eds. A.J. McMichael et al., Oxford University Press, pp.559-561.

6. Sutherland, S., Crawford, D.H., Wilson, S.A., Morgan, B., **Azim, T.** and Huehns, E.R. (1987) Production and characterisation of a human monoclonal antibody to cytomegalovirus and its use in an early nuclear fluorescence assay. J. Med. Virol., 22:245-255.

7. Morgan-Capner, P., Morris, J.A., McIllmurray, M.B., Thomas, J.A., Crawford, D.H. and **Azim, T.** (1986) Immunohistological studies of lymphoproliferative lesions in a fatal case of Epstein-Barr virus infection. J. Clin. Pathol., 39:1317-1322.

8. Berliner, N., Duby, A.D., Linch, D.C., Murre, C., Quetermous, T., Knott, L.J., **Azim, T.**, Newland, A.C., Lewis, D.L., Galvin, M.C. and Seidman, J.G. (1986) T cell receptor gene rearrangements define a monoclonal T cell proliferation in patients with T cell lymphocytosis and cytopenia. Blood, 67:914-918.

b) Dr. Pradip Kumar Bardhan

1. Molla A, Gyr K, Molla AM, **Bardhan PK**, Patra FC.(1986) Preserved exocrine pancreatic function in patients with acute cholera and acute non-cholera diarrhoea. Int J Pancreatology 1:259-264.

2. Islam A, Bardhan PK, Islam MR, Rahman N. (1986) A randomized double blind trial of aspirin versus placebo in cholera and non-cholera diarrhoea. J Trop Geographical Med 38:221-225.

3. Kehl O, Gyr K, Groetzinger U, Stalder G, Bardhan PK, Amman R, Kayasseh L. (1986) Diagnosis of achlorhydria by plasma secretion. Determination - a tubeless approach. Am J Gastroenterol 81:681-684.

4. Molla A, Lonnroth I, Jahan F, Bardhan PK, Molla AM, Holmgren J. (1985) Stool cyclic AMP in diarrhoea due to different organisms. Diarrhoeal Dis Res 3:199-204.

5. Butler T, Rahman H, Al-Mahmud KA, Islam M, Bardhan PK, Kabir I, Rahman MM. (1985) An animal model of haemolytic uremic syndrome in shigellosis: lipopolysaccharides of Shigella dysenteriae 1 and Shigella flexneri produce leucocyte mediated renal cortical necrosis in rabbits. J Exp Pathol 66:7-15.

6. Samadi AR, Ahmed SM, Bardhan PK, Huq MI, Islam MR, Wahed MA. (1985) Treatment of infantile diarrhoea with standard oral rehydration solution and early introduction of milk feeds. J Trop Paediatr 31:162-166.

7. Svennerholm AM, Gothefors L, Sack DA, Bardhan PK, Holmgren J. (1984) Local and systemic antibody responses and immunological memory in humans after immunization with cholera B subunit by different routes. Bull WHO 62:909-918.

8. Molla AM, Gyr K, Bardhan PK, Molla A. (1984) Effect of intravenous somatostatin on stool output in diarrhoea due to Vibrio cholerae. Gastroenterology 87:845-847.

9. Islam MR, Samadi AR, Ahmed SM, Bardhan PK, Ali A. (1984) Oral rehydration therapy: efficacy of sodium citrate equals to sodium bicarbonate for correction of acidosis in diarrhoea. Gut 25:900-904.

10. Butler T, Islam MR, Bardhan PK. (1984) The leukemoid reaction shigellosis. Am J Dis Child 138:162-165.

c) Dr. Laila N. Islam

1. Islam, L.N. and Wilkinson, P.C. (1988) Chemotactic factor-induced polarization, receptor redistribution and locomotion of human blood monocytes. Immunology, 64:501-507.

2. Wilkinson, P.C., Islam, L.N., Sinclair, D. and Dagg, J.H. (1988) The defect of lymphocyte locomotion in chronic lymphocytic leukaemia: studies of polarization and growth-dependent locomotion. Clin. Experim. Immunol., 71:497-501.

3. Wilkinson, P.C., Lackie, J.M., Haston, W.S. and Islam, L.N. (1988) Effects of phorbol esters on shape and locomotion of human blood monocytes. J. Cell Sci., 90:645-655.

4. Islam, L.N., McKay, I.C. and Wilkinson, P.C. (1985) The use of collagen or fibrin gels for the assay of human neutrophil chemotaxis. J. Immunol. Meth., 85:137-151.

5. Noor, L. and Khan, N.H. (1982) Studies on ninhydrin positive compounds present in some seeds. Dhaka Univ. Stud., Part-B, 30(1):99-105.

6. Khan, N.H., Noor, L. Begum, M. and Rahman, M. (1981) Studies on the antibacterial activity of some indigenous plant extracts. Bangladesh J. Biol. Sci., 10:31-38.

7. Islam, L.N. and Wilkinson, P.C. Evaluation of methods for isolating human peripheral blood monocytes: studies on chemotactic locomotion and other functional characteristics. J. Immunol. Meth. (in press)

8. Wilkinson, P.C. and Islam, L.N. Interleukin 4 and gamma interferon active locomotor capacity in human B lymphocytes. Immunology (in press).

d) Mr. M. A. Wahed

1. Alam, A.N., Abdal, N.M., Rao, B., **Wahed, M.A.**, Rahaman, M.M. et al. (1987) Plasma prostacyclin levels during the haemolytic-uraemic syndrome. In: Proceedings of 4th Asian Conference on Diarrhoeal Diseases, p.60.

2. Sarker, S.A., **Wahed, M.A.**, Rahaman, M.M., Alam, A.N., Khanom, A. and Jahan, F. (1986) Persistent protein losing enteropathy in postmeasles diarrhoea. Arch. Dis. Childhood, 61(8):739-743.

3. Sarker, S.A., **Wahed, M.A.**, Alam, A.N., Khanom, A. and Rahaman, M.M. (1985) Protein-losing enteropathy syndrome in postmeasles diarrhoea. In: Proceedings of 3rd Asian Conference on Diarrhoeal Disease, Bangkok, p.70.

4. Samadi, A.R., Ahmed, S.M., Bardhan, P.K., Huq, M.I. and **Wahed, M.A.** (1985) Treatment of infantile diarrhoea with standard ORS and early introduction milk feeds. J. Trop. Pediat., 31(3):162-166.

5. Ali, A. and **Wahed, M.A.** (1984) Preparation and quality control of hand packaged oral rehydration salt sachets. J. Diar. Dis. Res., 2(3):1162-167.

6. Samadi, A.R., Wahed, M.A., islam, R. and Ahmed, M. (1983) Consequence of hyponatreamia and hypernatreamia in children with acute diarrhoea in Bangladesh. Br. Med. J., 286:671-673.

7. Samadi, A.R., Wahed, M.A. and Islam, R. (1983) Comparison of osmolarity of milk feed with breastmilk. Nutr. Rep. Int., 28(5):111-114.

8. Rahaman, M.M. and Wahed, M.A. (1983) Direct nutrient loss and diarrhoea. In: Diarrhoea and Malnutrition, ed. Chen and Scrimshaw, pp.155-160.

FLOW CHART

trols 00	Malnutrition 50	Acute diarrhoea 50	Persistent diarrhoea 50	Recurrent diarrhoea 50	Acute shigellosis 50	Complications of shigellosis 50	Time 3 years (36 months)
0	10	10			10		0-6 months
0	20	10	10	10	10	10	6-12 months
0		10	10	10	10	10	12-18 months
0		10	10	10	10	10	18-24 months
0		10	10	10	10	10	24-30 months
	20		10	10		10	30-36 months

13. ITEMISED SPECIFIC TASKS FOR EACH LISTED INVESTIGATORS

a) Dr. Tasnim Azim

1) Lymphocyte studies

2) ELISA for IgE, IgA, IgG, IgM and specific antigens

3) Crossed immunoelectrophoresis for determination of complement activation

4) Electrophoresis for estimation of protein fractions

5) DTH studies

b) Dr. P.K. Bardhan

Clinical assessment and management of patients at ICDDR,B

c) Dr. Laila Noor Islam

Granulocyte studies

d) Mr. M. A. Wahed

1) Determination of serum complement levels by COBAS B10

2) Determination of IgM, IgG and IgA levels in plasma by COBAS B10.

3) Determination of plasma levels of iron, transferrin/ferritin and CRP by COBAS-BIO.

4) Determination of plasma zinc levels by AAS.

13. BUDGET

	1st yr	2nd yr	3rd yr
Personnel			
Tasnim Azim	5,625	6,188	6,807
Laila Islam	750	825	908
M. A. Wahed	1,808	1,989	2,188
Technician	4,500	4,950	5,445
Plastics	5,000	6,000	6,642
Chemicals	5,000	6,000	8,000
Media, antibiotics and other reagents	8,817	11,000	12,000
Stock items, such as pipette tips, glass slides, glasswares, etc.	4,000	6,000	7,000
Instruments, including automash, camera, camera accessories, finnpipettes	13,000	3,048	3,000
10% added costs	8,000	9,000	10,000
Miscellaneous, including mail, photocopying, transport, etc.	5,000	5,500	6,000
Total:	61,500	60,500	68,000

GRAND TOTAL: US$ 190,000

Development of an immunology laboratory and application immune function tests to persistent diarrhoea, recurrent diarrhoea, shigellosis and malnutrition

INVESTIGATORS: Dr. Tasnim Azim
Dr. P. K. Bardhan
Dr. Laila N. Islam
Mr. M. A. Wahed

RESPONSE TO REVIEWERS COMMENTS AND CHANGES TO MANUSCRIPT

REVIEWERS:

1. Prof. D. M. Roberton
   Department of Paediatrics
   University of Adelaide
   Australia

2. Dr. M. W. Turner
   Reader in Immunochemistry
   Hugh Greenwood Department of Immunology
   Institute of Child Health
   London
   U.K.

3. Dr. M. Bennish
   Co-Head, Tufts-ICDDR,B Shigella Project
   ICDDR,B
   Dhaka
   Bangladesh

PROF. D. M. ROBERTON

1. The reviewer comments that the assays described in the protocol should be straightforward provided major equipments, such as ß-counter, γ-counter, centrifuges are available. All these equipments are already present at the Centre and are in working condition.

2. The reviewer stresses the importance of establishing normal ranges of immunoglobulin (Ig) levels in different age groups in Bangladesh. For this purpose, the age range of our control group has been reduced from 0-12 to 0-5 years, and this has been further divided into 2 groups of 0-2 and 2-5 years. In addition, in order to establish normal IgA concentrations in saliva and faeces, correlation against total Ig in saliva and faeces will be done to allow for the differences in water content.

3. We have incorporated the following tests recommended by the reviewer:

   i) Measurement of Ig levels to diphtheria, tetanus and polio, in order to assess the response to antigens to which the children are likely to have been exposed previously.

   ii) Measurement of Ig secretion to specific bacterial antigens (such as *Shigella* antigens) following stimulation with pokeweed mitogen (PWM).

   iii) Measurement of plasma iron, transferrin/ferritin and zinc levels, as well as estimation of protein fractions in plasma for nutritional assessment.

4. The reviewer suggests that serum opsonic activity to specific organisms be compared in neutrophil iodination responses in the presence of autologous serum and in the presence of pooled heterologous serum. This has already been included in the study.

DR. M. W. TURNER

1. The reviewer emphasises the need to rigorously select the control group. For this purpose, the age range of children in the control group has been reduced from 0-12 years to 0-5 years. These children will again be divided into 2 groups; 0-2 years and 2-5 years. Such an age range has been chosen on the basis of immunological data available for

Australian children. The reviewer's suggestion that these children be non-parasitised individuals is impractical in Bangladesh. The reviewer also suggests that children with possible immunological abnormalities be excluded. The incidence of immunological abnormalities is very low even in developed countries and although in a country like Bangladesh these abnormalities would pass undetected, such children would not be able to survive the onslaught of infections. The control group will comprise hospitalised children who have no infection (viral or bacterial), e.g. children with fractures.

2. The reviewer stresses the need to ensure that the clinical groups are as homogeneous as possible, especially with regard to timing of samples. We have already mentioned that all samples from each clinical group will be collected at specified times, each group will be monitored clinically and assessed for malnutrition. We will adhere to these specifications.

3. According to the reviewers suggestion, a statistician has been consulted on whose advice a sample size of 50 for each group has been chosen and the t-test has been selected for the evaluation of data.

DR. M. BENNISH

1. The reviewer criticises a lack of hypotheses in the protocol. This is deliberate, because the study is a preliminary one. Ideally, to investigate a hypothesis regarding the immunopathology of any of these diarrhoeal illnesses, a longitudinal study with information about an individual's immune system prior to the illness will be necessary. It is hoped that this study will identify certain immunological markers in the different diarrhoeal illnesses on which hypotheses can be drawn and investigated in future studies.

2. In accordance with the reviewers comment that there is a lack of description regarding gut biopsies, we have now explained that these biopsies will be carried out when the facilities are available to the Centre. This is dependent largely on the return of a post-graduate student who will be trained in the Karolinska Institute, Sweden.

3. The reviewer stresses the need for a carefully selected control group. In addition, to our infection-free, age-matched control group, we have in-built controls for each diarrhoeal group. This is illustrated below:

	CLINICAL GROUP	CONTROLS
1.	Acute diarrhoea	Malnutrition "Healthy" controls
2.	Persistent diarrhoea	Acute diarrhoea Malnutrition "Healthy" controls
3.	Recurrent diarrhoea	Acute diarrhoea Malnutrition "Healthy" controls
4.	Acute shigellosis	Acute diarrhoea Malnutrition "Healthy" controls
5.	Complications of shigellosis	Acute shigellosis Malnutrition "Healthy" controls
6.	Malnutrition	"Healthy" controls

4. The reviewer queries the need for assessing immunity in malnourished children as these studies have already been carried out. We have included this group of children in our study as no such investigations have been conducted in Bangladesh and this group also constitutes another control group for children with diarrhoea and malnutrition. The reviewer recommends the inclusion of children with severe malnutrition within the group. However, we have excluded severely malnourished children from all our clinical groups for simplicity; immunosuppression that usually accompanies severe malnutrition may interfere with the interpretation of data.

5. The reviewer comments that our third reason for conducting this study, i.e. it will provide information that may direct immunisation strategies, will not be supported by any of the tests we have so far included. Taking this into account and his suggestion (as well as Prof. D. M. Roberton's), we have included estimations of Ig levels to diphtheria, tetanus and polio.

6. In response to the reviewers criticism, we have excluded the suggestion that one of the usefulness of measuring the immune status is that it provides an indirect measure for the nutritional status of an individual.

7. As the reviewer suggests, the three antigens that we planned to use in skin tests for delayed type hypersensitivity (DTH) are not adequate. Accordingly, we will now use a commercially available DTH kit (Multitest CMI kit), whereby 7 antigens will be tested.

8. According to the reviewers suggestion, we have incorporated titles for our tables.

9. The reviewer questions whether we will measure any trace elements. We will measure iron and zinc.

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July 12, 1989

RESEARCH PROJECT REVIEW

TITLE OF PROJECT : Development of an immunology laboratory and application of immune function tests to persistent diarrhoea, shigellosis and malnutrition

INVESTIGATORS : Dr. Tasnim Azim
Dr. Laila Noor Islam
Mr. M. A. Wahed

INSTITUTION : International Centre for Diarrhoeal Disease Research, Bangladesh

COMMENTS :

Aims of project

a) Investigation of immunological parameters: This project seeks to establish laboratory procedures which will measure several aspects of immunological competence and antigen-specific immune responses in children from infancy until age 12 years. These techniques will be used to perform a systematic evaluation of children with persistent diarrhoea (i.e. greater than 2 weeks duration); with shigellosis; and with malnutrition (with or without diarrhoea).

The assays described in the application are comprehensive and will provide for a thorough evaluation of systemic immunological responsiveness. Provision has also been made

for investigation of some parameters of mucosal immune responses [measurement of salivary IgA content; measurement of faecal immunoglobulin content; and phenotypic analysis of lymphocytes in gut (presumably duodenal) biopsies]. Establishment of the assays should be straightforward, provided that major items of equipment (β-counter, γ-counter; centrifuges are readily available). The assays described are in use routinely in many other laboratories in paediatric institutions worldwide, although response norms for varying geographic, racial and nutritional status groups are less readily available. Therefore, it will be important for the applicants' laboratories to establish their own normal ranges, particularly for age-related values (preferably as centiles) for immunoglobulin concentrations, including IgE. It will be necessary to establish normal ranges for IgA concentrations in saliva and faeces, and these will require correlation against total immunoglobulin or total protein content to allow for the differing secretory phases (water content) of saliva/faeces.

The assays described in the application are those which laboratories in paediatric institutions would use to detect the abnormalities of classical primary or acquired immunodeficiency disorders. These disorders are rare (e.g. severe combined immunodeficiency approximately 1:67,000 live births; SLA approximately 1:83,000 live births), but accurate diagnosis is essential to ensure appropriate

treatment and survival. However, detection of these disorders presumably will be of less priority in the investigations outlined in the proposal.

b) Investigation of immunological abnormalities in specific disorders: Of more relevance to the application is the detection of abnormalities which may occur in specific disease states. The investigators propose to use the assays described to evaluate immunological competence in three disorders which are prevalent in childhood in their community, i.e. persistent diarrhoea, shigellosis and malnutrition. The proposed studies will provide comprehensive information, particularly with respect to normal lymphocyte and neutrophil function, which will be relevant to the community which their institute serves. This information will be very valuable as a basis for further more specific studies. Any abnormalities detected in the disorders studied are likely to be secondary to malnutrition or specific infection rather than primary immunodeficiency disorders. Thus, the information gained will be of use in detecting the extent of the alterations of immunological responses in these disease states (and hence delineating the risk of further infection consequent upon these secondary immunological abnormalities). Detection of the secondary immunological abnormalities also may be of use in differentiating different disorders, e.g. there may be different patterns of non-specific immunological responses in malnutrition compared with shigellosis.

Investigation of antigen-specific immune responses may be helpful in these studies in order to provide greater sensitivity. It may be useful to determine quantitative antibody responses to antigens to which the children in the various study groups have been exposed previously, e.g. serum antibody responses to tetanus and diphtheria (exposure by systemic immunisation); salivary antibody responses to poliovirus (exposure by mucosal immunisation); isohaemogglutinins (an IgM function). Serum opsonic activity to specific organisms, e.g. *S. aureus* and *C. albicans* can be incorporated easily into the proposed assays of neutrophil metabolic activity by comparison of responses of the test subjects' iodination responses in the presence of autologous serum and responses in pooled human serum. The applicants propose to measure immunoglobulin production in supernatants from PWM-stimulated PBL; it will also be possible to determine the ability of antigen-stimulated PBL to produce antibody to specific antigens, e.g. *Shigella* somatic antigens. Are there age-related differences in the ability of PBL to produce antibody to various *Shigella* antigens, e.g. as for *H. influenzae* b PRP?

An important requirement of the above studies will be to have accurate data concerning nutritional status of the patients studied, e.g. weight for height, arm circumference, serum iron/folate/plasma zinc concentrations. Presumably such information will be available from the studies of

Drs. Bardhan and Bennish. Data from the immunological studies can then be assessed with respect to these parameters within the various study groups.

Assessment of protein loss/deficiency will be important and therefore data from protein electrophoresis studies should be incorporated. Studies for the future which can arise out of the methodologies proposed in this application will be able to be designed which will assess cytokine responses by PBL to specific antigens, e.g. γ-interferon production in response to *Shigella*; TNF α and β production.

SUMMARY :

This proposal should be supported, and will result in the establishment of a competent immunology laboratory with expertise in lymphocyte culture techniques, assessment of neutrophil function and immunoglobulin quantitation. The proposed studies will then be able to establish normal ranges (with respect to age where appropriate) for a sample of the paediatric population, and can investigate potential changes in children with specific disorders. The expertise and information gained will be of great value in further studies of immunological function designed to elucidate the pathophysiology of specific infections.

D. M. Roberton, MD, FRACP
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20th July, 1989

Dr. Saul Tzipori,
Associate Director,
Laboratory Sciences Division,
ICDDR, B,
GPO Box 128,
Dhaka 1000,
Bangladesh.

Dear Dr. Tzipori,

Research Project Grant - Dr. T. Azim et al

At the request of Dr. D. Roberton of the Royal Children's Hospital in Melbourne, I am forwarding to you some comments on the above application for a project grant. I hope that these, taken together with Dr. Roberton's more detailed evaluation, will be of help to you.

Yours sincerely,

M. W. TURNER,
Reader in Immunochemistry.

Enc.

SUPPLEMENTARY COMMENTS ON RESEARCH PROJECT REVIEW

TITLE OF PROJECT: Development of an immunology laboratory and application of immune function tests to persistent diarrhoea, shigellosis and malnutrition

INVESTIGATORS: Dr. Tasnim Azim
Dr. Laila Noor Islam
Mr. M. A. Wahed

INSTITUTION: International Centre for Diarrhoeal Disease Research, Bangladesh

As in many other countries with a high prevalence of these disorders, there is clearly a need for wide ranging immunological investigations in Bangladeshis with persistent diarrhoea, shigellosis and malnutrition. For this reason alone the application should be supported since it seeks to define for these subpopulations the functional ranges of several humoral/cellular tests commonly measured in the developed world. The application is, however, unclear in several critically important points and consideration should be given to these before laboratory work commences. Firstly, the control group is, at present, not adequately defined. It needs to encompass all age bands from 0-12 years and, as far as possible, be drawn from apparently healthy non-parasitised children. Any hospitalised individuals should be carefully selected cases in order to exclude possible immunological abnormalities. The interpretation of all the data from the clinical subgroups will be seriously impaired if the control group is not rigorously selected. The applicants must also attempt to ensure that the clinical groups are as homogeneous as possible - especially with regard to the timing of samples in relation to the particular illness.

There is no discussion of how the applicants propose to evaluate their data, nor do they appear to have sought the help of a statistician at the planning stage.

If the applicants prepare the ground carefully before embarking on this project I am sure the data they obtain for the three disease groups will be most valuable as a reference baseline for future studies and/or therapeutic trials.

M. W. TURNER
Reader in Immunochemistry
(University of London)

TO: DR. SAUL TZIPORI. AD, LSD

FROM: MICHAEL BENNISH, M.D. MKRB

DATE: 6 AUGUST, 1989

RE: REVIEW OF PROTOCOL ENTITLED "DEVELOPMENT OF AN IMMUNOLOGY LABORATORY AND APPLICATION OF IMMUNE FUNCTION TESTS TO PERSISTENT DIARRHOEA, SHIGELLOSIS AND MALNUTRITION" BY DRS TASNIM ISLAM AND LAILA NOOR ISLAM, AND MR. WAHED.

Thank you for asking me for my comments on the above named protocol. In general I think that the protocol is a good one, and have only the following limited comments.

GENERAL COMMENTS

This is a well written and important protocol. It attempts to establish for the first time at the Centre the techniques for assessment of immune function. It has been written by a scientist with excellent training in a Ph.D programme in the UK. Although her Ph.D was not in clinical immunology, she is conversant with many of the techniques necessary for this type of study. The same is true of her co-investigators. They are admirably suited to establish and carry out the planned laboratory investigations.

In general I thought that the strength of the protocol as it is currently written is in the laboratory methods that have been detailed in the protocol. The investigators have outlined a series of investigations into immune function that are state of the art, targeted for the problems seen in Dhaka, and adapted for use with small volumes of blood, making them appropriate for use in children, who will be the subjects in most of the planned studies. In addition, I think that as the tests are outlined in the protocol, and given the skills of the investigators, they can be established successfully in Dhaka, an important consideration given the previous difficulties in establishing, and sustaining, many laboratory techniques at the Centre.

The weakness of the protocol as it now stands is with the specific hypotheses that attempt to relate abnormalities in immune function to disease pathogenesis in three groups of patients: (1) patients with persistent diarrhea; (2) patients with shigellosis and complications of shigellosis; and (3) patients with malnutrition. For the most part the protocol

proposes to describe the immune status of such patients, rather than test specific hypothesis.

SPECIFIC COMMENTS

Pages 4-5. Persistent diarrhea.

As discussed above, what specific immune defect do the authors postulate patients with chronic diarrhea have? Which of the tests that the authors are proposing to establish would shed light on the pathogenesis of the persistent diarrhea? I don't think it sufficient to state, as the authors do, that "Children with persistent diarrhea may have lowered immunity". Statements such as that are simply too vague. Given that most patients that we see with persistent diarrhea are severely malnourished, it is to be expected that they will have altered immune function, especially alterations in cellular immunity. The authors need to at least postulate on the mechanisms whereby a specific immune abnormality might lead to persistent diarrhea.

Let me take the liberty of putting on paper a few examples of hypotheses that come to mind sitting here in front of the computer screen in Boston. Persistent diarrhea is likely to result from a heterogeneous set of pathologic processes. Some patients with persistent diarrhea will have small bowel overgrowth, or perhaps infection with enteroadherent Escherechia coli. Do such patients, when compared to patients with persistent diarrhea who do not have an identified infection of the small bowel, or patients of similar nutritional status without persistent diarrhea, have diminished immune function? Specifically, do they have lower concentrations of serum or gut secretory immunoglobulins or diminished delayed type hypersensitivity? It would seem that if an immune defect were to contribute to persistent gut infection, that it would most likely (by analogy with patients with chronic giardiasis or rotavirus infection) be due to a defect in immunoglobulin quantity or quality (or less likely T-cell function) rather than a neutrophil defect.

If gut biopsies are to be done, what specifically will be looked for? Will the mononuclear cells in the mucosa that are to be phenotyped - be compared between patients with persistent diarrhea and demonstrated infection, patients with persistent diarrhea and without infection, and patients without persistent diarrhea? Although this is also descriptive in nature, it is important to define the control groups that will be used for comparison. The selection of appropriate control groups will allow the investigators to make associations between observed abnormalities in immune function, and specific disease states. Without carefully selected control patients, it will only be

possible to enumerate and describe, and not to define associations.

Page 5-7. Shigellosis.

In this section the authors focus primarily on the specific immune response to shigellosis. However, as with the previous section they fail to generate specific hypothesis. What specific questions might be asked? I think there are a number of important ones. In patients with leukemoid reaction, do these large numbers of neutrophils function normally? Are there differences between neutrophil function during over time in these patients - before, during and after the development of the leukemoid reaction? These questions stem from two hypotheses. One hypothesis is that the leukemoid reaction in these patients is a consequence of dysfunctional neutrophils, and that as a result there is no negative feedback. That is, the stimulus for white cell production and release remains turned on as a result of an absence of negative feedback. The factors regulating neutrophil production and release from the marrow are not completely elucidated, but showing that these white cells are dysfunctional would by implication show that abnormalities in both function and regulatory mechanisms have occurred.

The second hypothesis that could be tested by examining neutrophil function in these patients is to attempt to correlate neutrophil number and activity with the extent of colitis. If biopsies are to be done, one could grade the extent of the colitis and see if it directly relates to white cell number - is the leukemoid reaction simply a function (or vice versa) or the extent of mucosal damage? It would also be possible to look at soluble enzymes of neutrophil elastase - and see if these correlate with the extent of mucosal injury. This would not necessarily answer the question of what is chicken and what is egg - which is goru and which is gari - are the white cells responsible for the tissue injury that occurs, or are they simply recruited in response to tissue injury. However if a negative correlation, or no correlation between the extent of tissue damage and the number and function of white cells is found, this would imply that factors other than tissue injury are responsible for the leukocytosis that occurs in these patients.

The authors refer to "chronic shigellosis". Although this is an ill-defined entity, it is known, as pointed out by the authors, that when they are compared to patients with watery diarrhea, excessive mortality occurs in patients with dysentery and shigellosis subsequent to their discharge from the hospital. Is this because they have a greater degree of immunosuppresion when compared to patients of similar nutritional status? What is the nature of this immunosuppresion? Is it simply loss of

immunoglobulins through a leaky GI tract?

The above are only "off-the-cuff" (and not detailed) examples of hypotheses that might be tested using the techniques the investigators are proposing to establish. I am not proposing that these are necessarily the hypotheses to test - only that the background and research plan need to include more specific hypotheses than have been included in this version of the proposal.

Pages 7-8. Malnutrition.

A large number of descriptive studies of immune function during malnutrition have already been done. What do the authors propose to do that will be new? Also, if malnutrition is to be studied, why exclude (as the authors propose on page 3) patients with severe malnutrition? It would seem to me that those would be the patients that you would be most interested in.

Pages 8-9. Usefulness of the study.

I agree with the first two reasons that the investigators put forth as to why this study would be useful- namely that such investigations will provide important prognostic information and will provide insight into the immunopathology of specific disease conditions. The next three reasons put forth are a bit more problematic - namely that they will provide important information about immunization strategies (numbers 3 and 5) or that the tests are a more sensitive or useful measure of nutritional status than are traditional measures such as anthropometry or determination of serum albumin concentrations. The serologic response to immunization is not a part of the protocol as currently written, and thus it is not likely that this study will provide any new information on the efficacy of immunization of malnourished children. In general, the available data suggest that immunizations included as part of the standard EPI regimen are effective even in malnourished children, although they, to my knowledge, have never been evaluated in children malnutrition as severe as that frequently encountered in Dhaka.

To use of these assessments of immune function as an indirect measure of nutritional status seems to me to be stretching a point. It could not be argued that they are easier or less costly to perform. In addition although they might be a better predictor of outcome than anthropometry (which would be an important, and not entirely unexpected finding) but not necessarily because they are assessing nutritional status. What they asses is immune status, which very well be a more important determinant of outcome than is body mass or serum albumin.

Methods. Pages 10-17.

As mentioned above, this is the strength of the protocol as it now stands. The methods are well described and outlined. I have only a couple of comments. Under "III. Gut Biopsy" no details are given of where, how, and from whom the biopsies will be obtained from. Under "IV.DTH" the three antigens that will be looked for are not likely to be sufficient. Most children tested will not have been immunized with tetanus toxoid, and thus are unlikely to have a DTH response to tetanus. The same holds true for PPD - most children coming to the Treatment Centre will not have been immunized with BCG, and will not have been infected with Mycobacterium tuberculosis. There will thus be only one antigen, Candida albicans that they should mount a DTH to, and this probably is insufficient to judge responsiveness. There are other antigens, such as streptococcal antigens and other ubiquitous fungi, such as tricophytan, that are available that may be of use, as well as commercially available multiple test sets.

Tables. Pages 16-20.

The tables are nicely set up and informative, but could benefit from having titles. Do the investigators plan to measure trace elements as is outlined in Table 2? It is not mentioned in the text.

References. Pages 20-25.

The ones selected are appropriate and relevant.

Publication of the investigators. Pages 26-30.

As discusses above the investigators seem admirably suited to carry out the investigations listed, and this is borne out in their publication record.

Specific tasks. Page 31.

This is a very useful addition to the protocol format, and helps to clearly define what the principal investigators will be doing.

Budget. Page 32.

This appears appropriate for the proposed laboratory investigations.

CONCLUSIONS.

This protocol is designed to establish techniques to assess immune function that will be of fundamental importance to investigations carried out at the Centre. The ability to perform

such tests will now allow us to look at the pathogenesis of infections from the side of the host, rather than simply from the perspective of the infecting microbe, or by epidemiologic relationships. As such it is deserving of support as it now stands. The investigators are all laboratory based researchers, and this is reflected in the lack of precise hypotheses regarding pathophysiology. The latter should develop as the techniques are established, and the investigators have more opportunity to discuss some of the issues at hand with clinicians working on these problems. I think that this is almost a two phase protocol - the first, and basic step is to develop the laboratory techniques, and the next step is to apply them to the problems at hand. Given a proper interchange between scientists, I am convinced that more precisely defined investigations will be elaborated in the coming months. In the meantime I think the efforts to establish the techniques assume primary importance, and should proceed.