RRC/F

Attachment 1.

Date 11.89

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator TASNIM AZIM Trainee Investigator (if any)

Application No. 89-014 Supporting Agency (if Non-ICDDR,B)

Title of Study STUDY OF THE IMMUNE RESPONSE TO S.DISENTERIAE I IN AN EFFORT TO IDENTIFY ABNORMALITIES LEADING TO THE DEVELOPMENT OF UREMOID REACTION

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 NA
   (f) Right to refuse to participate or to withdraw from study (Yes) No
   (g) Confidential handling of data (Yes) No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No NA
5. Will signed consent form be required:
   (a) From subjects Yes (No)
   (b) From parent or guardian (if subjects are minors) (Yes) No
6. Will precautions be taken to protect anonymity of subjects (Yes) No
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ✓ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

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

(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.

Tasnim Azim
Principal Investigator

Trainee

APPLICATION FOR PROJECT GRANT

P.I.

1. INVESTIGATORS : Dr. Tasnim Azim
Dr. Laila Noor Islam
Dr. Firdausi Qadri
Dr. M. A. Salam
Mr. M. A. Wahed

2. TITLE OF PROJECT : Study of the immune response to *S. dysenteriae* 1 in an effort to identify abnormalities leading to the development of leukemoid reaction

3. STARTING DATE : As soon as possible

4. COMPLETION DATE : 3 years from starting date

5. TOTAL BUDGET REQUESTED : US$ 207,392

6. FUNDING SOURCE :

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

8. AIMS OF PROJECT :

a) GENERAL AIM

To carry out a systematic examination of the immune response of children with dysentery from *S. dysenteriae* 1 in order to identify possible immunological abnormalities leading to complications such as leukemoid reaction.

b) SPECIFIC AIMS

1) Assessment of the immune response to *S. dysenteriae* 1 in children who recover from shigellosis and those who develop leukemoid reaction.

2) Identification of possible alterations in granulocyte and lymphocyte responses in children with leukemoid reaction arising from shigellosis.

c) SIGNIFICANCE

A systematic study of the immune response of children with shigellosis may identify cellular immunological changes that predict complications as well as provide insights into the immunopathogenesis of these conditions.

9. ETHICAL IMPLICATION

The following children will be studied:

Disease condition	No. of patients	Age of patients (yrs)	Samples	Source of patients
Uncomplicated shigellosis	150	1-5	P.blood Stool Saliva	ICDDR,B
Complications of shigellosis	30	1-5	P.blood Stool Saliva	ICDDR,B
Malnutrition	30	1-5	P.blood Stool Saliva	Possibly from Save the Children Fund, Shishu Hospital
Controls	30	1-5	P.blood Stool Saliva	Possibly from Pongu Hospital

*P.blood = peripheral blood

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

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

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

From each of these children stool and saliva samples will be collected and peripheral blood will be obtained by venepuncture. Delayed type hypersensitivity (DTH) will also be tested on the forearm of all children.

Children (1-5 yrs) with complaints of dysentery and with a stool microscopy showing >20 leucocytes/HPF will initially be enrolled in the study. We will include one hundred and fifty children with culture confirmed *S. dysenteriae* 1. It is difficult to estimate the number of patients infected with *S. dysenteriae* 1, however, from the ongoing study of Bennish

et al.. it has been estimated that approximately 367 of children with a presumptive diagnosis of shigellosis will have to be enrolled initially to obtain 150 children infected with *S. dysenteriae* 1. Also from the same study, it has been calculated that 18% of children infected with *S. dysenteriae* 1 develop leukemoid reaction. Enrollment of 150 children with shigellosis will allow us to study approximately 30 children with leukemoid reaction. However, as these numbers can be very variable, we may have to enroll more children for the study to obtain 30 with leukemoid reaction.

Upto 5 mls of blood will be drawn from children infected with *S. dysenteriae* 1 approximately 24 hrs after admission, i.e. on confirmation of diagnosis, sometime between days 3-5 after admission and 14 days after discharge. Saliva and stool will also be collected on the same days. DTH will be tested during the acute illness. Thus not more than 10 ml of blood will be obtained from children in 1 week. Since most children in this study will be 1-5 yrs old, their blood volume will be over 400 mls. Therefore, drawing of 10 mls of blood in 1 week will be harmless. Control children with malnutrition but without infection will be selected by matching every case child with a control child of the same weight for age within 5%. Control children without malnutrition or infection (i.e. healthy children) will be selected by matching for age such that for children <2 years control children will be within 2 months, and for children >2 years, control children will be within 4 months.

Five mls of blood will be drawn once from these children. All children will be assessed for malnutrition by anthropometry.

Children with shigellosis will be clinically evaluated by a daily physical examination, measurements of weight, fluid, and stool output. Patients will have a standard six-hourly monitoring of rectal temperature, heart rate and blood pressure. Microscopic examination for cellular elements in stool will be carried out. The haemoglobin percentage and total and differential leucocyte count will be measured in blood.

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

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. Shigellosis is a common cause of morbidity and mortality in Bangladesh. 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).

The immune mechanisms against shigellosis are not well understood. There is evidence to suggest that both cellular and humoral defense mechanisms are involved in recovery from shigellosis. Thus, serum Immunoglobulins (Ig) of the A, G and M isotypes 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 (Winsor *et al.*, 1988), saliva (Schultsz *et al.*, ongoing work at the Centre), intestinal fluid (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). Initial studies on a T cell clone responsive to *S. 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.

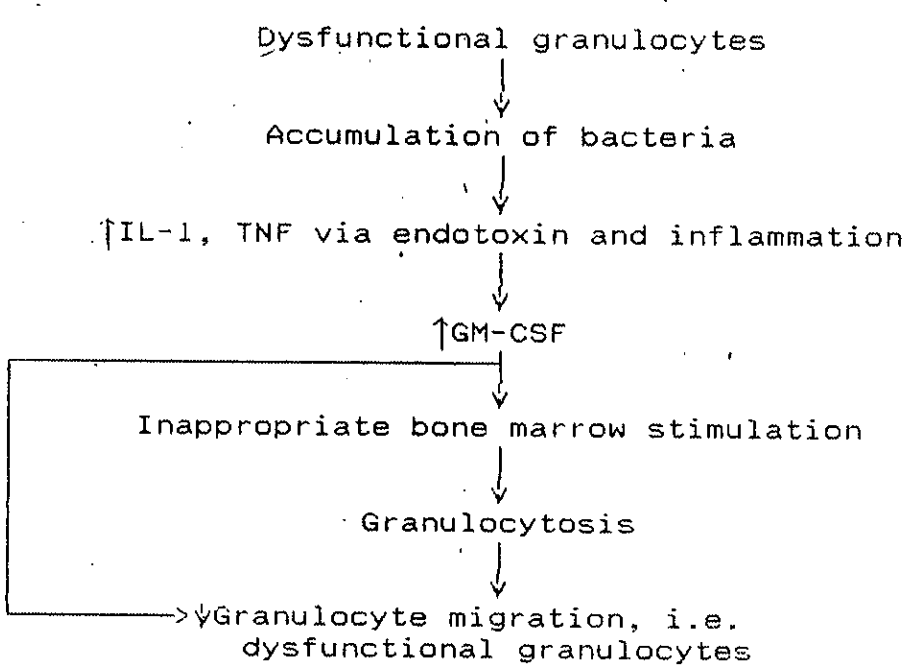
Infection from Shigella can lead to a wide variety of complications. Thus, patients may develop a chronic illness with associated malnutrition, shigellaemia, leukemoid reaction or haemolytic uremic syndrome (HUS). The leukemoid reaction is associated with a WBC count of ≥40,000/cu mm, granulocytosis and an increase in immature neutrophils (Rahaman *et al*, 1974; Butler *et al*, 1984). HUS consists of a triad of haemolytic anaemia, thrombocytopenia and acute renal failure which may be related to the deposition of immune complexes (Koster *et al*, 1978). However, the cause/causes for these severe systemic complications is/are 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 IL1 alpha and beta, tumour necrosis factor (TNF) in the pathogenesis of shigellosis. Although cytokines may be involved in these complications, their release may be secondary to cellular dysfunction. Our study proposes to investigate possible abnormalities in the cellular immune response prior to the onset of leukemoid reaction and during leukemoid reaction by answering the following questions:

1) Are there dysfunctional granulocytes in leukemoid reaction?

Granulocytes are produced in the bone marrow from progenitor cells which are pluripotent stem cells. Pluripotent stem cells differentiate into metamyelocytes which mature into band forms and eventually into functioning granulocytes. This developmental process is controlled by various haemopoietic factors present in the microenvironment of the bone marrow (Clark and Kamer, 1987). The haemopoietic factors include granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin-3 (IL-3). Other factors such as IL-1, IL-4 and tumour necrosis factor (TNF) also control haemopoiesis. From the bone marrow mature granulocytes enter the circulation and they are then available for defence against infection. For optimal antibacterial activity, neutrophils must first adhere to endothelial cells, migrate through the blood vessels, engulf the organisms and degranulate.

The excessive numbers of granulocytes, especially immature granulocytes, in leukemoid reaction suggest an inappropriate stimulation of the bone marrow the cause for which is not known. It is possible that in children who develop leukemoid reaction granulocytes do not function adequately with a resultant accumulation of bacteria. A greater bacterial load causes more tissue damage and more secretion of bacterial endotoxin and both these factors enhance the release of IL-1 and TNF. IL-1 and TNF stimulate GM-CSF production which stimulates the bone marrow thus causing granulocytosis. Although GM-CSF stimulates differentiation of early progenitors, it induces margination of mature granulocytes and inhibits their migration *in vivo* (Addison *et al.*, 1989). Thus, leukemoid reaction may occur because of dysfunctional granulocytes which cause excessive GM-CSF release leading to enhanced production of granulocytes that are functionally inept *in vivo* (shown below):



2) Are there immature T cells in leukemoid reaction?

Immature T cells emerge from the bone marrow and enter the thymus where they differentiate into mature T cells. About 95-99% bone marrow derived T cells undergo cell-death in the thymus, the rest acquire T cell antigens such as the T cell receptor (TCR), CD2, CD3, CD4 and CD8. TCR+, CD3+, CD4+ (helper/suppressor-inducer) and TCR+, CD3+, CD8+ (suppressor/cytotoxic) cells which are functionally competent T cells leave the thymus and circulate to different areas of the body. These mature T cells, on activation by antigen, release soluble factors which activate other T cells and B cells. Activated T cells can also provide B cell help and mediate suppressor and cytotoxic activities. Thus, altered T cell function can occur as a result of defects at several levels. There may be defects in lymphoid progenitors, i.e. in the bone marrow, in the thymus, in molecular events that regulate T cell antigens or in the T cell activation process. In patients with leukemoid reaction from *S. dysenteriae* 1 a proportion of circulating T cells are immature as they do not express the CD2 antigen (Jackson *et al.*, 1979). However, CD2 expression can be induced by thymopoietin suggesting abnormalities in the bone marrow or the thymus. Jackson *et al.* (1979) hypothesize that factors, such as endotoxin, released by *S. dysenteriae* 1 adversely affect the bone marrow or the thymus causing the release of immature T cells. We intend to investigate the

hypothesis further by characterising T cells using monoclonal antibdies to CD3, CD4 and CD8 and assessing their functional response to mitogens and antigens.

3) Are there functional B cells in leukemoid reaction?

Like T cells and granulocytes, B cells are also produced in the bone marrow. B cells differentiate from stem cells to cells that express surface IgM (sIgM) which enter the circulation. On exposure to antigen, sIgM+ B cells become activated, switch to other Ig isotypes, proliferate and give rise either to memory cells or Ig-secreting plasma cells. Activation of B cells by antigen may be T-dependent or T-independent. In the former case, antigen-specific B cells are activated by T cells themselves and by interleukins secreted by T cells (IL-2, IL-4, IL-5 and IL-6). Even when B cells are activated independently of T cells, i.e. directly by antigen, B cells often require T cell factors. Thus, altered B cell responses may be due to defects in the differentiation process occurring in the bone marrow or due to alterations of T cell functions. Both these factors may operate in leukemoid reaction from *S. dysenteriae* 1.

4) Does malnutrition contribute to the development of leukemoid reaction?

Nutrition has profound effects on immunity and in malnutrition there is an overall suppression of the immune response (Tables 1 and 2). When shigellosis is accompanied by malnutrition, there is increased risk of development of a chronic

illness in children (Keusch, 1982) and fatality in infants (Streulens *et al.*, 1985). However, there appears to be no association between malnutrition and the development of leukemoid reaction in shigellosis (Rahaman *et al.*, 1975). This may be because of the overall immune suppression in malnutrition which may not allow the development of a leukemoid reaction. On the other hand, a decrease in granulocyte numbers has not been reported in malnutrition although there is diminished granulocyte function. It is, therefore, possible that dysfunctional granulocytes and reduced T cells in malnutrition could predispose to the development of leukemoid reaction. We, therefore, intend to examine the relationship between malnutrition and leukemoid reaction in shigellosis by not only comparing immune responses of children with and without leukemoid reaction, but also by assessing *in vitro Shigella*-specific responses of granulocytes and lymphocytes from nutritionally matched children without shigellosis

A detailed plan of study is given below.

Materials to be studied include:

I. Peripheral blood
II. Saliva and stool
III. 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 peripheral 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 (5 ml maximum)

—> 1.0 ml in glass tubes for SERUM to be used for:
1. C_3, C_4 levels
2. Autologous serum for use in neutrophil iodination
3. 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. Autologous plasma for PWM and PHA stimulation and MLC
4. Ig to causative organism and its antigens
5. 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. Stimulation by Shigella antigens —> 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 BIO). Anti-C3 and anti-C4 antibodies will be obtained commercially.

2. Protein fractions will be estimated by electrophoresis.

B PLASMA

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

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

3. Igs to *Shigella* antigens will be estimated by ELISA.

4. Igs to diphtheria and tetanus will be measured by a neutralisation assay and ELISA respectively.

5. 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. Stimulation by *Shigella* antigens (LPS, toxin) etc. patients mononuclear cells will be cultured in the presence of *Shigella* antigens with autologous plasma heterologous plasma, or calf serum for 5 days. Proliferation and antibody secretion will then be measured by ^{3}HTDR incorporation and ELISA.

5. Phenotyping by indirect immunofluorescence will be carried out for determining proportions 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 source), 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 gamma-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), *Shigella* 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 microscope.

4. Morphological polarisation of neutrophils-neutrophils will be incubated with FMLP, *Shigella* antigens for 30 mins at 37°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. All samples will be obtained at least an hour after a meal in the morning. 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 Shigella antigens. Igs to poliovirus will also be determined using a neutralisation assay.

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 used for measuring Ig as for saliva. Shiga toxin will also be assayed in stool by ELISA.

III. 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. Tuberculin 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

↓ IL1 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

TEST	SIGNIFICANCE
A. SERUM	
1. Complement C3, C4 levels	C3 ↓ malnutrition, C4 is normal.
2. 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. CRP	↑ in bacterial infections although not in viral infections.
3. 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. 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.

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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. Laila N. Islam

1. Islam, L.N. and Wilkinson, P.C. (1989) Evaluation of methods for isolating human peripheral blood monocytes. J. Immunol. Meths. 121:75-84.

2. Wilkinson, P.C. and Islam, L.N. (1989) Recombinant IL-4 and IFN-γ activate locomotor capacity in human B lymphocytes. Immunol, 67:237-243.

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

4. 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.

5. 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 lymphocytes. J. Cell Sci., 90:645-655.

6. 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.

7. 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.

8. 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.

c) 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.

12. FLOW CHART

Study population	No. of children	Time (3 years)
Shigellosis (*S. dysenteriae*)	~150	Children will be enrolled whenever available, preferably by 1½ years
Leukemoid reaction	30	As above
Malnutrition	30	Samples will be obtained from children at similar times to that from children with shigellosis
Healthy controls	30	As above

13. ITEMISED SPECIFIC TASKS FOR EACH LISTED INVESTIGATORS

a) Dr. Tasnim Azim

1) Lymphocyte studies

2) DTH studies

b) Dr. M.A. Salam

Clinical assessment and management of patients at ICDDR,B

c) Dr. Laila Noor Islam

Granulocyte studies

d) Dr. Firdausi Qadri

1) ELISAs for IgE, IgA, IgG, IgM and bacterial antigens in plasma, saliva and stool

2) Determination of Shiga toxin in stool

e) 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-B10

4) Determination of plasma zinc levels by AAS

5) Electrophoresis for estimation of protein fractions

13. BUDGET

	1st yr	2nd yr	3rd yr
Personnel			
Tasnim Azim	10,908	11,999	13,199
Laila Islam	2,000	2,200	2,442
M.A. Wahed	603	664	730
Technicians (2)	9,000	9,900	10,890
Plastics	5,000	5,500	5,904
Chemicals	5,000	5,500	6,500
Media, antibiotics and other reagents	8,092	10,000	11,500
Stock items, such as pipette tips, glass slides, glásswares, etc.	4,000	5,000	6,000
Instruments, including automash, camera, camera accessories, finnpipettes	9,500	2,851	2,000
10% added costs	8,000	9,000	10,000
Miscellaneous, including mail, photocopying, transport, etc.	4,000	4,500	5,000
Total:	66,103	67,114	74,175

GRAND TOTAL: US$ 207,392

Terminal date: 31.12.94

ICDDR,B, DHAKA (AN INCOMPLETE FORM WILL NOT BE ACCEPTED: SL.NOS.9 & 13 MUST)

ETHICAL REVIEW COMMITTEE: PROTOCOL E X T E N S I O N/T E R M I N A T I O N FORM
RESEARCH REVIEW COMMITTEE:

PRINCIPAL INVESTIGATOR : Dr. Tasnim Azim, LSD Protocol No: 89-014

Title: "Study of the immune response to S.dysenteriae 1 in an effort to identify abnormalities leading to the development of leukemoid reaction".

Starting dates of Protocol: Original planned: 20.6.90 Actual: 20.6.90

(1) Status of Protocol :

~~Was~~/will be completed as scheduled [✓] Will require an extension [ ]

(2) Results achieved :

Publication(s) : Yes [ ] No [✓]

Manuscript(s) : Yes [✓] No [ ]

If "Yes", please provide one copy each of papers, abstracts &/or manuscripts.

If no article or manuscript is available, please provide brief summary achieved:

MANUSCRIPT ATTACHED. ALSO SEE ABSTRACT OF STUDY.

(3) If an extension is needed, state length of extension required and reason extension needed (e.g. 6 months, field work completed but analysis work outstanding). CALCULATE EXTENSION FROM ORIGINAL EXPECTED COMPLETION DATE.

_____ months/year Reasons:

Original expected completion date : _____

(4) State if you have added any methods or procedures not originally in the protocol:

(5) Any adverse effects in cases where the study involves human subjects:

(6) Additional data analysis/procedures not originally in the protocol is planned:

Yes [ ] No [ ]

(7) If "Yes", give details :

(8) Name of Division involved LSD

(9) Date on which plans for extension was approved in the Division: _____

(10) Additional budget needed: Yes [ ] No [ ]

(11) If yes, give details:

(12) Signature of the Principal Investigator: Tasnim Azim Date: 11.12.94

(13) Signature of A/Associate Director of the Division: [signature] Date: 11.12.94

* If space is insufficient to a particular question, please use seperate sheet.

Interim/Final

SUMMARY COMPLETION FORM FOR PROTOCOLS

e : Study of the immune response to S. dysentériae 1 in an effort to identify abnormalities leading to the development of leukemoid reaction.

estigator(s) : Dr. Tasnim Azim, Laboratory Sciences Division

dget Code # : 20 55 11 (Protocol No.89-014)

dings (Abstract) :

SEE ATTACHED

ficy implications :

NOT APPLICABLE

ssemination plans:

Publications in journals, presentations at meetings and conferences.

te: 11.12.94

Signature of the P.I. Tasnim Azim

FINDINGS (ABSTRACT) OF STUDY

The immune response of three groups of children including children with uncomplicated and complicated *S. dysenteriae* 1 infection and age-matched healthy controls have been compared. Aspects of immunity studied include functional responses of peripheral blood granulocytes (polarisation, phagocytosis and chemotaxis), phenotype and proliferation in response to mitogens of peripheral blood mononuclear cells (PBMs), cell-mediated immunity as measured by skin tests, antibody titres to the lipopolysaccharide (LPS) of *S. dysenteriae* 1 in stool, saliva and plasma, and levels of tumour necrosis factor α (TNFα) and interleukin 6 (IL6) in stool. In most cases, each study group has been divided into 2 nutritional subgroups - $\leq$65% and >65% (weight-for-age as a percentage of the NCHS median).

Significant increases were observed in some aspects of granulocyte functional responses (polarisation and locomotion) between children with *S. dysenteriae* 1 infection (whether uncomplicated or complicated) and healthy controls (P=0.00), but there was no difference between children with uncomplicated and complicated *S. dysenteriae* 1 infection. Phagocytosis was similar in all 3 study groups. Thus, these aspects of granulocyte function were not altered in children with complicated versus uncomplicated *S. dysenteriae* 1 infection.

Children with complicated *S. dysenteriae* 1 infection had significantly lower percentages of circulating helper T (CD4+) lymphocytes than children with uncomplicated infection and

healthy controls (P<0.05). These children also had markedly depressed CMI responses when compared to children with uncomplicated infection (P=0.00). However, proliferation by PBMs in response to mitogens were similar in the 3 study groups.

Antibody titres to the LPS of *S. dysenteriae* 1 were higher in children with *S. dysenteriae* 1 infection (whether uncomplicated or complicated) than in controls (P<0.05). Antibody titres were lower in children with complicated versus uncomplicated infection although this was not statistically significant. However, there was a significant rise in antibody titres from the day of enrollment (d0) to 3-5 days later (d3-5), in children with uncomplicated infection but not in children with complicated infection. These findings suggest that antibodies to the LPS of *S. dysenteriae* 1 are suppressed in children with complicated infection.

Finally, both stool TNFα and IL6 levels were markedly reduced in children with complicated versus uncomplicated *S. dysenteriae* 1 infection (P<0.05). In children with uncomplicated infection, both cytokines were higher than in healthy controls (P<0.05).

In conclusion, some aspects of the immune response are altered in children with complicated infection. Thus, these children have lowered number of circulating helper T lymphocytes and depressed CMI responses to specific antigens, reduced antibody response to the LPS of *S. dysenteriae* 1 and lower stool TNFα and IL6 levels.

TA:mh/T3A:STUDY.ABS

Cytokines in the Stools of Children with Complicated Shigellosis

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Running head: Stool cytokines in complicated shigellosis

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ABSTRACT

Serious, often fatal, complications may accompany shigellosis particularly in children below 5 years of age. These complications include leukemoid reaction, hemolytic uremic syndrome, toxic megacolon and septicemia. Excessive production of proinflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 6 (IL6) have been suggested as possible factors in the pathogenesis of these complications. We measured TNFα and IL6 in stools of 97 children aged 12-60 months. These children included those with uncomplicated *Shigella dysenteriae* 1 infection (n=40), complicated *S. dysenteriae* 1 infection (n=18), uncomplicated *Shigella flexneri* infection (n=6), mixed *Shigella* and *Salmonella* infections (n=4) and age matched healthy controls (n=29). Higher stool TNFα and IL6 concentrations were found in children with uncomplicated *S. dysenteriae* 1 infection than in healthy controls ($p<0.05$). The numbers of children with *S. flexneri* infection and mixed infections were low, but in either of these groups of children, stool concentrations of both cytokines were similar to those in healthy controls. Children with complicated *S. dysenteriae* 1 infection as a group had lower stool cytokine concentrations than children with uncomplicated infection ($p<0.05$). However, subgroup analyses of children with complicated *S. dysenteriae* 1 infection showed that those with weight for age ≤65% (of the National Center for Health Statistics median) had higher stool cytokine concentrations than those with weight for age >65% ($p=0.004$). It is concluded that complicated

infection is not associated with higher stool concentrations of proinflammatory cytokines. TNFα and IL6. It is not clear how the levels of stool cytokines were related to the pathogenesis of complications in shigellosis.

INTRODUCTION

Shigellosis, particularly due to *Shigella dysenteriae* 1 is associated with a number of complications which are usually seen in children below 5 years of age. These, often fatal, complications include: leukemoid reaction, where, there is an increase in the white blood cell count to more than 40,000/cmm, granulocytosis and an increase in immature granulocytes (4); hemolytic uremic syndrome (HUS) which consists of a triad of hemolytic anemia, thrombocytopenia and acute renal failure (13); septicemia (17) and toxic megacolon (1). The pathogenesis of these complications remains to be understood.

Shigellae first adhere to (12) and then invade the colonic epithelial cells (9) where they multiply and then spread to adjacent cells (14). This process is accompanied by an intense inflammatory response with infiltration of large numbers of neutrophils and macrophages in the lamina propria (10). In other inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, activated macrophages in the lamina propria secrete proinflammatory cytokines such as interleukin 1 (IL1), interleukin 6 (IL6) and tumor necrosis factor α (TNFα) which can be detected in the colonic mucosa (15). High concentrations of IL6 and TNFα have been found in the serum and stool of children with uncomplicated *S. dysenteriae* 1 and *Shigella flexneri* infections (5,11). Higher concentrations of IL6, but not TNFα have been demonstrated in the serum of children with complicated

shigellosis. However, no data are available on the concentrations of cytokines in corresponding stool samples (5). As there is evidence that stool TNFα is a reliable marker for intestinal inflammation (3) and as high concentrations of IL6 are detected in stools of children with shigellosis (5,11), we compared stool IL6 and TNFα concentrations among healthy control children and children with complicated and uncomplicated shigellosis to investigate the possible roles these cytokines may play in the pathogenesis of complications in shigellosis.

MATERIALS AND METHODS

Study population. Children aged 12-60 months who were admitted to the Clinical Research and Service Centre (CRSC) of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, with acute dysentery (visible blood in stool with or without fever) were enrolled in the study. Stools were examined microscopically and were cultured for shigellae (18). Only children who were culture positive for *Shigella* were enrolled in the study. Samples of stool were collected for cytokine analysis from these children on the day of enrollment, 3-5 days later, and at convalescence (at least 14 days after discharge). A single stool sample was also obtained from control healthy children, matched for age attending the Nutrition Follow-Up Unit of ICDDR,B. These control children were free from any infection for at least one month prior to enrollment.

Collection and preparation of stool samples. Freshly passed stools were collected in sterile containers, weighed and 1 gm of stool was mixed with 1 ml of sterile phosphate buffered saline (PBS) (pH 7.2). The mixture was centrifuged at 20,000 *g* and the supernatant was filtered through a 0.45 mm filter (Sartorius, Goettingen, Germany). Aliquots of filtrates were stored at -70°C until use.

Cytokine assays. Assays for IL6 and TNFα were carried out using ELISA kits (Endogen Inc., Boston, MA, USA) in duplicate. The detection limit for TNFα and IL6 was 5 pg/ml and 4 pg/ml, respectively. Concentrations of both cytokines were calculated by extrapolation using a standard curve and were expressed as pg/g of stool.

Statistical analyses. Multiple regression analysis was carried out to evaluate the effects of duration of diarrhea prior to enrollment, presence of other infections, sex and nutritional status (weight for age as a percentage of the National Center for Health Statistics [NCHS] median) on stool IL6 and TNFα concentrations. After controlling for the confounders (duration of diarrhea and nutritional status), comparisons between the patient groups were done by one way ANOVA, and the Bonferroni test was then applied to assess significance of differences between 2 groups of children. The Mann-Whitney U test was also used to compare differences between the nutritional subgroups. Comparisons between the acute stage and follow-up days were made between paired samples by using the Wilcoxon Matched Pairs Signed

Rank test. The impact of the duration of diarrhea on the nutritional status was evaluated using Pearson's correlation. Differences were considered to be significant when p was ≤ 0.05. SPSS for Windows (version 6.0) was used for these analyses.

RESULTS

Study population: Ninety seven children were enrolled in the study. The study groups and assays performed are described in Table 1. The number of convalescent stool samples was very few (n=6 and 3, for uncomplicated and complicated *S. dysenteriae* 1 infection, respectively), for statistical comparisons. All but 1 child with mixed infections developed complications. The duration of diarrhea prior to enrollment, nutritional status, and proportion of males and females were comparable among the study groups (Table 2).

Correlation between stool cytokines and clinical parameters. Multiple regression analysis indicated that neither sex nor the presence of other infections (respiratory tract infections or middle ear infection) influenced stool TNFα and IL6 concentrations. However, stool concentrations of both cytokines were affected by nutritional status in that cytokine concentrations were higher in children with a lower weight for age. For this reason, children were divided into 2 subgroups: weight for age $\leq 65\%$ and $>65\%$. Stool IL6 and TNFα concentrations were further influenced by the duration of diarrhea prior to enrollment; a longer history of diarrhea was associated with lower cytokine concentrations in stool. However, the nutritional

status correlated negatively with the duration of diarrhea ($r=-0.2623$, $p=0.000$) so that undernourished children had a longer duration of diarrhea prior to enrollment. As a result of this negative correlation, the effect of duration of diarrhea was automatically removed after controlling for the nutritional status.

The numbers of infected children with *S. flexneri* and mixed *Shigella* and *Salmonella* were low. Therefore, the main comparisons were carried out between healthy control children, children with uncomplicated *S. dysenteriae* 1 infection and children with complicated *S. dysenteriae* 1 infection.

Stool TNFα and IL6 concentrations. Figs. 1 and 2 show concentrations of stool TNFα and IL6 respectively, on enrollment, by patient group and their nutritional subgroup.

Poorly nourished children (weight for age ≤65%) with complicated *S. dysenteriae* 1 infection had higher stool TNFα (n=7; 65-1013 pg/g; median=129) and IL6 (n=7; 38-5370 pg/g; median=517) concentrations than better nourished children (weight for age >65%) (for TNFα: n=11; 0-266 pg/g; median=33; for IL6: n=10; 2-88 pg/g; median=31), ($p=0.004$). The nutritional status had no effect on stool cytokine concentrations in healthy controls or children with uncomplicated *S. dysenteriae* 1 infection.

Significant differences in the levels of both cytokines were observed among healthy control children, children with

uncomplicated *S. dysenteriae* 1 infection and children with complicated *S. dysenteriae* 1 infection in both nutritional subgroups ($p<0.000$ for a vs b vs c in all four comparisons - 2 nutritional subgroups x 2 cytokines) (Figs. 1 and 2, A and B). Further analysis showed that stool TNFα concentrations were higher in children with uncomplicated *S. dysenteriae* 1 infection than in healthy controls ($p<0.05$) and children with complicated *S. dysenteriae* 1 infection ($p<0.05$) in both nutritional subgroups (Figs.1, A and B). However, TNFα concentrations were similar in healthy controls and children with complicated *S. dysenteriae* 1 infection (Figs. 1, A and B).

For IL6, the difference in concentrations between study groups varied with nutritional status. In the poorly nourished group (Fig. 2A), the levels were significantly higher in children with both uncomplicated (n=16; 2-10000 pg/g; median=1124; $p<0.05$) and complicated (n=7; 38-5370 pg/g; median=517; $p<0.05$) *S. dysenteriae* 1 infection, compared to healthy controls (n=17; 0-471 pg/g; median=16). The levels in poorly nourished children with uncomplicated and complicated *S. dysenteriae* 1 infection were similar ($p>0.05$). In the better nourished group (Fig. 2B), the levels were higher in children with uncomplicated *S. dysenteriae* 1 infection (n=17; 4-10000 pg/g; median=4140) than in healthy controls (n=12; 0-51 pg/g; median=9) ($p<0.05$). However, the levels in children with complicated *S. dysenteriae* 1 infection (n=10; 2-88 pg/g; median=31) were similar to that in healthy controls ($p>0.05$).

Most children with uncomplicated *S. dysenteriae* 1 infection showed a decline in stool TNFα (Figs.3. A and B) and IL6 (Figs. 4. A and B) concentrations from the day of enrollment to 3-5 days later. Thus, when median concentrations were compared between these study periods, the declines were significant for TNFα (p=0.002 and 0.042 for children with weight for age ≤65% and >65%, respectively) and IL6 (p=0.033 and 0.006 for children with weight for age ≤65% and >65%, respectively). In children with complicated infection, stool cytokine concentrations remained similar or showed a rise from the day of enrollment to 3-5 days later (Figs. 3 and 4, C and D). However, in better nourished children with complicated infection, no significant differences in median stool TNFα (p=0.124) (Fig. 3D) and IL6 (p=0.058) (Fig. 4D) concentrations were observed between the study periods. In poorly nourished children, there were not enough paired stool samples for statistical comparison (Fig. 3C and 4C).

Comparisons (not statistically compared as numbers were too small) between paired stool samples taken 3-5 days after enrollment and at convalescence (14 days or more after discharge) showed no differences in TNFα and IL6 concentrations in children with uncomplicated *S. dysenteriae* 1 infection in either of the nutritional subgroups (data not shown). There were not enough convalescent stool samples from children with complicated *S. dysenteriae* 1 infection for comparison.

In children with *S. flexneri* infection, stool TNFα (n=6, 0-252 pg/g) and IL6 (n=5, 5-410 pg/g) concentrations were similar

to those in healthy controls. No changes were detected in stool TNFα (n=3. 0-671 pg/g) and IL6 (n=2. 9-73 pg/g) concentrations 3-5 days later. In children with mixed *Salmonella* and *Shigella* infections, stool TNFα (n=4: 0-1498 pg/g) and IL6 (n=4, 17-993 pg/g) concentrations were similar to those in healthy controls.

DISCUSSION

In this study we found higher concentrations of both TNFα and IL6 in stools of children with uncomplicated *S. dysenteriae* 1 infection than in healthy controls and there was considerable individual variation, corroborating previous findings (5). Higher concentrations of these cytokines have also been measured in the sera of children with uncomplicated *S. dysenteriae* 1 infection (5). It is known that some of these cytokines are increased locally in inflamed tissues in active inflammatory bowel disease (15). The possible sources of proinflammatory cytokines in dysenteric stool are, therefore, several including the blood present in stool, local production by neutrophils and macrophages in stool, and leakage from inflamed colonic mucosa.

The main finding in this study was the lower stool concentrations of TNFα and IL6 in most children with complicated compared with uncomplicated *S. dysenteriae* 1 infection. Lower production of proinflammatory cytokines by whole blood and mononuclear cells have been reported in seriously ill patients, such as those with complicated typhoid fever (8), sepsis (7) and Familial Mediterranean fever (16). It is suggested that lower

concentrations of cytokines are due to exhausted production capacity possibly as a result of continued endotoxin stimulation (7) or that cells switch from producing proinflammatory to antiinflammatory cytokines (8). However, in complicated *S. dysenteriae* 1 infection, high serum concentrations of TNFα and IL6 have been reported earlier (5). The reason(s) for the difference between serum and stool concentrations of TNFα and IL6 are not clear. A similar situation occurs in children with active inflammatory bowel disease where separate studies have shown low concentrations of IL6 in stools (11) and high concentrations in plasma (15). The suggested explanation for these findings was that there are inhibitors for IL6 in stool (11). It is possible that stools of children with complicated *S. dysenteriae* 1 infection contain higher levels of inhibitors for IL6 and TNFα. It may also be hypothesized that there is a switch from a proinflammatory to an antiinflammatory cytokine profile locally due to continued endotoxin stimulation which may not be seen systemically.

Stool TNFα and IL6 declined from the day of enrollment to 3-5 days later in most children (19/23 for TNFα; 18/25 for IL6) with uncomplicated *S. dysenteriae* 1 infection (Figs. 3A and 4A). The reason why stool cytokine concentrations increased in some children is not clear.

In the present study, we did not find high concentrations of these cytokines in the stools of children with *S. flexneri* infection. These findings are contrary to earlier reports

(5.11). Reasons for discrepancies may include that the number of children in this group was low. We also found that in children with mixed *Salmonella* and *Shigella* infections, stool TNFα and IL6 concentrations were similar to those in healthy controls. Concomitant *Salmonella* infection, therefore, does not appear to increase stool cytokine concentrations. However, concomitant infections with *S. dysenteriae* 1 (3/4 children) caused a more serious illness with complications which may explain why stool cytokine concentrations were not higher in these children compared with healthy controls.

Finally, we have demonstrated a negative correlation between stool TNFα and IL6 concentrations and the nutritional status of children. However, this effect was only seen in children with complicated *S. dysenteriae* 1 infection. Higher cytokine concentrations in these children may either be a cause or effect of malnutrition. TNFα can cause cachexia as well as stimulate IL6 production (2). It may be that some severely ill children produce more TNFα which then leads to more IL6 production which exacerbates acute malnutrition. Alternatively, plasma TNFα concentrations are raised in conditions of stress and malnutrition (6); therefore, more cytokines may be released in severely ill and undernourished children.

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Table 1. Description of study groups and assays performed

Study group	TNFα		IL6	
	On the day of enrollment	3-5 days later	On the day of enrollment	3-5 days later
Healthy controls	29		29	
Uncomplicated *S. dysenteriae* 1	40	26	33	27
Complicated *S. dysenteriae* 1	18	12	17	12
leukemoid reaction	11	9	10	9
leukemoid reaction + HUS	2	1	3	1
toxic megacolon + others	3	1	2	1
septicemia	2	1	2	1
Uncomplicated *S. flexneri*	6	3	6	2
Shigella + Salmonella	4	3	4	4

Table 2. Characteristics of the study groups of children with and without *Shigella* infection

Characteristics	Control	Uncomplicated *S. dysenteriae* 1	Complicated *S. dysenteriae* 1	Uncomplicated *S. flexneri*	*Shigella* and *Salmonella*	*P* value
	n=30	n=41	n=19	n=6	n=4	
Duration of diarrhea prior to enrollment (days) Mean (SD)	0 (0)	8.6 (6.3)	6.9 (2.4)	6.5 (3.0)	6.8 (2.2)	NS*
Weight/age (% of the NCHS median) Mean (SD)	64.3 (6.8)	65.4 (13.4)	70.9 (12.0)	57.4 (11.1)	71.0 (11.1)	NS
Gender (no. of children) M:F	16:14	21:20	8:11	6:0	3:1	NS

*NS=Not significant

LEGEND TO FIGURES

Fig. 1. Stool TNFα concentrations on enrollment in control children (a) and in children with uncomplicated (b) and complicated (c) *S. dysenteriae* 1 infection. Horizontal bars represent median values. A) Poorly nourished children with weight for age ≤65%, and B) Better nourished children with weight for age >65%. (Numbers of dots do not match numbers of children as some values are similar or too close so that they appear as single dots.)

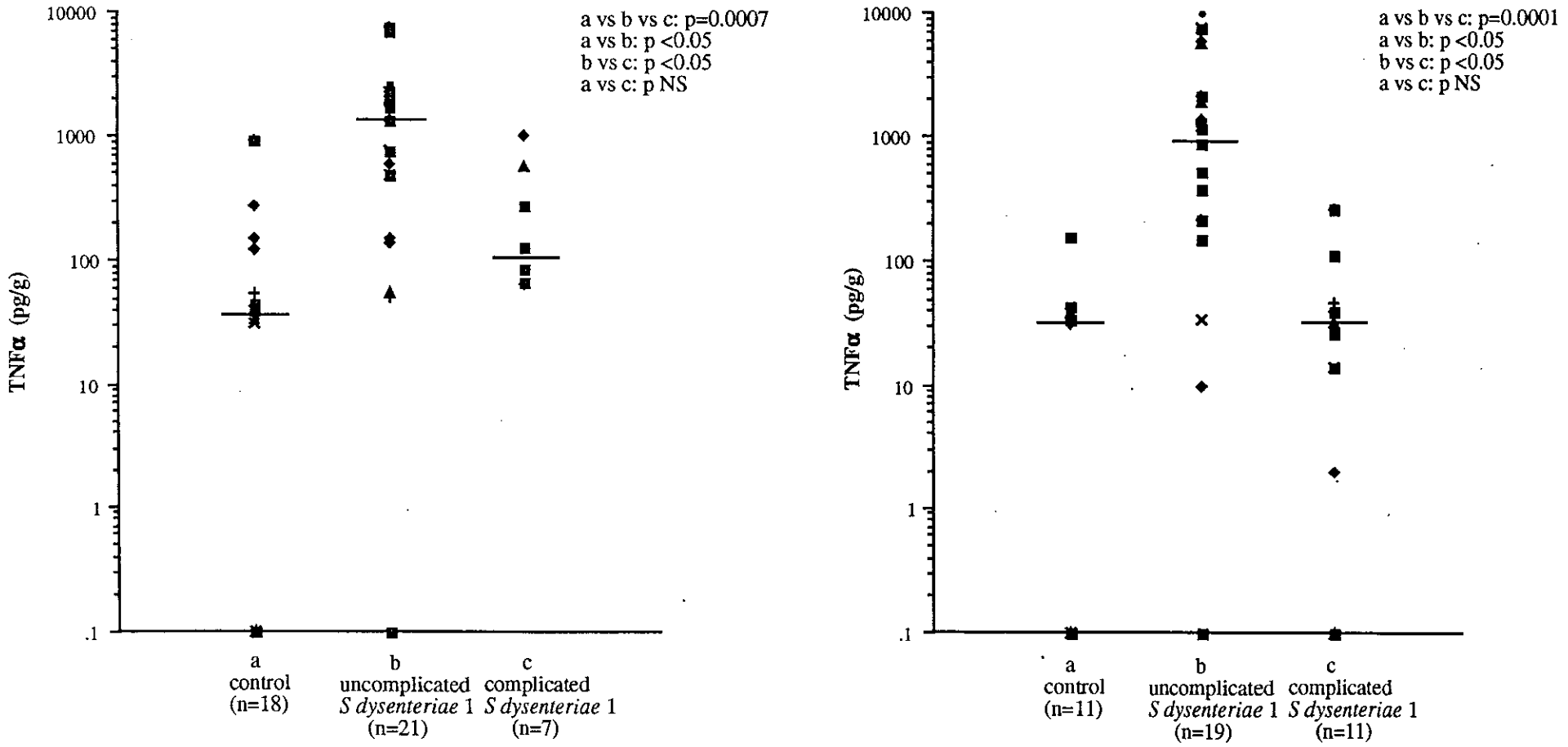
Fig. 2. Stool IL6 concentrations on enrollment in control children (a) and in children with uncomplicated (b) and complicated (c) *S. dysenteriae* 1 infection. Horizontal bars represent median values. A) Poorly nourished children with weight for age ≤65%, and B) Better nourished children with weight for age >65%. (Numbers of dots do not match numbers of children as some values are similar or too close so that they appear as single dots.)

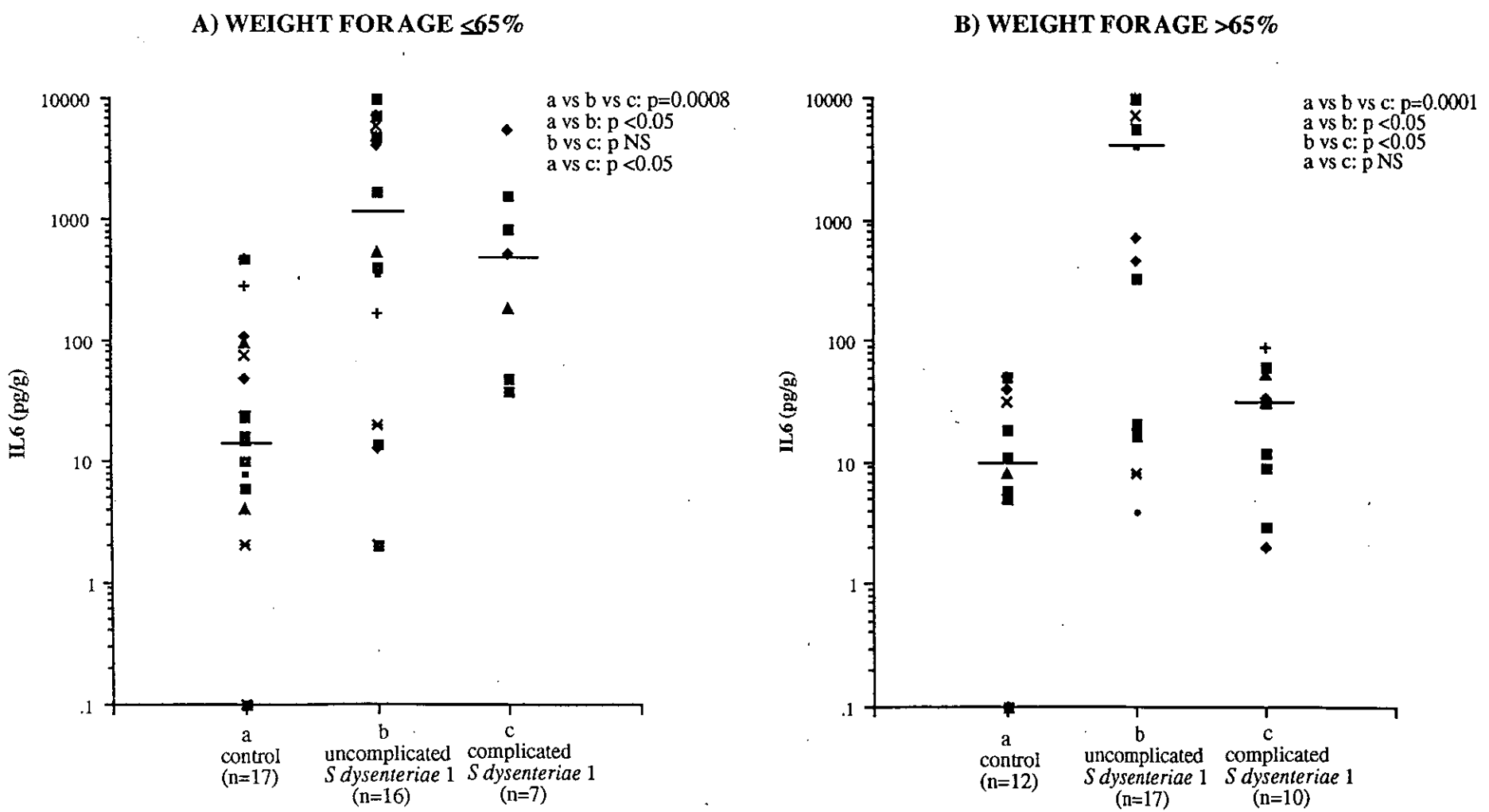
Fig. 3. Stool TNFα concentrations in children with uncomplicated and complicated *S. dysenteriae* 1 infection in whom samples were available on the day of enrollment (d0) and 3-5 days later (d3-5). Each symbol represents a single patient. A) Poorly nourished

children with uncomplicated *S. dysenteriae* 1 infection. B) Better nourished children with uncomplicated *S. dysenteriae* 1 infection. C) Poorly nourished children with complicated *S. dysenteriae* 1 infection. and D) Better nourished children with complicated *S. dysenteriae* 1 infection.

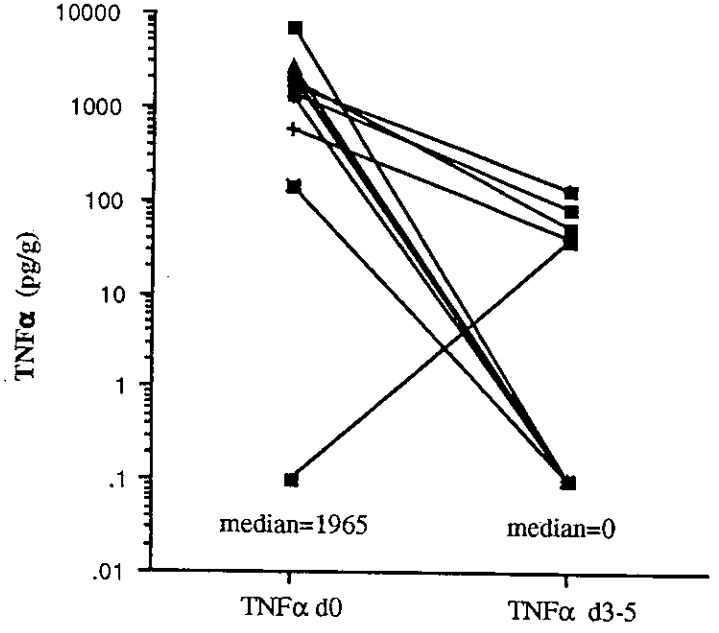
Fig. 4. Stool IL6 concentrations in children with uncomplicated and complicated *S. dysenteriae* 1 infection in whom samples were available on the day of enrollment (d0) and 3-5 days later (d3-5). Each symbol represents a single patient. A) Poorly nourished children with uncomplicated *S. dysenteriae* 1 infection. B) Better nourished children with uncomplicated *S. dysenteriae* 1 infection. C) Poorly nourished children with complicated *S. dysenteriae* 1 infection. and D) Better nourished children with complicated *S. dysenteriae* 1 infection.

TA:mh/T7:CYTOKINE.PAP

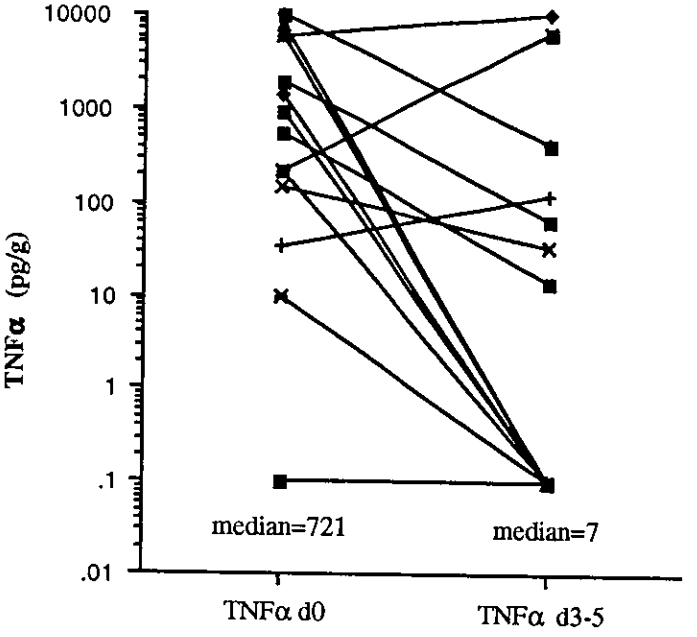

A) WEIGHT FOR AGE ≤65%
B) WEIGHT FOR AGE >65%
TNFα (pg/g)
10000
1000
100
10
1
.1
a vs b vs c: p=0.0007
a vs b: p <0.05
b vs c: p <0.05
a vs c: p NS
a vs b vs c: p=0.0001
a vs b: p <0.05
b vs c: p <0.05
a vs c: p NS
a control (n=18)
b uncomplicated S dysenteriae 1 (n=21)
c complicated S dysenteriae 1 (n=7)
a control (n=11)
b uncomplicated S dysenteriae 1 (n=19)
c complicated S dysenteriae 1 (n=11)


A) WEIGHT FOR AGE ≤65%
IL6 (pg/g)
10000
1000
100
10
1
.1
a vs b vs c: p=0.0008
a vs b: p <0.05
b vs c: p NS
a vs c: p <0.05
a
control
(n=17)
b
uncomplicated
S dysenteriae 1
(n=16)
c
complicated
S dysenteriae 1
(n=7)
B) WEIGHT FOR AGE >65%
IL6 (pg/g)
10000
1000
100
10
1
.1
a vs b vs c: p=0.0001
a vs b: p <0.05
b vs c: p <0.05
a vs c: p NS
a
control
(n=12)
b
uncomplicated
S dysenteriae 1
(n=17)
c
complicated
S dysenteriae 1
(n=10)

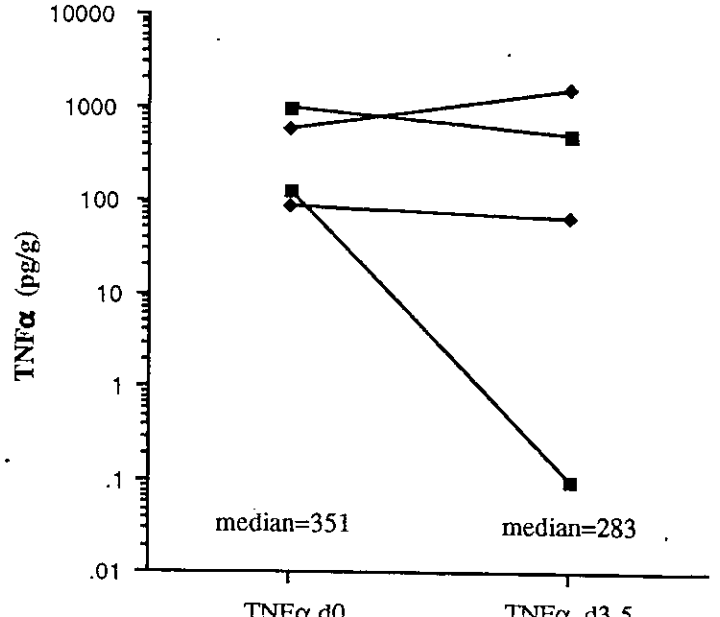
A) UNCOMPLICATED *S dysenteriae* 1
WEIGHT FOR AGE ≤65%



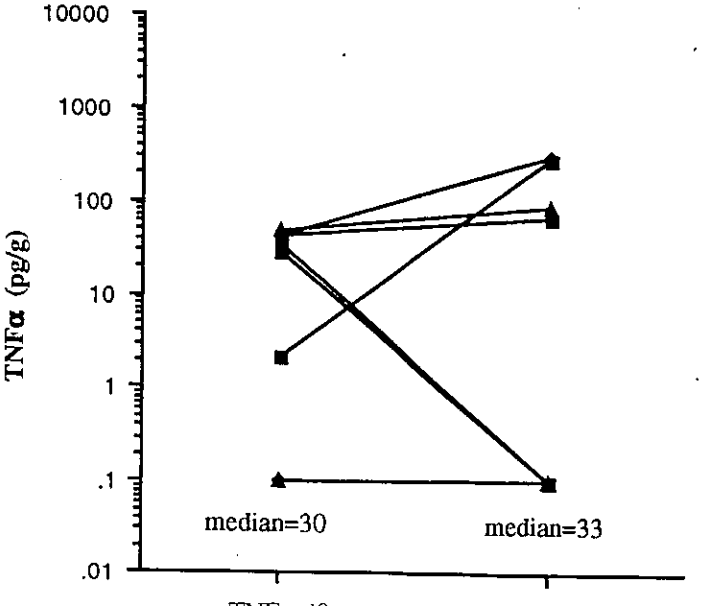
B) UNCOMPLICATED *S dysenteriae* 1
WEIGHT FOR AGE >65%



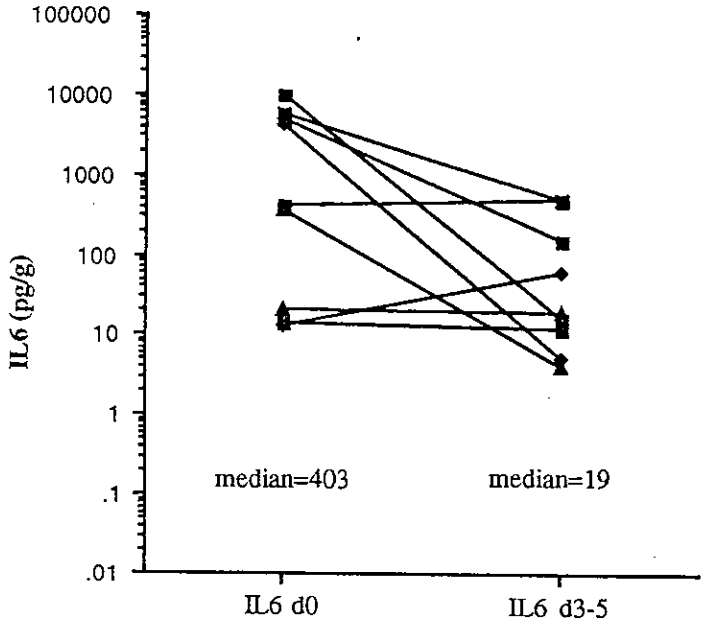
C) COMPLICATED *S dysenteriae* 1
WEIGHT FOR AGE ≤65%



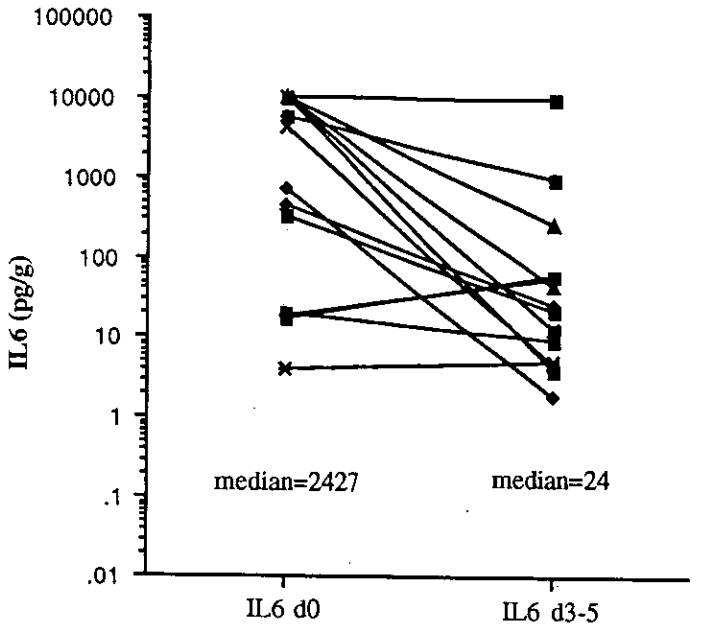
D) COMPLICATED *S dysenteriae* 1
WEIGHT FOR AGE >65%



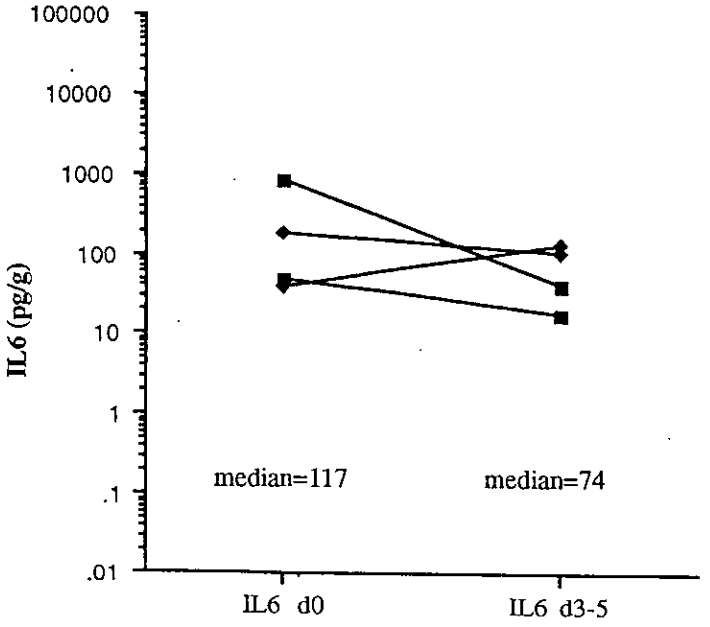
A) UNCOMPLICATED *S dysenteriae* 1
WEIGHT FOR AGE ≤65%



B) UNCOMPLICATED *S. dysenteriae* 1
WEIGHT FOR AGE >65%



C) COMPLICATED *S. dysenteriae* 1
WEIGHT FOR AGE ≤65%



D) COMPLICATED *S. dysenteriae* 1
WEIGHT FOR AGE >65%