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SECTION I : RESEARCH PROTOCOL

NO.88-001

1. Title: THE ROLE OF CYTOKINES IN THE PATHOGENESIS OF SHIGELLOSIS

A Collaborative Study between ICDDR,B and New England Medical Centre, Tufts University School of Medicine and Hospital Erasme, the Free University of Brussels

2. Principal Investigators:

Michael Bennish, M.D.
International Research Associate, ICDDR,B
Co-head, Tufts - ICDDR,B Shigella Project

Assistant Professor of Pediatrics and Medicine
New England Medical Center
Tufts University School of Medicine

Dr. M.A. Salam
Chief Physician
Dhaka Treatment Centre

Co-investigators:

Dhaka: Dr. F. Qadri
Scientist
Laboratory Sciences Division

Boston: Gerald Keusch, M.D.
Professor of Medicine
New England Medical Center
Tufts University School of Medicine
Chief, Division of Geographic Medicine and Infectious Disease

Charles Dinarello, M.D.
Professor of Medicine
Division of Geographic Medicine and Infectious Disease
New England Medical Center

Brussels: Marc Struelens, M.D.
Department of Bacteriology and Laboratory Medicine
Hospital Erasme
The Free University of Brussels

3. Starting Date: As soon as approval is granted

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6. Scientific Programme Head: Professor Roger Eeckels, Acting Associate Director, Clinical Sciences Division

Signature: [signature]

Date: 22.12.87

7. Abstract Summary:

Cytokines are polypeptide products of lymphocytes or monocytes which have diverse biological effects in man that could underlie many clinical events and complications of *Shigella* infection. Among these effects are the production of fever, induction of acute phase proteins, the stimulation of granulocyte proliferation in the bone marrow, protein catabolism and amino acid release in muscle tissue, the inhibition of albumin synthesis in the liver, the stimulation of insulin release by the pancreas, a decrease in the renal reabsorption of sodium, and the regulation of cellular interactions in the immune system.

Dysenteric illness is probably currently responsible for the majority of diarrhea related deaths in Bangladesh (annex 1), and is primarily due to *Shigella* infection. Even with current standard therapy, such as provided in our diarrhea Treatment Centre in Dhaka, mortality rates remain high. During the period 1983 through 1986 289(9%) of the 3,235 patients admitted to our Treatment Centre with *Shigella* infection died, and another 311 (10%) were discharged on "risk bond" (against medical advice), many of them already moribund (annex 2).

The reasons for this high fatality rate remain poorly understood. Although the site of *Shigella* infection and bacterial multiplication is in the gut epithelium, systemic disease manifestations are generally prominent. These systemic manifestations include anorexia and fever in nearly all patients, and in some patients the leukemoid reaction, hypoglycemia, hypoalbuminemia, hyponatremia, hemolysis, uremia and cachexia. Because cytokines can cause effects similar to the systemic complications of shigellosis in vitro, or in in-vivo in experimental animals, there is reason to think that cytokines play a role in many of the systemic manifestations of this illness. We will therefore examine the relationship between

cytokines in plasma and disease manifestations in shigellosis, and correlate these with levels of circulating levels of endotoxin, acute phase proteins and Shiga toxin in 250 patients admitted to the ICDDR,B hospital with documented *Shigella* infection. We will sample patients on admission, and, in those patients who remain hospitalized, on days 3 and 7. Patients will be requested to return for follow-up 30 days after discharge in order to determine post-discharge outcome and to obtain a convalescent serum sample. In a selected group of patients we will measure cytokine response to a DPT immunization at the time of discharge and at the 30 day follow-up. During hospitalization clinical and anthropometric data will be recorded daily in order to evaluate the magnitude of colitis, fever and anorexia, as well as the occurrence of specific complications such as hypoalbuminemia, hyponatremia, leukemoid reaction, and hypoglycemia. The assay of cytokines will take advantage of a newly developed radio-immunoassay from our laboratory, which is both more sensitive and requires a much smaller sample volume than the previous bio-assay technique. In addition to measuring circulating levels of cytokines, *in vivo* production in response to a defined stimulus, concanavalin A, will be measured with a new, simple, whole blood cell assay developed in our laboratory. Assay results will be correlated with the severity of the colitis, nutritional changes, specific systemic complications and death in an attempt to determine if the cytokines play a role in the pathogenesis of shigellosis. If they do, then it may become possible in the future to improve the outcome of severe shigellosis by controlling cytokine production, or their effects.

8. Reviews:

i. Ethical Review Committee _____ _____

ii. Research Review Committee _____ _____

iii. Director's signature & remarks, if any -

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SECTION II - RESEARCH PLAN

1. INTRODUCTION

1. Objectives

This project is an intensive three year study of the relationship of cytokine production and action to severe and life-threatening events in shigellosis. We propose to study in orderly fashion the many events that cytokines can mediate in this disease, because these molecules have so many relevant activities. If such mechanisms can be established patients at risk of complications may be identified and better treatment or prevention measures used to modulate the cytokine response.

More specifically, this study will attempt the following:

a. to measure the production of the cytokines interleukin 1 alpha (ILa), interleukin 1 beta (ILb), cachectin - tumor necrosis factor (TNF), granulocyte and granulocyte-macrophage colony stimulating factor (G-CSF and GM-CSF), as well as representative acute phase proteins, endotoxin and Shiga toxin in patients with shigellosis.

b. to determine whether a correlation exists between these biologically active host and microbial product mediators of the non-specific host-response and the severity of colitis, fever, leukocytosis and weight loss, or the development of specific complications, such as leukemoid reaction, hyponatremia, or hypoglycemia.

c. to determine if patients recovering from a *Shigella* infection have a normal cytokine response to a defined stimulus, a DPT immunization.

2. Background

In Bangladesh dysenteric illness is probably responsible for the majority of diarrhea related deaths when dehydration can be effectively treated (annex 1), and the majority of dysenteric illness is due to *Shigella* infection. Even in our diarrhea Treatment Centre in Dhaka mortality rates remain high, with 289 (9%) of the 3,235 patients admitted with *Shigella* infection during the period 1983 to 1986 dying, and another 311 (10%) being discharged on risk bond, many of them moribund (annex 2).

The pathogenesis of bacillary dysentery involves invasion of the colonic epithelium by *Shigellae*, intracellular multiplication of the pathogen and epithelial cell death. These events trigger a local and general inflammatory reaction. *Shigella* colitis is characterized by epithelial sloughing and ulceration associated with mucosal infiltration by polymorphonuclear and mononuclear phagocytes. Luminal exudation and bleeding produce the "dysenteric stools." The general inflammatory response is manifested by fever and leukocytosis with neutrophils predominating. Whether the latter two manifestations are beneficial to the host in this (or in other) infections remains an open question. Nevertheless, clinical studies have shown that the case-fatality rate in severe shigellosis is inversely related to body temperature and is increased in patients with leukocytosis above 50,000 WBC/ml (Struelens 1985, Bennish, Butler). Body temperature was predictive of outcome in multivariate analysis which included other risk factors like age and nutritional status (Struelens 1985). Infancy and malnutrition are associated with increased risk of death from *Shigella* infection (Struelens 1985, Bennish, Butler, Keusch 1982). The

interrelations between these variables (age, nutritional status, fever, leukocytosis) have not been systematically explored, although a weak correlation between febrile response and anthropometric indicators of nutritional status was noted in one study (Struelens 1985). Protein-energy malnutrition (PEM) is also associated with prolonged illness due to *Shigella* (Keusch 1982). In addition, shigellosis exerts a strong negative effect on nutritional balance, both through intestinal protein exudation and the catabolic cost of the inflammatory response (Keusch 1982, Black). Thus there is a complex inter-relationship between PEM and *Shigella* infection. Specifically, chronic *Shigella* infection compromises a child's nutritional and immunologic status. Conversely, chronic PEM from any cause may predispose a patient to infection.

Host defense mechanisms responsible for recovery from shigellosis have not been elucidated. It is presumed, but not proven, that secretory immunoglobulin A may terminate further invasion of epithelial cells from the gut lumen. Complement-mediated serum bactericidal activity may help localize mucosal infection and a majority of *Shigella* isolates are rapidly killed by normal serum but less effectively so by serum from malnourished patients with bacteremia (Struelens 1984). Infants with protein-energy malnutrition have been shown to be deficient in activity of the alternative pathway of complement, and for opsonic activity for gram negative bacteria (Keusch 1984). Polymorphonuclear cells can phagocytize and kill *Shigellae* opsonized with immunoglobulins (Reed). The importance of this system has not been investigated in patients with shigellosis. However the markedly increased susceptibility of malnourished patients to *Shigella* infection is unlikely to be due to impairment of this system which is only marginally depressed in malnutrition (Keusch 1980). In contrast, there

is evidence from in-vitro experiments that cell-mediated immunity may contribute to host defense against *Shigellae.* Human peripheral blood killer-lymphocytes and monocytes were found to exert antibody dependent cytotoxicity on *Shigellae* at effector: target ratios over 100 : 1 (Lowell, Morgan). Colostral lymphocytes also had natural killer (NK) activity (Morgan). HeLa cells are selectively killed by NK cells when invaded by *Shigella* (Niesel). Interferon pretreatment of epithelial cell lines makes the cell refractory to invasion by *Shigellae* (Klimpel). Protein-energy malnutrition induces a marked depression of cell-mediated immune responses that could in part explain the enhanced susceptibility of the malnourished to severe dysentery (Keusch 1987).

IL-1, TNF and GM-CSF are a group of polypeptide products of monocytes, fibroblasts, endothelial cells and T-cells which have a variety of biologic effects, and have been the subject of intense scrutiny in recent years (Dinarello 1984, 1987, Beutler 1987, Metcalf, Clark). IL-alpha and IL-beta are structurally related proteins that share most, but not all, biologic activities. Although produced by numerous cell types, monocytes are their main source of production, and production is stimulated by antigens, toxins, injury and other inflammatory processes (Dinarello 1986). The principal biological activities of IL-1 alpha and beta include : fever induction secondary to PGE2 production acting on the hypothalamic thermo-regulatory center; B and T lymphocyte activation; hypoferremia due to lactoferrin release from PMN's; granulocyte synthesis, release and activation, probably by stimulating GM-CSF transcription and translation; upregulation of hepatic synthesis and release of acute phase proteins and downregulation of hepatic albumin synthesis; muscle proteolysis and

fibroblast proliferation; and increased pancreatic insulin release (Baracos, Comens, Dinarello 1984a, 1987). The metabolic changes induced by IL-1 appear to be mediated via endocrine changes. In animals, IL-1 stimulates the release of insulin, glucagon and glucocorticoid hormones, thus permitting hepatic gluconeogenesis and peripheral glucose utilization to meet the increased metabolic demand of the acute inflammatory reaction (Besedovsky). Evidence from animal experiments also incriminates IL-1 as a mediator of anorexia (MacCarthy). In the rat model IL-1 markedly increases renal excretion of sodium (Caverzasio).

The physiological role of cachectin is in many ways similar to that of IL-1 (Beutler 1986, 1987). It is believed to be a proximal mediator in endotoxic shock via endothelial alterations resulting in increased permeability and procoagulant activity (Beutler 1987). It suppresses adipocyte anabolism, and can induce production of GM-CSF as well as acting as an endogenous pyrogen (Broudy).

Not only is it likely that cytokines are produced during infection with *Shigella* but their altered or abnormal regulation may also play a role in the pathogenesis of the disease, and especially of its associated complications. Translocation of bacterial antigens from the gut flora to the circulation occurs through the altered mucosa in *Shigella* colitis. This is manifested by circulating immune complexes and endotoxemia during acute *Shigella* infection, as has been shown in a previous study conducted at the ICDDR,B. (Koster). These substances are potent inducers of IL-1 production, and endotoxin stimulates TNF release by macrophages which is in turn an inducer of IL-1 (Dinarello 1984, Beutler 1987, Movat).

Controlled interleukin-1 secretion during infection is likely to

be beneficial to the host. The peptide is highly conserved in evolution and thus may be advantageous for survival. Several of its biological effects may contribute to recovery from a bacterial infection such as shigellosis: (i) the bacteriostatic effect of interleukin-1 induced hypoferremia, (ii) fever enhancement of phagocytic functions, production of interferon and activation of B-cells and NK-cells; (iii) recruitment and activation of PMNs (Dinarello 1984, Movat).

On the other hand, excessive and/or sustained secretion of IL-1 may become detrimental in view of its catabolic effects, anorexia and glucocorticoid-mediated immunosuppression (Besedovsky). Similarly, cachectin secretion may contribute to adverse effects of infection such as catabolism and shock. Experimentally, all of the manifestations of septic shock can be reproduced by IL-1 or TNF administration, and passive immunization with antibody to TNF protects against lethal endotoxin challenge (Tracey 1986, 1987, Beutler 1985). Therefore, even if the infection were eliminated, deleterious or even life threatening processes may continue if cytokine production were improperly regulated (Beutler 1987).

Recent studies have begun to elucidate more specifically the role that cytokines may play in disease processes. TNF was originally defined in the course of studies to elucidate the mechanism of cachexia in T. brucei infection in cattle. TNF production has been found to be essential for the development of malaria in a murine model (Grau). A review of nematode infections in cattle responsible for diarrhea and weight loss in these animals, suggested that TNF production, rather than fecal protein loss, was more likely to be responsible for the

weight loss that is seen during these infections (Hammerberg). The role of cytokines in human diseases has also been studied. In one study TNF was measured by a bioassay in the serum of 73 patients with meningococcal disease. 10 of 11 patients who died had detectable levels of TNF, compared to only 8 of 68 patients who survived (Waage). In another study, 67% of patients with kala-azar and 70% of patients with malaria were found to have elevated levels, compared to only 8% of patients with neoplastic disease (Scuderi). Lymphocyte activating factor (IL-1) was produced spontaneously by lymphocytes of 6 patients with Crohn's disease, but not by 10 healthy control patients (Satsangi). Sera from patients with Kawasaki's disease lysed vascular endothelial cells only after those cells were exposed to IL-1 and TNF (Leung). Such patients may may have abnormally high circulating levels of TNF and IL-1 following treatment with aspirin, which is known to augment the release of TNF from macrophages (Larrick).

Production of cytokines and biological responses may not always be coordinate. Protein-deprived rabbits display a dissociated reaction to *P.multocida* infection: they have a normal neutrophil response but decreased febrile and hypoferremic responses, and diminished production of endogenous pyrogen by monocytes (Hofmann-Goetz). Adult patients with protein deficiency exhibit a similar defect in mediator production during infection (Keenan). In the rat model the effect of protein malnutrition is independent of age, however (Bradley).

Immunizations are known to elicit an acute phase response that is similar to that seen during infection. In their classic studies on the pathogenesis of fever Atkins and Wood showed that immunization with typhoid vaccine elicited the production of endogenous pyrogen (a previously used term for IL-1), that endogenous pyrogen was produced in

both subjects that had been previously immunized as well as those receiving their primary immunization, and that the endogenous pyrogen response was maximal at two hours (Atkins). A subsequent study of volunteers immunized with combined parenteral typhoid-cholera vaccine showed that when compared to control patients, immunized patients had a significant increase in both protein degradation and synthesis, results that are consistent with stimulation of IL-1 production in the immunized group (Garlick). And in a provocative field study conducted in the Punjab, investigators found that children under six months of age who were immunized with live virus vaccines (BCG, smallpox, polios and DPT + polio) had a significant reduction in their weight in the 30 days following immunization when compared to unimmunized control patients, a finding that is also consistent with the induction of cytokines by immunization (Kielmann).

Cytokines are known to stimulate the production of a number of other biologically active peptides and proteins. Among these are the colony-stimulating factors (CSF's). The colony-stimulating factors (CSF's) are a group of distinct proteins that induce differentiation and proliferation of white cell (and perhaps erythrocyte) precursors (Clark, Metcalf). Four human CSF's have now been identified and cloned. These four are granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3. GM-CSF and IL-3 tend to act at earlier stages of white cell differentiation than do G-CSF and M-CSF. However in higher concentrations the effects of the latter two proteins tend to overlap with effects of the former two. This may have to do with the relative affinity of receptors for

different CSF's on the membranes of the progenitors of the various cell lines. Febrile, neutrophilic responses during hemodialysis have been shown to be associated with increased plasma levels of TNF (Lonneman) which may have been stimulated by sodium acetate that is used as the exchange-fluid buffer (Bingel).

CSF's are produced by a variety of cells, including fibroblasts, endothelial cells, and monocytes. Even though their production is stimulated by IL-1 and TNF (Broudy, Mochizuki), CSF's are present in only trace amounts, making isolation and purification difficult and limiting the opportunity for clinical trials. These trace amounts however are very potent biologically. The recent cloning and expression of the complementary DNA for all four human CSF's, resulting in recombinant peptides, has made clinical and therapeutic trials possible, and a number of such trials have been initiated recently. Continuous infusion of GM-CSF into normal monkeys elicited a dose dependent increase in circulating white cells, with counts rising from the normal level of 8,000 - 10,000 to over 50,000 within 24 to 48 hours of the initiation of the infusion (Donahue). This rapid rise in peripheral leukocyte counts resembles the situation in *S. dysenteriae* type 1 infection, where peripheral white blood cell counts can also rise in such a rapid fashion.

Acute phase reactants (APR) are generally considered to be a group of proteins that are normally not present in plasma, or are present in trace amounts, and are detectable in increased levels in the plasma during inflammatory processes (Kushner). The first of these proteins to be described was C-reactive protein, so named because it reacts with the C polysaccharide of pneumococci (McCarty). It, like alpha 2 macrofetoprotein, is a true acute phase reactant, in that it is not

normally detectable in plasma. Other proteins, such as serum amyloid A, fibrinogen, haptoglobin, ceruloplasmin, C3 (the third component of complement), alpha-1 antitrypsin, alpha-1 antichymotrypsin and alpha-1 acid glycoprotein are all normally present (albeit often in very low concentrations) but increase in concentration several fold to several hundredfold in response to an inflammatory stimulus (Kushner). Some normal liver derived plasma proteins, such as albumin and transferrin, actually decrease in concentration in response to an inflammatory stimulus. The physiologic role of APR's in host defense, especially CRP, remain to be more completely defined, although a number of studies suggest that they play a role in modulating the immune response (Li, Mold, Mortensen).

There is considerable evidence that cytokines play a role in the induction of the synthesis of some of these proteins by the liver. This evidence is strongest for serum amyloid A protein (Sztein, Sipe, McAdam 1978, 1980, 1982), but studies have also suggested that production of CRP and other APPs may also be either directly or indirectly controlled by cytokines (Bornstein, Mackiewicz, Merriman, McAdam 1978, Perlmutter).

3. Rationale

Although we have considerable understanding of the pathogenesis of the colonic lesions in shigellosis, there remains little knowledge of the pathogenesis of systemic complications, or factors leading to death from this infection. In *in-vitro* and animal models cytokines and CSF's are known to induce many of the systemic abnormalities that we witness in shigellosis; leukemoid reaction, hyponatremia, hypoglycemia, a shock

like state, anorexia and cachexia It is therefore reasonable to ask whether the abnormal production or regulation of such biologically active proteins play a role in some of the above mentioned complications. It is also possible that the cytokines might play a role in determining outcome in patients with shigellosis - that is whether resolution of infection, and more importantly resolution of illness, is likely to be dependent on more than just specific immune responses. Better understanding of these relationships can lead to not only to a better understanding of the the pathogenesis of shigellosis, but to new and improved therapeutic approaches. Such new approaches are clearly necessary, because even with appropriate antibiotic therapy, 10% of patients admitted to the inpatient unit of the Dhaka Treatment Center still die from their *Shigella* infection, suggesting that treatment of the disease requires more than simply eradication of infection.

B. SPECIFIC AIMS and HYPOTHESIS

The specific hypothesis underlying this protocol is that dysregulation of cytokines is responsible for a number of the complications that occur in shigellosis, and that this dysregulation may in part be due to the effects of Shiga toxin. Based upon what we know of the clinical features of shigellosis, and the action of cytokines, in this clinical study we hope to gain information on ten specific questions on the role of cytokines in the pathogenesis of shigellosis. It is important to recognize that other studies are underway to learn how to regulate and modulate the cytokine response,

and that such measures could be applied to high risk *Shigella* patients in the future.

Question 1: Is the leukemoid reaction in shigellosis due to abnormally high and sustained circulating levels of GM-CSF or G-CSF?

GM-CSF is known to be a potent inducer of macrophage and neutrophil precursors, and G-CSF of later stage neutrophil precursors. Most inflammatory processes lead to an increase in the number of circulating neutrophils - few produce the abnormally high WBC levels seen so frequently in *S. dysenteriae* type 1 infections. Is the abnormal WBC response seen in some patients with shigellosis due to to abnormally high levels of G-CSF or GM-CSF? By comparing G-CSF and GM-CSF levels in *Shigella* infected patients with and without the leukemoid reaction who have equivalent degrees of colitis, we will be able to answer this question.

Question 2: Are IL-1 and or TNF responsible for high GM-CSF levels in patients with *S. dysenteriae* type 1 infection and the leukemoid reaction, and are IL-1 and TNF production in turn related to circulating Shiga toxin?

IL-1 and TNF are known to stimulate GM-CSF production, so it is logical to determine if the levels of these two cytokines correlate with GM-CSF levels. These two cytokines are produced during many inflammatory processes however, but in most infections, even when treatment is delayed or not provided, downregulation presumably results in close enough control that the leukemoid reaction rarely occurs. What makes *S. dysenteriae* type 1 different from most other infections? Two possibilities stand out. One is that *S. dysenteriae* type 1

produces Shiga toxin in large amounts, and that Shiga toxin in turn stimulates IL-1 and TNF, or stimulates GM-CSF directly. We will measure Shiga toxin in the serum and stool of patients and will then compare levels in patients with and without the leukemoid reaction, and we will measure effects of toxin directly on IL-1, TNF and CSF production *in vitro*.

The other possible mechanism by which the leukemoid reaction might be induced is circulating endotoxin absorbed through an inflamed colonic epithelium. Endotoxin is a known potent stimulus of IL-1 and TNF production, and IL-1 and TNF could in turn stimulate GM-CSF production. In a previous study conducted at the ICDDR,B endotoxin was detected in the serum of patients with *S. dysenteriae* infection, and was associated with the development of the hemolytic-uremic syndrome. That study was done however with less specific endotoxin assays than are now available. To us endotoxin seems a less likely candidate than Shiga toxin as the initiator of the leukemoid reaction, as patients with *S. flexneri* infection can also develop severe colitis, and presumably would absorb endotoxin in the same manner, but rarely if ever develop leukemoid reaction. It is also possible that Shiga toxin acts synergistically with endotoxin, or in particular *S. dysenteriae* type 1 LPS in causing the effect seen. By measuring endotoxin and Shiga toxin, as well as cytokines and GM-CSF, we will hopefully be able to elucidate the mechanism underlying the leukemoid reaction in these patients.

Question 3: Is sustained interleukin 1 and TNF production responsible for the hypoalbuminemia that is seen during *Shigella* infection?

In a previous study we demonstrated that low levels of serum

albumin were strongly associated with a fatal outcome, and in a multivariate analysis this association was independent of nutritional status as assessed by weight for age criteria (Bennish). This finding suggests that factors affecting albumin synthesis and loss during the illness might be as important as prior nutritional status in determining illness outcome. Factors during the illness that might affect serum albumin levels are albumin loss through an inflamed colon, (Wahed), decreased nutrient intake due to anorexia, increased protein catabolism due to the metabolic demands of fever and infection, and decreased albumin synthesis. At least three of these processes, (anorexia, protein catabolism, and decreased albumin synthesis) are known to be mediated by both IL-1 and TNF in experimental animal models and probably, (but not definitively proven) in humans as well. We will measure caloric (with nasogastric and per oral feedings recorded separately) intake, prealbumin levels, and stool alpha-1 antitrypsin levels and correlate these with plasma cytokine and serum albumin levels. We will thus have at least indirect indicators of four possible ways that serum albumin levels might be effected by the disease process: 1. increased nitrogen catabolism and loss due to increased levels of IL-1 and TNF; 2. decreased availability of substrate because of decreased caloric and protein intake; 3. increased loss through the gut (indirectly measured by spot alpha-1 antitrypsin levels) 4. decreased synthesis as measured by pre-albumin, which is an albumin precursor that is known to be downregulated by IL-1. All of these methods are indirect indicators of the processes that we are actually interested in. For instance oral intake is only an indirect measure of protein and calorie availability, as we will not be measuring how much of the intake is absorbed; stool anti-1 alpha trypsin is a proxy

measure of the amount of serum protein being lost in the gut; and prealbumin is again only an indirect measure of altered priorities in hepatic protein synthesis that we are not able to measure directly. Nonetheless these studies will provide insights into which of these processes might be involved, they are easily measured with available methods, and determining them will help to define in more detail research goals for future studies.

Question 4: Is the inability of some patients to mount a fever during *Shigella* infection a reflection of an inability to respond to IL-1 and TNF, or rather an inability to produce them?

In previous studies we demonstrated that shigellosis patients without fever were at much greater risk of death than shigellosis patients with fever (Struelens 1985, Bennish). As with hypoalbuminemia, this association was independent of nutritional status as assessed by anthropometric indicators. IL-1 and TNF are the major "endogenous pyrogens"; i.e. substances that cause the setpoint of the hypothalamic regulatory center to be raised. Since there is no evidence that the inflammatory stimulus in patients without fever is any less than in patients with fever, we have to assume that the inability of some patients to mount a febrile response is either due to their inability to produce IL-1 and TNF, or to an inability to respond to TNF and IL-1. We will test the first possibility by measuring unstimulated and stimulated IL-1 and TNF plasma levels. The second possible explanation for the lack of a febrile response, the inability to respond normally to IL-1 and TNF, is somewhat more difficult to test. IL-1 and TNF act to increase prostaglandin E levels in the hypothalamus - and it is the prostaglandin E which in turn is responsible for increasing the

thermoregulatory set point. The best measure of hypothalamic response to IL-1 and TNF is to measure prostaglandin E levels in the CSF - something that we are not prepared to do. Nonetheless it is possible to assess the ability of individuals to respond to other effects of IL-1 and TNF; specifically by measuring some representative acute phase reactants known to be synthesized in response to inflammation, for example serum amyloid A which is known to be synthesized in direct response to IL-1 (MacAdam).

Question 5: Is IL-1 responsible for hypoglycemia in patients with *S. flexneri infection*?

In a recently completed study we found that patients with shigellosis are at increased risk of hypoglycemia when compared with the general hospital population. 35 of 100 patients with hypoglycemia had *Shigella* infection, compared to 10% of all Treatment Centre inpatient admissions (Bennish, unpublished data). IL-1 is known to be a potent inducer of insulin secretion in various animal models (Comens). By measuring IL-1 levels in shigellosis patients with and without hypoglycemia, we will be able to determine if IL-1 correlates with and can explain the hypoglycemia that is seen in patients with shigellosis.

Question 6: Is IL-1 induced natriuresis responsible for the hyponatremia seen in patients with *S. dysenteriae* type 1 infection?

Patients with *S. dysenteriae* type 1 infection can have hyponatremia that is disproportionate to the degree of dehydration that they have (Rahman). In animal models IL-1 is known to decrease renal sodium reabsorption (Caverzasio). This may explain the hyponatremia

that is seen in these patients, and is consistent with the preliminary results of our study of such patients, which showed that urinary concentrations of sodium are inappropriately high. In this study we will compare the levels of IL-1 in patients with and without hyponatremia to see if increased levels of IL-1 are associated with hyponatremia.

Question 7: Are sustained high levels of IL-1 and TNF responsible for the wasting that is seen in some patients with *Shigella* infection?

Most patients with severe shigellosis are anorectic, and often become cachectic if their illness is prolonged. Although the colitis itself might to some degree contribute to the anorexia by directly affecting gut motility and function, it is also probable that IL-1 and TNF, which are known to induce both anorexia and a catabolic state, contribute to the weight loss that occurs in many patients with shigellosis. We will measure change in weight, IL-1 and cachectin-TNF levels, and caloric intake during the period of the study to see if sustained high levels of IL-1 and TNF are associated with more severe weight loss, or decreased caloric intake.

Question 8: Is Shiga toxin, rather than endotoxin, responsible for abnormal levels of IL-1 and TNF in patients with shigellosis?

If we find abnormally high levels of IL-1 and TNF in certain patients with shigellosis, as we suspect that we will, the question than becomes what induces these abnormally high levels. Two possible inducers stand out. One is endotoxin, which is known to be a potent inducer of IL-1 and TNF, and which has been shown in previous studies at the ICDDR,B to be found in the plasma of patients with *S. dysenteriae* type 1 infection. However, as was discussed in Question

1, we see many patients with *S. flexneri* infection whose colitis is as severe as it is in patients with *S. dysenteriae* type 1 infection, yet they rarely develop many of the specific complications that occur in patients with *S. dysenteriae* type 1 infection. As the presumed mechanism for the circulation of endotoxin in these patients was absorption through a inflamed intestine, we should see patients with *S. flexneri* infections who develop similar complications - something that we rarely find. Moreover, patients with endotoxemia due to other gram negative bacteria do not develop the same complications as the *Shigella* patients. Therefore a more attractive hypothesis is that Shiga toxin, rather than endotoxin, might be the initiator of a sequence of events that results in these specific complications, a pathogenetic sequence that is likely to include cytokines. We will measure levels of both endotoxin and Shiga toxin in patients with shigellosis, and determine which more closely correlates with cytokine levels.

Question 9: Do patients with shigellosis have a normal cytokine response to a standard inflammatory stimulus?

Immunization with toxoid vaccines is known to elicit fever and a cytokine response within hours of injection, even in malnourished children (Atkins, Keusch unpublished data). As part of the new Child Health Program, we will be providing routine immunizations to many children upon their discharge from the Treatment Centre. In 30 patients who are hospitalized for seven days or longer, we will measure levels of IL-1 and TNF as well as antibodies to diphtheria and tetanus before and after a DPT injection and compare them with 30 age and nutritional status matched control patients who were hospitalized for watery diarrhea.

Question 10: Does the development of the the hemolytic uremic syndrome correlates more closely with the circulating or stool Shiga toxin levels than it does with endotoxin levels?

A previous study from the ICDDR,B showed that patients with hemolytic-uremic syndrome (HUS) were more likely to have detectable circulating endotoxin levels than did patients with shigellosis patients who did not have endotoxemia (Koster). In that study the authors did not control for the degree of colitis however. In subsequent studies conducted in the North America and Europe it has been found that HUS in those countries is most commonly associated with infection by a specific serotype of *Escherichia coli*, *E. coli* 0157: H7 (Karmali., Neill, Grandsen). *In-vitro E. coli* 0157:H7 produces a toxin that that is identical to Shiga toxin (Calderwood, Seidah). As we have discussed, there are many diseases that cause severe colonic inflammation (ulcerative colitis, *S. flexneri* infection, but it is notable that the HUS is most closely associated epidemiologically with infection by two organisms that share in common the ability to produce *in-vivo* large amounts of Shiga toxin. Indeed in one patient from a previous study we found high serum levels, but low urine levels, of toxin, suggesting that perhaps circulating toxin is bound to renal epithelium, and therefore not excreted in the urine.

C. METHODS OF PROCEDURE

1. Patient selection

Our plan for patient selection is guided by the perception that it is likely that cytokine levels prior to the onset of a

specific complication are more important in the pathogenesis of that complication than levels at the time the complication is already present. Therefore we cannot simply enroll patients with the specific complications that we are interested in, and compare them with an equal number of patients without those complications, but rather must enroll patients prior to the onset of the complication that we are interested in.

Patients ages 1 through 12 years admitted to the ICDDR,B hospital with a presumptive clinical diagnosis of shigellosis based on complaints of dysentery and confirmed by stool microscopy (>20 leukocytes/HPF) will be eligible to participate in the study. We will attempt to enroll a total of 250 patients with culture-confirmed shigellosis. Initial enrollment will be somewhat greater because patients initially enrolled in the study who do not have Shigella isolated from their stool will be excluded from analysis.

In those 250 patients, if we were to take them consecutively, we anticipate that we would have approximately 100 patients each with *S. flexneri* and *S. dysenteriae* type 1 infections: the rest would be infected with other *Shigella* species. The exact number of patients with each type of infection would depend on the prevalence of the two types of infection during the period that the study was conducted. Prevalence rates for the two species have varied by as much as 50% over the last few years, and the number of patients with each type of infection could vary accordingly.

The number of patients with specific complications will vary as well, but based on previous patterns and with the chosen sample size we estimate that we should be able to enroll approximately 15

patients with leukemoid reaction (WBC > 50,000 mm^3), 25 patients who have a fatal outcome, 10 with hypoglycemia (plasma glucose < 2.2 mmoles/ml), 20 with hyponatremia (serum sodium <125 meq/l), and 10 with HUS (serum creatinine >200 mmoles/l and either a fall in hematocrit $\geq$ 10% or a hematocrit < 20%. Other outcomes that we are interested in, such as wasting (as determined by weight loss) can be compared in all patients enrolled in the study.

2. Clinical Evaluation

Clinical evaluation of patients during their hospital course will include a daily physical examination, as well as daily measurements of weight, fluid, calorie and protein intake, and stool output. Height and arm circumference will be measured upon admission to the Treatment Centre. Patients will also have standard six-hourly monitoring of rectal temperature, heart rate, and blood pressure. Stool samples for microscopic examination for enumeration of cellular elements, and blood for hemoglobin concentration, total leukocytes and differential, platelets, creatinine and total protein levels will be obtained on admission and days 3 and 5, at the time of a convalescent visit 30 days after discharge, and at other times as clinically indicated.

The major clinical and laboratory variables that we are interested in will be recorded on a standard data entry form which will be computerized. A copy of this computerized data entry form is attached (annex 4).

3. Laboratory studies

The following special laboratory studies will be done as part of

this protocol: measurement of plasma or serum levels of IL-1 alpha and beta, TNF, GM-CSF and G-CSF, endotoxin, Shiga toxin, pre-albumin, albumin, serum amyloid A and CRP; measurement of stool levels of Shiga toxin and alpha-1 anti-trypsin. Because the ability of monocytes to synthesize cytokines ("primed monocytes") may be a more important indicator of cytokine function than actual circulating levels, we will measure cytokines both before and after exposure to a defined stimulus, concanavalin A (Con A). We have developed methods that allow this to be done on a single sample of blood, as is described in detail below in the section on laboratory methods. In addition to these special studies, we will also perform standard clinical studies as indicated. These would include blood cultures, measurement of serum electrolytes and creatinine, complete blood counts, stool microscopic examination and stool cultures. The schedule for tests during the study will be as follows;

a. Admission

Standard Studies

1. Blood culture.
2. Complete blood count.
3. Serum electrolytes and creatinine.
4. Stool culture.
5. Stool microscopic examination.
6. Glucose if dextrose screen is less than 2.2 mmole/ml.

Special Studies

1. IL-1 and TNF plasma levels (before and after *in-vitro* Con A stimulation).
2. GM-CSF and G-CSF plasma levels.
3. Endotoxin plasma levels.
4. Albumin and pre-albumin serum levels.
5. SAA and CRP serum levels.
6. Shiga toxin serum levels.

7. Stool Shiga toxin and alpha-1 anti-trypsin concentration.

Day 3 (48 hours after admission).

1. Complete blood count.
2. Serum electrolytes and creatinine.
3. Stool microscopic examination.

Special Studies

1. IL-1 and TNF plasma levels (before and after *in-vitro* Con A stimulation).
2. GM-CSF and G-CSF plasma levels.
3. Endotoxin plasma levels.
4. Albumin and pre-albumin serum levels.
5. SAA and CRP serum levels.
6. Shiga toxin serum levels.
7. Stool sample for Shiga toxin and alpha-1 anti-trypsin level.

Day 5 (after 96 hours of admission).

Patients who are still hospitalized after 96 hours will have the following tests done:

1. Complete blood count.
2. Serum electrolytes and creatinine.
3. Stool microscopic examination.

Special Studies

1. IL-1 and TNF plasma levels (before and after *in-vitro* Con A stimulation).
2. GM-CSF and G-CSF plasma levels.
3. Endotoxin plasma levels.
4. Albumin and pre-albumin serum levels.
5. SAA and CRP serum levels.
6. Shiga toxin serum levels.
7. Stool sample for Shiga toxin and alpha-1 anti-trypsin level.

The volume of blood to be drawn on enrollment, including both routine and special studies, is 8.3 ml. This breaks downs as follows:

Test	Volume of whole blood required for test	Test performed on:
1. Creatinine and electrolytes	0.2 ml	serum
2. Blood culture;	1.0 ml	in isolator tube
3. Blood count:	0.1 ml	EDTA anti-coagulated
4. IL-1 and TNF	2.0 ml	"
5. GM-CSF and G-CSF	2.0 ml	"
6. Albumin, prealbumin, CRP SAA, diptheria and tetanus	2.0 ml serum	

antibodies, Shiga toxin

7. Endotoxin 1.0 ml heparinized plasma

Since blood cultures will not be obtained subsequently per protocol, the volume will be 1.0 ml less, or 7.3 ml. Thus the total amount required for the three planned blood draws during the period of hospitalization will be approximately 24 ml. In the 30 patients in whom we will determine response to DPT immunization, up to an extra 5 ml of blood will be drawn. Thus the total blood draw during the period of hospitalization will not exceed 30 ml in any patient, and in most patients will not exceed 24 ml. This volume during a one week period poses no danger. The additional volume required for the 30 day follow-up will be 7.3 cc. Since we are limiting the study to patients 1 year of age or older the smallest volume of blood in any of our patients should be approximately 640 mls (8 kg x 8% circulating blood volume). Most patients in this study will have blood volumes in excess of 640 ml. In any event patients who have blood smear evidence of iron-deficiency will be given oral iron therapy during convalescence.

4. Response to DPT injection.

The first 30 patients entered into the study who are discharged improved will be have an additional aliquot of 5.0 ml of blood taken 4 hours after their DPT immunization, which will be given as part of the new DANIDA Child Health Immunization Program. For patients who are discharged on day 3 or day 5 this blood drawing will be done in conjunction with the blood drawing that is scheduled for that day. For patients who are discharged on day 2 or 4, or after day 5, two additional blood draws (baseline and post-DPT) will be done on those days. We will compare post-

immunization cytokine levels with those obtained just prior to immunization, and will also compare levels on discharge with those obtained at follow-up, 30 days latter.

In addition to comparing the response to immunization at the time of discharge and at a 30 day follow-up, we will also compare the response in these patients with *Shigella* infection to that of 30 patients being discharged after having watery diarrhea, and who will be matched for age and nutritional status. Age matching will be done within the following ranges: 13 months-24 months, 25 months-59 months. Patients older than 59 months will not be entered into this part of the study. Matching for nutritional status will be on the basis of weight for height, and will follow the conventional classifications of mild, moderate, and severe malnutrition. We will attempt to identify a suitable control patient the same day a case patient is discharged, and will select patients from either the inpatient ward, or the rehydration unit. Watery diarrhea patients will by definition have less than 20 WBC's/HPF and 5 RBC/HPF on stool microscopic examination, and will not have had *Shigella* isolated from a culture of their stool.

At the time of their return visit 30 days after discharge patients will also have cytokines measured before and after a second injection of DPT. On one of these samples patients will also have antibodies to tetanus and diptheria toxins measured. We will also request control patients to return after 30 days, and will as well measure pre and post DPT immunization cytokine and diphtheria and tetanus antibody levels in these patients.

4. Follow up

All patients will be requested to return for a follow up visit 30 days after discharge. At this time a repeat clinical evaluation will be done, and another sample of blood obtained. Tests done on this visit will be:

1. Complete blood count
2. Serum electrolytes and creatinine
3. Stool culture
4. Stool microscopic examination
5. IL-1 and TNF (before and after concanavalin A stimulation).
6. GM-CSF and G-CSF
7. Endotoxin
8. Albumin and pre-albumin
9. SAA and CRP
10. Shiga toxin
11. Stool Shiga toxin and alpha-1 anti-trypsin concentration.

Patients who do not return for their follow up visit will be visited by a home health worker who will encourage them to return for this follow-up visit.

4. Laboratory methods

a. Assay of IL-1, TNF, G-CSF and GM-CSF

Sample collection and preparation:

For the assay of these cytokines 2 ml of blood will be added to each of two purple top EDTA containing Vacutainer tubes to which the protease inhibitor aprotinin has been added (to inhibit enzymatic degradation of cytokines) . The tubes will be immediately put on ice, and then centrifuged at approximately 3,000 RPM for five minutes. The plasma (excluding the buffy coat) will be aspirated, and transferred to a 1.5 ml Eppendorf tube, and then be subjected to a hard spin (11,000 RPM) in a microfuge in

order to completely sediment any remaining white cells or platelets. The supernatant will then again be aspirated, placed in storage vials, and stored at -70^{o} C until assayed for cytokine levels.

Cytokines will also be measured after *in-vitro* stimulation of peripheral blood leukocytes with concanavalin A. This will be done as follows. After the plasma has been pipetted off from the two EDTA containing vacutainer tubes, an equal amount of endotoxin free normal saline will be added back to the cells. One of these solutions will contain Con A in a concentration of 10 ug/ml. The samples will then be mixed by inversion and incubated overnight at 22^{o} C. The tubes will then be spun at 3,000 RPM for 10 minutes and the supernatant then pipetted off into Eppendorf tubes, which will then receive a fast spin in a microfuge. The cell free supernatant from these two tubes will then be extracted, which will allow us to measure basal cytokine production in the absence of Con A as well as stimulated production in the presence of Con A. Although it is normal to use a tissue culture media (e.g. RPMI) for the overnight incubation of cells, recent studies from our laboratory have shown that endotoxin free saline performs just as well (Annex 3). This considerably simplifies the procedure, as well as reduces the cost.

Assay procedure

Assay of IL-1 alpha and beta, TNF and G-CSF and GM-CSF will be done in the laboratory of Dr. Charles Dinarello, using a radioimmunoassay method that was developed in that laboratory (Lisi, Lonnemann 1987a, 1987b, van der Meer). Sensitivities of

the assays are typically 80 pg/ml (IL-1 beta) 40 pg/ml (IL-1 alpha) and 10 pg/ml (C/TNF). There is no cross reactivity of the antibodies used in these assays with other leukocyte-derived factors. A similar assay system is currently being developed for GM-CSF and G-CSF and will be standardized with the soft agar murine bone marrow colony-plaque assay.

b. Endotoxin

Sample collection.

1.0 ml of blood will be collected into a heparin containing vacutainer tube (green top) and immediately placed on ice. The sample will then be centrifuged at slow speed (200 x g for 10 minutes), the platelet rich plasma extracted and stored at -70° C until the assay is performed.

Assay method.

Endotoxin will be assayed using a limulus amoebocyte lysate assay with a chromogenic substrate to amplify the reaction (Hass, Tamura) Commercially available reagents will be used in carrying out the assay (Coatest Endotoxin, KabiVitrum Diagnostica, Stockholm).

c. CRP, albumin and prealbumin

Specimen collection

2 ml of blood will be collected into a Vacutainer tube without additive (redtop tube). The blood will be allowed to sit for 1 hour at room (air-conditioned room) temperature, the sample centrifuged and the serum extracted.

Assay method

All three will be done lasernephelometry on an automated Beckmann

system.

d. Serum amyloid A

A 25 microliter aliquot of serum (collected as outlined above) will be used for this radioimmunoassay, which will be done with reagents developed in our laboratory.

e. Serum and stool Shiga toxin

Shiga toxin will be measured using a ELISA method that we have previously described (Donahue-Rolfe). Serum shiga toxin will be measured on a 200 microliter aliquot of serum. Toxin levels in stool will be measured using the same ELISA method. Stool samples will be collected in 50 ml Falcon tubes, vortexed for five minutes, and than centrifuged at 3000 RPM for 15 minutes. The supernatant will be extracted and frozen at minus 70° C until the assay is performed. Prior to performing the assay the specimen will be transferred to a Eppendorf tube, centrifuged at high speed in a microfuge, the supernatant extracted, and filtered through a 450 millimicron filter.

f. Diphtheria and tetanus antibodies

This will be done on serum that will be collected as detailed above, and the assay will be performed by a standard hemagglutination method (Schubert).

5. Data analysis.

The mean concentration of cytokines, acute phase reactants, endotoxin and Shiga toxin in the groups under consideration, as detailed in the specific aims section, will be compared. As admission values will be available on all patients, we will use these values for the initial comparisons. Follow up values will

be used in an attempt to discern patterns of response - for instance do patients with leukemoid reaction have persistently high values, while patients with *S. dysenteriae* type 1 infection and without complications have high values initially, and subsequently have lower levels? Or do HUS patients have high values only initially, establishing a sequence of pathologic events which continue even if cytokine levels return to normal?

It is anticipated that levels of acute phase reactants and endotoxin should correlate closely with those of cytokines. If levels of one or more of the acute phase reactants do not correlate with cytokine production, than it will raise the question of whether there is perhaps not only abnormal regulation of cytokine production, but an abnormal, subnormal or selective response to cytokines that are circulating.

There are a number of potentially confounding variables that have to be taken into account - these include age, duration of illness, and nutritional status. If, after initial examination of the results it appears that cytokine response correlates closely with any of these factors, and if the groups under comparison differ as to age, nutrition, or duration of illness, it will be necessary to analyze the results after stratifying for these latter factors. If in univariate analysis a number of factors appear to be associated with a particular outcome, (say age, nutritional status and IL-1 alpha levels), it may be helpful to use a multi-variate analysis to determine which factors are independently associated.

There is little to help guide us as to precisely what levels

of cytokines we should expect in these patients. Therefore we have not performed formal sample size calculations. Rather we have determined the target sample size based on the expected number of patients we anticipate will provide the required number of patients in each subgroup under study. It has to be kept in mind that this study is unlike most treatment studies where the outcome in one group can be predicted with some certainty - that we do not in this study have a group where we can predict with relative certainty the outcome - but that we rather are looking for patterns of response. Based on the findings of this initial study we intend to propose additional detailed protocols when we have the initial data to guide us and select particular patient populations for further study and therapeutic interventions when available.

Becuase we are uncertain of the pattern and magnitude of the cytokine response in these patients we will perform an interim analysis after 30 patients have been enrolled in the study.

D. SIGNIFICANCE

The potential significance of this study lies in a number of areas. It's most immediate significance is the high likelihood it will provide a better understanding of the mechanism of the pathogenesis of shigellosis and it's complications. Since the study done by Koster et. al. here at the ICDDR,B in the mid-seventies, little work has been done anywhere to study the mechanisms of disease in patients with shigellosis. Given the fact that *Shigella* is probably the primary cause of diarrhea related mortality in this country, and that there is a high mortality rate even among patients who come to treatment centers for care, we need to understand more about the lethal complications of this disease to devise better treatment.

Most of the recent work on pathogenesis (Sansonetti, Formal and others) has looked at the pathogenesis of tissue invasion or fluid secretion at a cellular level or in animal models. It is clear that we need to know more about what is happening in humans, especially as regards complications that cannot be explained by the immediate epithelial cell - bacteria interaction, complications for which there is currently no effective therapy. This study aims to not only re-examine work previously initiated at the ICDDR,B, but also to look at a number of host-response factors which have been elucidated only during the last decade.

The ultimate determinant of the significance of clinical studies such as this is what impact the study might have on patients who are seriously ill with shigellosis. It is clear that our current therapeutic approach to the patient with shigellosis is appropriate, but insufficient. Antibiotics can effectively eradicate infection, but do not necessarily halt the progression of disease. If an aberrant host response is involved in the disease process, than immunomodulation might be a useful therapeutic

modality in addition to the appropriate use of antimicrobial agents. Immunomodulation can range from treatment with classically used agents, such as steroids, to more specific means of immunomodulation that are likely to become available in the coming years, such as monoclonal antibodies directed at specific type of lymphocytes or their products, or eicosanoid regulation of IL-1 *in-vivo* in humans (Cannon, Dinarello, unpublished data). This study will help provide a better understanding of aspects of the host-response that might be amenable to such therapy. It will also help elucidate the role of Shiga toxin in the pathogenesis of disease, something that has not been as yet resolved. If it does appear that Shiga toxin has an important role in the pathogenesis of some of these lethal complications, it has important implications for vaccine development.

E. FACILITIES REQUIRED

This study will be done using existing facilities. These will include the research wards of the Dhaka Treatment Centre, the newly constructed research ward laboratory, and the office space assigned to the Tufts-New England *Shigella* Invasive Diarrhea Research Team.

F. COLLABORATIVE ARRANGEMENTS

This study has been developed in collaboration with the Division of Geographic Medicine and Infectious Diseases at the New England Medical Center, Tufts University School of Medicine, Boston, Mass. Dr. Dinarello will do the cytokine assays, and Dr. Keusch's laboratory will assist with the assays of Shiga toxin. Dr. Dinarello at this institution has been a pioneer in the identification of interleukin 1, and his laboratory is one of the leading groups in the world for the study of IL-1 as well as other

cytokines. The laboratory of Dr. Keusch is one of the foremost groups working on Shiga toxin, and indeed discovered its action on the gut and proposed its involvement in pathogenesis. A fellow from the Division of Geographic Medicine and Infectious Disease of Tufts New England Medical Center, Dr. Judith Nerad, will take an active role in the study. ICDDR,B now has an official collaborative relationship with this Division, which is headed by Professor Gerald Keusch.

There will be national counterparts at the fellows level as well. We anticipate that Dr. K. Jahan, and M.P.H from the Harvard School of Public Health, will accept a position to work on the project, and she will work closely with Dr. Nerad and other members of the Tufts-ICDDR,B team.

In addition Dr. Marc Struelens, an alumnus of ICDDR,B, currently working in the Department of Bacteriology and Serology at Erasme Hospital at the Free University of Brussels, will participate. The assay of acute phase reactants will be performed at this centre.

The C.V.'s of these collaborating scientists are attached (Annex 5).

In terms of the procedures and assays that will be used in the study, it is anticipated that the Shiga toxin assay (both the ELISA and the cytotoxicity assay) will be done in Dhaka, and that the assay of cytokines, which are now being done only in the laboratory in Boston, can be established in Dhaka once the newer, simpler assay methods that we will be using for this study are well validated against the older, more cumbersome methods. Such assays could then be used in a variety of studies that look at the complications and pathogenesis in patients with invasive diarrheas.

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ABSTRACT SUMMARY

1. The patient population for this study will be patients admitted to the ICDDR,B Treatment Centre who are suspected of having shigellosis at the time of their entry into the study. Only patients with culture proven shigellosis will be analyzed however. Only patients older than 1 year of age will be entered into the study. Children are included because the complications under study, as well as death, occur almost exclusively in children.

2. There are no risks associated with the study procedure. A small amounts of blood will be sampled aseptically by venipuncture. In most patients this will amount no more than 25 ml within a one week period, and 7.3 ml a month later. In some patients an additional 5 ml will be required. These small volumes are made possible thanks to the recent development of micromethods for the study of cytokines and should pose no risk to any of the children in this study, the smallest of whom are likely to have a minimum blood volume of about 480 ml (6 kg x 8% blood volume), and most of whom are likely to have much greater blood volumes. Where appropriate patients will be provided with iron therapy during convalescence.

3. No risk is involved to the patient, so that the question (minimizing potential risk) does not apply.

4. All clinical records will be handled only by investigators. Code numbers will be used instead of patients name.

5. Signed consent will be obtained from the patient or his/her parent or guardian after a clear description of the study methods and purpose.

6. No special interviews will be required.

7. If the pattern of non specific host response is found to influence the severity of shigellosis, this may lead to original preventive or therapeutic approaches, as we have described in the significance section of the protocol. With a death rate of over 10% among our patients hospitalized with *Shigella* infection it is clear that we need to find more effective therapies. To do so we need to understand more about the disease process, which is what this study attempts to do.
8. The study will use only human plasma or serum stool and medical records.

INFORMED CONSENT FORM

Your child has bloody dysentery which is probably caused by a germ named *Shigella*. Diarrhea caused by this germ can be very serious, and can lead to a number of complications and problems that we do not see with other types of diarrhea. We do not know how and why this germ causes the problems and complications that it does, and to understand more about the illness that it causes we are conducting a study. We would like your permission to enroll your child into this study.

The study is a quite simple. During the time that your child is in the Treatment Centre we will provide him with the care that we would normally provide for this serious illness, and we will record his progress during hospitalization. Your child will not have to be hospitalized any longer than they otherwise would have been. In addition to the normal tests that we do, we will obtain an extra 7.3 cc of blood (a little more than 1 teaspoon) on three occasions over 7 days in order to perform some special tests. These tests will tell us how effectively your child is fighting the infection. Taking this extra amount of blood will not in any way harm your child. We will ask you to bring your child back 30 days after his discharge in order to assess how he has progressed. At that time we will take another blood sample of similar volume. All the information that we record for study purposes will be kept by the investigators and no one else will have access to this information. By allowing your child to enter into the study we will hopefully be able to learn more about this infection which is one of the major causes of diarrheal illness and death in Bangladesh and similar poor countries. Hopefully by learning more about the illness we will be able in the future to more effectively treat patients with this infection.

We might also give your child an immunization against diptheria, tetanus, and whooping cough at the time he is discharged from the hospital. If so, we will measure how effective the immunization was by taking an extra sample of blood (about half a teaspoon) both before and after the immunization. We will do this again at the time of the follow-up visit 30 days after discharge.

If you would prefer to not have your child enter into this study he (or she) will receive the same care as they usually. If you do agree to let your child participate in this study please sign (or give your left thumb impression) below.

Justification for budgetary items.

1. Project co-head.

This position is intended for a national scientist who will serve as the co-director of the project. The person who fills this position should be someone at the senior medical officer level or above, who has familiarity with the clinical problems of patients coming to the Treatment Centre, who has some background in clinical research, and is now ready to devote the majority of their time to research.

2. Medical officer.

This will be a physician at a more junior level than the project co-head, who will actively participate in the clinical and research activities of the study.

3. Laboratory technician

The responsibility of this person will include learning and carrying out certain assay procedures that will be part of the study. These include both the ELISA and cytotoxicity assays for Shiga toxin. In addition this person will process specimens, arrange their storage in the freezer, and order supplies as necessary.

4. Community health workers.

These two persons (preferably women) will coordinate the follow-up activities. This means obtaining a detailed address of patients who are enrolled in the study so that they can visit the home if the patient does not return for the scheduled follow-up, visiting the homes of those patients who do not return for follow-up, and assisting with patients during their hospitalization.

5. Computer entry technician/programmer

This person will be responsible for developing a computer data entry form, making corresponding patient data collection forms, entering data into the computer, and assisting with the computer based data analysis. This work will be done using Stat-pac data base and statistical analysis program on pre-existing computer equipment.

6. Vacutainer tubes for blood collection

Although somewhat (2x) more expensive than using plan test tubes without additives, these tubes possess the advantage of being endotoxin free, having the additive (i.e. EDTA, heparin) pre-added, and having a standard volume draw.

7. Microtainer tubes for CBC.

These tubes have the advantage of collecting a micro-sample with good preservation of RBC morphology, which is critical for judging hemolysis.

8. Nunc tubes for specimen storage.

This tubes are again slightly more expensive than glass tubes for storage of specimens, but our experience in previous studies has been that they are more reliable than glass tubes, which often break in transport, or microcentrifuge tubes, which at times do not have sufficient volume for the sample, and which are more likely to lose their labels.

9. ELISA reader, washer and multi-channel pipet.

This will be used for the SHIGA toxin ELISA, a method which we will establish in the laboratories in Dhaka.

10. -65^0 C freezer.

This will be used for safe and secure storage of the specimens that are being batched for assay.

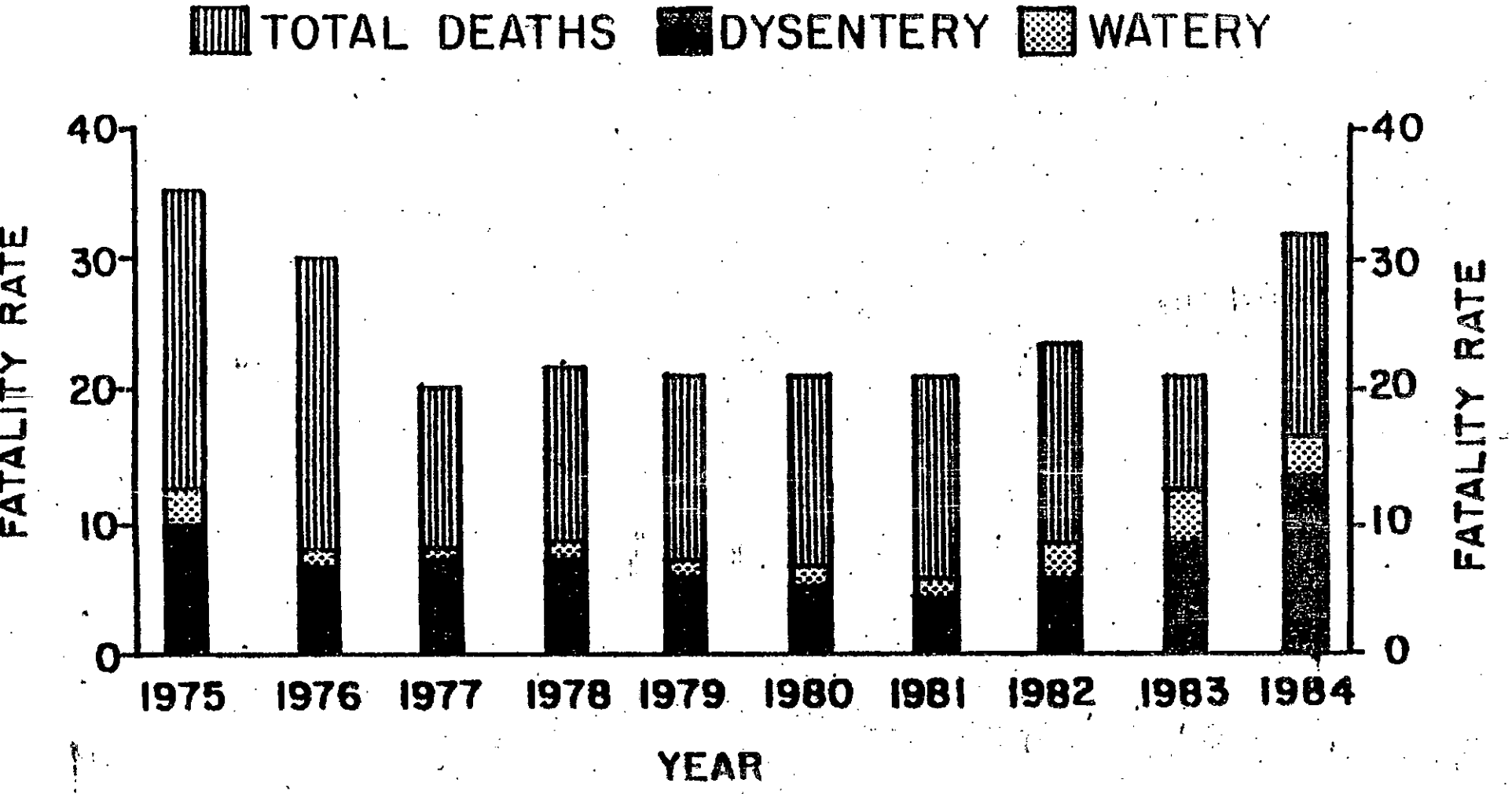
11. Ice maker

Blood for endotoxin assay should be kept cold while awaiting processing. This is true even the the period from collection to processing is very short. An ice maker in the Treatment Centre will allow us to do this.

ANNEX 1

FATALITY RATES BY TYPE OF DIARRHOEA : MATLAB 1975 - 84

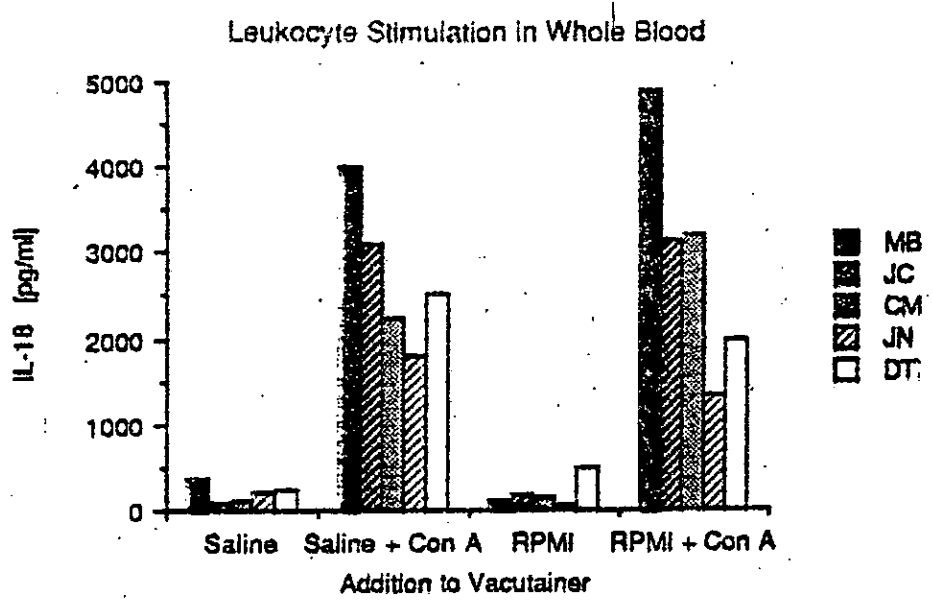
RATES PER 1000 CHILDREN AGE 1-4 YEARS

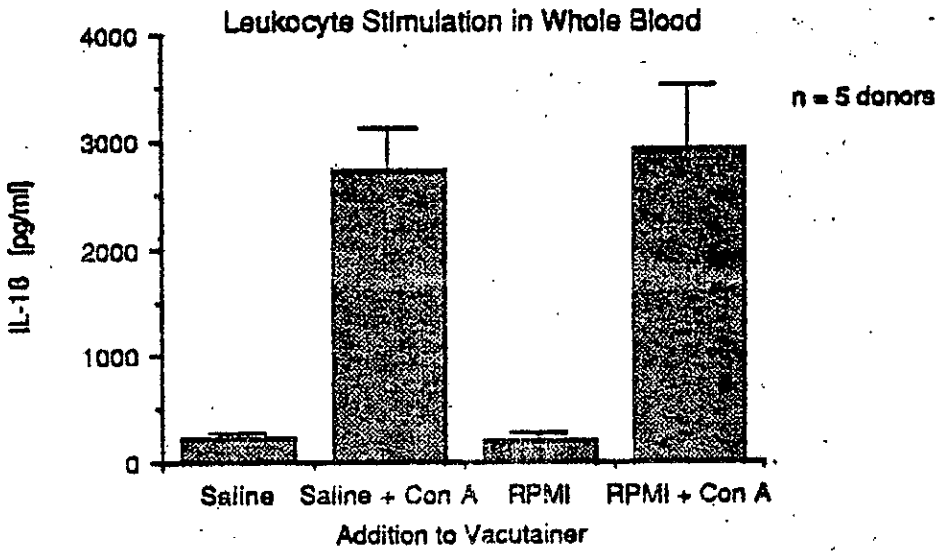


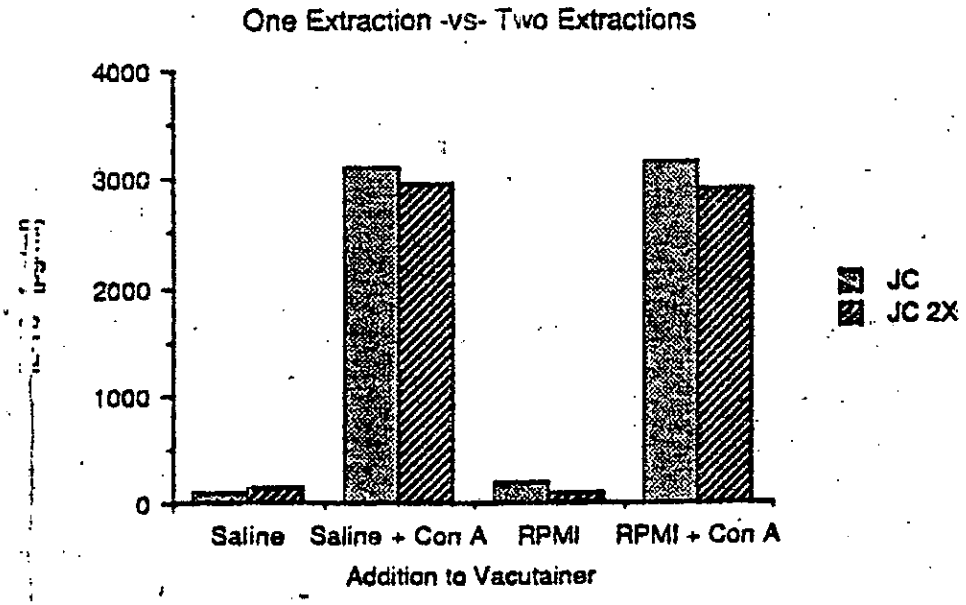
ANNEX 2: SHIGELLA ADMISSIONS AND DEATHS

ICDDR,B - 1983-1986

Species or serotype	Total admissions	Discharged alive	DORB	Referred to other hospital	Died in hospital
S. flexneri	1740	1390 (80)	162 (9)	20 (1)	168 (10)
S. dysenteriae type 1	1070	835 (78)	118 (11)	35 (3)	82 (8)
S. dysenteriae types 2-10	105	88 (84)	4 (4)	1 (1)	12 (11)
S. boydii	208	169 (81)	18 (9)	1 (0)	20 (10)
S. sonnei	112	94 (84)	9 (8)	2 (2)	7 (6)
Total	3235	2576 (80)	311 (10)	59 (2)	289 (9)


Leukocyte Stimulation in Whole Blood
IL-1β [pg/ml]
5000
4000
3000
2000
1000
0
Saline
Saline + Con A
RPMI
RPMI + Con A
Addition to Vacutainer
MB
JC
CM
JN
DT


Leukocyte Stimulation in Whole Blood
IL-1β [pg/ml]
4000
3000
2000
1000
0
Saline
Saline + Con A
RPMI
RPMI + Con A
Addition to Vacutainer
n = 5 donors


One Extraction -vs- Two Extractions
4000
3000
2000
1000
0
Saline
Saline + Con A
RPMI
RPMI + Con A
Addition to Vacutainer
JC
JC 2X

Codebook listing - A:GTO.COD

Variable # 1 - HOSPITAL NO
Start column = 1 Number of columns = 6 Type = Numeric

Variable # 2 - STUDY NO
Start column = 7 Number of columns = 4 Type = Numeric

Variable # 3 - SEX
Start column = 11 Number of columns = 1 Type = Alpha

1=MALE
2=FEMALE

Variable # 4 - AGE (MONTHS)
Start column = 12 Number of columns = 3 Type = Numeric

Variable # 5 - HX TAKEN HOSPITAL DAY
Start column = 15 Number of columns = 2 Type = Numeric

Variable # 6 - HX HRS OF DIARRHEA
Start column = 17 Number of columns = 4 Type = Numeric

Variable # 7 - STOOL CHAR ADMISSION
Start column = 21 Number of columns = 1 Type = Alpha

1=WATERY
2=WATERY/MUCOID
3=MUCOID
4=BLOODY/MUCOID
5=NORMAL

Variable # 8 - HX STOOL CHAR ONSET
Start column = 22 Number of columns = 1 Type = Alpha

1=WATERY
2=WATERY/MUCOID
3=MUCOID
4=BLOODY/MUCOID
5=NORMAL

Variable # 9 - HX ST FREQ PRO 24 HR ADM
Start column = 23 Number of columns = 2 Type = Numeric

Variable # 10 - HX HRS FEVER
Start column = 25 Number of columns = 4 Type = Numeric

Variable # 11 - HX HRS VOMITING
Start column = 29 Number of columns = 4 Type = Numeric

Variable # 12 - HX # VOMITING LAST 24
Start column = 33 Number of columns = 2 Type = Numeric

Variable # 13 - HX FOOD INTAKE
Start column = 35 Number of columns = 1 Type = Alpha

1=USUAL
2=>USUAL
3=<USUAL
4=NONE

Variable # 14 - HX THIRST
Start column = 36 Number of columns = 1 Type = Alpha

1=USUAL
2=>USUAL
3=<USUAL

Variable # 15 - HX URINE OUTP
Start column = 37 Number of columns = 1 Type = Alpha

1=USUAL
2=>USUAL
3=<USUAL
4=NONE

Variable # 16 - HX ABD PAIN
Start column = 38 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 17 - HX STRAINING
Start column = 39 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 18 - HX RECT PRO
Start column = 40 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 19 - HX MYALGIA
Start column = 41 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 20 - HX ARTHRALG
Start column = 42 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 21 - HX HEADACHE
Start column = 43 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 22 - HX MENT STAT
Start column = 44 Number of columns = 1 Type = Alpha

1=NORMAL
2=IRRITABLE
3=LETHARGIC
4=COMATOSE
5=SEIZURES
6=OTHER

Variable # 23 - HX DRUG RX
Start column = 45 Number of columns = 1 Type = Alpha

1=NONE
2=ANTIBIOTIC
3=OTHER ALLOPATHIC
4=HOMEOPATHIC
5=AYORDEVIDIC
6=TAKEN,NOT KNOWN
7=INCLUDING ANTIBX
8=NO ANTIBIOTIC

Variable # 24 - ADM TEMP
Start column = 46 Number of columns = 4 Type = Numeric

Variable # 25 - ADM HEART RT
Start column = 50 Number of columns = 3 Type = Numeric

Variable # 26 - ADM RESP RATE
Start column = 53 Number of columns = 3 Type = Numeric

Variable # 27 - ADM BP SYS
Start column = 56 Number of columns = 3 Type = Numeric

Variable # 28 - ADM RAD PULSE
Start column = 59 Number of columns = 1 Type = Alpha

1=NORMAL
2=WEAK/FEEBLE
3=ABSENT

Variable # 29 - ADM DEHYD
Start column = 60 Number of columns = 1 Type = Alpha

1=NONE
2=MILD
3=MODERATE
4=SEVERE

Variable # 30 - ADM SHOCK
Start column = 61 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 31 - ADM PEDAL EDEMA
Start column = 62 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 32 - ADM CHEST
Start column = 63 Number of columns = 1 Type = Alpha

1=CLEAR
2=ABNORMAL

Variable # 33 - ADM BOWEL SDS
Start column = 64 Number of columns = 1 Type = Alpha

1=ACTIVE
2=SLUGGISH
3=ABSENT

Variable # 34 - ADM ABD DISTEN
Start column = 65 Number of columns = 1 Type = Alpha

1=NONE
2=PRESENT,SOFT
3=PRESENT,FIRM

Variable # 35 - ADM ABD TEND
Start column = 66 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 36 - ADM RECT PRO
Start column = 67 Number of columns = 1 Type = Alpha

1=NONE
2=SELF-REDUCIBLE
3=PRESSURE RED
4=NON-REDUCIBLE

Variable # 37 - ADM MENT STAT
Start column = 68 Number of columns = 1 Type = Alpha

1=ALERT
2=AGITATED
3=DELIRIOUS
4=OBTUNDED
5=STUPOROUS
6=COMA
7=SEIZURES

Variable # 38 - ADM WT (KG)
Start column = 69 Number of columns = 4 Type = Numeric

Variable # 39 - WT/AGE
Start column = 73 Number of columns = 3 Type = Numeric

Variable # 40 - HEIGHT
Start column = 76 Number of columns = 3 Type = Numeric

Variable # 41 - WT/HT
Start column = 79 Number of columns = 3 Type = Numeric

Variable # 42 - ADM CXRAY
Start column = 82 Number of columns = 1 Type = Alpha

1=NOT DONE
2=NORMAL
3=CONSOLIDATION
4=INFILTRATE
5=ABNORMAL OTHER

Variable # 43 - ADM ABD XRAY
Start column = 83 Number of columns = 1 Type = Alpha

1=NOT DONE
2=NORMAL
3=SUSPECT ABNORMAL

Variable # 44 - ADM WBC
Start column = 84 Number of columns = 3 Type = Numeric

Variable # 45 - ADM HCT
Start column = 87 Number of columns = 2 Type = Numeric

Variable # 46 - ADM BANDS
Start column = 89 Number of columns = 2 Type = Numeric

Variable # 47 - ADM POLYS
Start column = 91 Number of columns = 2 Type = Numeric

Variable # 48 - ADM LYMPHS
Start column = 93 Number of columns = 2 Type = Numeric

Variable # 49 - ADM NA+
Start column = 95 Number of columns = 3 Type = Numeric

Variable # 50 - ADM K+
Start column = 98 Number of columns = 3 Type = Numeric

Variable # 51 - ADM BICARB
Start column = 101 Number of columns = 2 Type = Numeric

Variable # 52 - ADM CL-
Start column = 103 Number of columns = 3 Type = Numeric

Variable # 53 - ADM CREAT
Start column = 106 Number of columns = 3 Type = Numeric

Variable # 54 - ADM PROT
Start column = 109 Number of columns = 2 Type = Numeric

Variable # 55 - ADM STOOL WBC
Start column = 111 Number of columns = 3 Type = Numeric

Variable # 56 - ADM STOOL RBC
Start column = 114 Number of columns = 3 Type = Numeric

Variable # 57 - ADM GLUCOSE
Start column = 117 Number of columns = 4 Type = Numeric

Variable # 58 - ADM ALB
Start column = 121 Number of columns = 4 Type = Numeric

Variable # 59 - ADM CRP
Start column = 125 Number of columns = 4 Type = Numeric

Variable # 60 - ADM SAA
Start column = 129 Number of columns = 4 Type = Numeric

Variable # 61 - ADM PRE-ALB
Start column = 133 Number of columns = 4 Type = Numeric

Variable # 62 - ADM ENDO TOX
Start column = 137 Number of columns = 4 Type = Numeric

Variable # 63 - ADM SH TOX
Start column = 141 Number of columns = 4 Type = Numeric

Variable # 64 - ADM IL1A UN-STIM
Start column = 145 Number of columns = 5 Type = Numeric

Variable # 65 - ADM IL1A STIM
Start column = 150 Number of columns = 5 Type = Numeric

Variable # 66 - ADM IL1B UN-STIM
Start column = 155 Number of columns = 5 Type = Numeric

Variable # 67 - ADM IL1B STIM
Start column = 160 Number of columns = 5 Type = Numeric

Variable # 68 - ADM TNF UN-STIM
Start column = 165 Number of columns = 5 Type = Numeric

Variable # 69 - ADM TNF STIM
Start column = 170 Number of columns = 5 Type = Numeric

Variable # 70 - ADM G-CSF
Start column = 175 Number of columns = 6 Type = Numeric

Variable # 71 - ADM GM-CSF
Start column = 181 Number of columns = 6 Type = Numeric

Variable # 72 - ADM ST SHIG TOX
Start column = 187 Number of columns = 6 Type = Numeric

Variable # 73 - ADM ST-A1ANT
Start column = 193 Number of columns = 6 Type = Numeric

Variable # 74 - ADM E HISTO
Start column = 199 Number of columns = 1 Type = Alpha

1=TROPHOZOITE
2=CYST
3=NEG

Variable # 75 - ADM GIARDIA
Start column = 200 Number of columns = 1 Type = Alpha

1=TROPHOZOITE
2=CYST
3=NEG

Variable # 76 - ADM OTHER STOOL PATH
Start column = 201 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 77 - ADM STOOL PH
Start column = 202 Number of columns = 1 Type = Alpha

1=ACID
2=ALKALINE

Variable # 78 - ADM STOOL COLOR
Start column = 203 Number of columns = 1 Type = Alpha

1=YELLOW
2=BROWN
3=OTHER

Variable # 79 - ADM STOOL CHAR
Start column = 204 Number of columns = 1 Type = Alpha

1=LIQUID
2=LOOSE
3=OTHER

Variable # 80 - ADM STOOL CX
Start column = 205 Number of columns = 1 Type = Alpha

1=S.DYS-1
2=S.DYS-2-10
3=S.FLEX
4=S.BOYD
5=S.SONNEI
6=NO SHIG ISO

Variable # 81 - AMPI RESIST
Start column = 206 Number of columns = 1 Type = Alpha

1=NO
2=YES
3=NOT TESTED

Variable # 82 - NAL ACID RESIST
Start column = 207 Number of columns = 1 Type = Alpha

1=NO
2=YES
3=NOT TESTED

Variable # 83 - TETRA RESIST
Start column = 208 Number of columns = 1 Type = Alpha

1=NO
2=YES
3=NOT TESTED

Variable # 84 - CHLOR RESIST
Start column = 209 Number of columns = 1 Type = Alpha

1=NO
2=YES
3=NOT TESTED

Variable # 85 - GENT RESIST
Start column = 210 Number of columns = 1 Type = Alpha

1=NO
2=YES
3=NOT TESTED

Variable # 86 - SXT RESIST
Start column = 211 Number of columns = 1 Type = Alpha

1=NO
2=YES
3=NOT TESTED

Variable # 87 - WT IN KG DAY-3
Start column = 212 Number of columns = 4 Type = Numeric

Variable # 88 - ABD XRAY DAY-3
Start column = 216 Number of columns = 1 Type = Alpha

1=NOT DONE
2=NORMAL
3=SUSPECT ABN

Variable # 89 - WBC DAY-3
Start column = 217 Number of columns = 3 Type = Numeric

Variable # 90 - HCT DAY-3
Start column = 220 Number of columns = 2 Type = Numeric

Variable # 91 - BANDS DAY-3
Start column = 222 Number of columns = 2 Type = Numeric

Variable # 92 - POLYS DAY-3
Start column = 224 Number of columns = 2 Type = Numeric

Variable # 93 - LYMPHS DAY-3
Start column = 226 Number of columns = 2 Type = Numeric

Variable # 94 - Na+ DAY-3
Start column = 228 Number of columns = 3 Type = Numeric

Variable # 95 - K+ DAY-3
Start column = 231 Number of columns = 3 Type = Numeric

Variable # 96 - BICARBONATE DAY-3
Start column = 234 Number of columns = 2 Type = Numeric

Variable # 97 - CL- DAY-3
Start column = 236 Number of columns = 3 Type = Numeric

Variable # 98 - CREAT DAY-3
Start column = 239 Number of columns = 3 Type = Numeric

Variable # 99 - SER PROT DAY-3
Start column = 242 Number of columns = 2 Type = Numeric

Variable # 100 - STOOL WBC DAY-3
Start column = 244 Number of columns = 3 Type = Numeric

Variable # 101 - STOOL RBC DAY-3
Start column = 247 Number of columns = 3 Type = Numeric

Variable # 102 - GLUCOSE DAY-3
Start column = 250 Number of columns = 4 Type = Numeric

Variable # 103 - ALB DAY-3
Start column = 254 Number of columns = 4 Type = Numeric

Variable # 104 - CRP DAY-3
Start column = 258 Number of columns = 4 Type = Numeric

Variable # 105 - SAA DAY-3
Start column = 262 Number of columns = 4 Type = Numeric

Variable # 106 - PRE-ALB DAY-3
Start column = 266 Number of columns = 4 Type = Numeric

Variable # 107 - ENDO TOX DAY-3
Start column = 270 Number of columns = 4 Type = Numeric

Variable # 108 - SH TOX DAY-3
Start column = 274 Number of columns = 4 Type = Numeric

Variable # 109 - IL1A UN-STIM D-3
Start column = 278 Number of columns = 5 Type = Numeric

Variable # 110 - IL1A STIM D-3
Start column = 283 Number of columns = 5 Type = Numeric

Variable # 111 - IL1B UN-STIM D-3
Start column = 288 Number of columns = 5 Type = Numeric

Variable # 112 - IL1B STIM D-3
Start column = 293 Number of columns = 5 Type = Numeric

Variable # 113 - TNF UN-STIM D-3
Start column = 298 Number of columns = 5 Type = Numeric

Variable # 114 - TNF STIM D-3
Start column = 303 Number of columns = 5 Type = Numeric

Variable # 115 - G-CSF DAY-3
Start column = 308 Number of columns = 6 Type = Numeric

Variable # 116 - GM-CSF DAY-3
Start column = 314 Number of columns = 6 Type = Numeric

Variable # 117 - ST SHIG TOX D-3
Start column = 320 Number of columns = 6 Type = Numeric

Variable # 118 - ST-A1ANT DAY-3
Start column = 326 Number of columns = 6 Type = Numeric

Variable # 119 - WI IN KG DAY-5
Start column = 332 Number of columns = 4 Type = Numeric

Variable # 120 - ABD XRAY DAY-5
Start column = 336 Number of columns = 1 Type = Alpha

1=NOT DONE
2=NORMAL
3=SUSPECT ABN

Variable # 121 - WBC DAY-5
Start column = 337 Number of columns = 3 Type = Numeric

Variable # 122 - HCT DAY-5
Start column = 340 Number of columns = 2 Type = Numeric

Variable # 123 - BANDS DAY-5
Start column = 342 Number of columns = 2 Type = Numeric

Variable # 124 - POLYS DAY-5
Start column = 344 Number of columns = 2 Type = Numeric

Variable # 125 - LYMPHS DAY-5
Start column = 346 Number of columns = 2 Type = Numeric

Variable # 126 - Na+ DAY-5
Start column = 348 Number of columns = 3 Type = Numeric

Variable # 127 - K+ DAY-5
Start column = 351 Number of columns = 3 Type = Numeric

Variable # 128 - BICARBONATE DAY-5
Start column = 354 Number of columns = 2 Type = Numeric

Variable # 129 - CL- DAY-5
Start column = 356 Number of columns = 3 Type = Numeric

Variable # 130 - CREAT DAY-5
Start column = 359 Number of columns = 3 Type = Numeric

Variable # 131 - SER PROT DAY-5
Start column = 362 Number of columns = 2 Type = Numeric

Variable # 132 - STOOL WBC DAY-5
Start column = 364 Number of columns = 3 Type = Numeric

Variable # 133 - STOOL RBC DAY-5
Start column = 367 Number of columns = 3 Type = Numeric

Variable # 134 - GLUCOSE DAY-5
Start column = 370 Number of columns = 4 Type = Numeric

Variable # 135 - ALB DAY-5
Start column = 374 Number of columns = 4 Type = Numeric

Variable # 136 - CRP DAY-5
Start column = 378 Number of columns = 4 Type = Numeric

Variable # 137 - SAA DAY-5
Start column = 382 Number of columns = 4 Type = Numeric

Variable # 138 - PRE-ALB DAY-5
Start column = 386 Number of columns = 4 Type = Numeric

Variable # 139 - ENDO TOX DAY-5
Start column = 390 Number of columns = 4 Type = Numeric

Variable # 140 - SH TOX DAY-5
Start column = 394 Number of columns = 4 Type = Numeric

Variable # 141 - IL1A UN-STIM D-5
Start column = 398 Number of columns = 5 Type = Numeric

Variable # 142 - IL1A STIM D-5
Start column = 403 Number of columns = 5 Type = Numeric

Variable # 143 - IL1B UN-STIM D-5
Start column = 408 Number of columns = 5 Type = Numeric

Variable # 144 - IL1B STIM D-5
Start column = 413 Number of columns = 5 Type = Numeric

Variable # 145 - TNF UN-STIM D-5
Start column = 418 Number of columns = 5 Type = Numeric

Variable # 146 - TNF STIM D-5
Start column = 423 Number of columns = 5 Type = Numeric

Variable # 147 - G-CSF DAY-5
Start column = 428 Number of columns = 6 Type = Numeric

Variable # 148 - GM-CSF DAY-5
Start column = 434 Number of columns = 6 Type = Numeric

Variable # 149 - ST-SHIG TOX D-5
Start column = 440 Number of columns = 6 Type = Numeric

Variable # 150 - ST-A1ANT D-5
Start column = 446 Number of columns = 6 Type = Numeric

Variable # 151 - DAYS OF F-UP VISIT
Start column = 452 Number of columns = 2 Type = Numeric

Variable # 152 - WT IN KG F-UP
Start column = 454 Number of columns = 4 Type = Numeric

Variable # 153 - ABD XRAY F-UP
Start column = 458 Number of columns = 1 Type = Alpha

1=NOT DONE
2=NORMAL
3=SUSPECT ABN

Variable # 154 - WBC F-UP
Start column = 459 Number of columns = 3 Type = Numeric

Variable # 155 - HCT F-UP
Start column = 462 Number of columns = 2 Type = Numeric

Variable # 156 - BANDS F-UP
Start column = 464 Number of columns = 2 Type = Numeric

Variable # 157 - POLY F-UP
Start column = 466 Number of columns = 2 Type = Numeric

Variable # 158 - LYMPHS F-UP
Start column = 468 Number of columns = 2 Type = Numeric

Variable # 159 - Na+ F-UP
Start column = 470 Number of columns = 3 Type = Numeric

Variable # 160 - K+ F-UP
Start column = 473 Number of columns = 3 Type = Numeric

Variable # 161 - BICARBONATE F-UP
Start column = 476 Number of columns = 2 Type = Numeric

Variable # 162 - CL- F-UP
Start column = 478 Number of columns = 3 Type = Numeric

Variable # 163 - CREAT F-UP
Start column = 481 Number of columns = 3 Type = Numeric

Variable # 164 - SER PROT F-UP
Start column = 484 Number of columns = 2 Type = Numeric

Variable # 165 - STOOL WBC F-UP
Start column = 486 Number of columns = 3 Type = Numeric

Variable # 166 - STOOL RBC F-UP
Start column = 489 Number of columns = 3 Type = Numeric

Variable # 167 - GLUCOSE F-UP
Start column = 492 Number of columns = 4 Type = Numeric

Variable # 168 - ALB F-UP
Start column = 496 Number of columns = 4 Type = Numeric

Variable # 169 - CRP F-UP
Start column = 500 Number of columns = 4 Type = Numeric

Variable # 170 - SAA F-UP
Start column = 504 Number of columns = 4 Type = Numeric

Variable # 171 - PRE ALB F-UP
Start column = 508 Number of columns = 4 Type = Numeric

Variable # 172 - ENDO TOX F-UP
Start column = 512 Number of columns = 4 Type = Numeric

Variable # 173 - SH TOX F-UP
Start column = 516 Number of columns = 4 Type = Numeric

Variable # 174 - IL1A UN-STIM F-UP
Start column = 520 Number of columns = 5 Type = Numeric

Variable # 175 - IL1A STIM F-UP
Start column = 525 Number of columns = 5 Type = Numeric

Variable # 176 - IL1B UN-STIM F-UP
Start column = 530 Number of columns = 5 Type = Numeric

Variable # 177 - IL1B STIM F-UP
Start column = 535 Number of columns = 5 Type = Numeric

Variable # 178 - TNF UN-STIM F-UP
Start column = 540 Number of columns = 5 Type = Numeric

Variable # 179 - TNF STIM F-UP
Start column = 545 Number of columns = 5 Type = Numeric

Variable # 180 - G-CSF F-UP
Start column = 550 Number of columns = 6 Type = Numeric

Variable # 181 - GM-CSF F-UP
Start column = 556 Number of columns = 6 Type = Numeric

Variable # 182 - ST SHIG TOX
Start column = 562 Number of columns = 6 Type = Numeric

Variable # 183 - ST-A1ANT F-UP
Start column = 568 Number of columns = 6 Type = Numeric

Variable # 184 - HC WBC MAX
Start column = 574 Number of columns = 3 Type = Numeric

Variable # 185 - HC TEMP MAX
Start column = 577 Number of columns = 4 Type = Numeric

Variable # 186 - HC HCT <20
Start column = 581 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 187 - HC HCT DROP >10
Start column = 582 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 188 - HC FRAGMENT MAX
Start column = 583 Number of columns = 3 Type = Numeric

Variable # 189 - HC LOWEST GLUC
Start column = 584 Number of columns = 4 Type = Numeric

Variable # 190 - HC LOWEST NA+
Start column = 592 Number of columns = 3 Type = Numeric

Variable # 191 - HC MAX CREAT
Start column = 595 Number of columns = 3 Type = Numeric

Variable # 192 - HC LOWEST PROT
Start column = 598 Number of columns = 2 Type = Numeric

Variable # 193 - HC CONJUNCT
Start column = 600 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 194 - HC ARTHRIT
Start column = 601 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 195 - HC ILEUS
Start column = 602 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 196 - HC RECT PRO
Start column = 603 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 197 - HC BLD CX
Start column = 604 Number of columns = 1 Type = Alpha

1=POS
2=NEG
3=NOT DONE

Variable # 198 - HC CSF CX
Start column = 605 Number of columns = 1 Type = Alpha

1=POS
2=NEG
3=NOT DONE

Variable # 199 - HC CSF PLEOCYT
Start column = 606 Number of columns = 1 Type = Alpha

1=NEG
2=YES,>1POLY/7WBCS
3=NOT DONE

Variable # 200 - HC SEIZURE
Start column = 607 Number of columns = 1 Type = Alpha

1=NO
2=YES

```
Variable # 201 - HC WORST MENT STAT
Start column = 608    Number of columns = 1    Type = Alpha

        1=ALERT
        2=AGITATED
        3=DELIRIOUS
        4=OBTUNDED
        5=STUPOROUS
        6=COMA
        7=SEIZURES
        8=LETHARGIC
-------------------------------------------------------------
Variable # 202 - HC SHOCK
Start column = 609    Number of columns = 1    Type = Alpha

        1=NO
        2=YES
-------------------------------------------------------------
Variable # 203 - HC HUS
Start column = 610    Number of columns = 1    Type = Alpha

        1=NO
        2=YES
-------------------------------------------------------------
Variable # 204 - HC CXRAY
Start column = 611    Number of columns = 1    Type = Alpha

        1=NOT DONE
        2=NORMAL
        3=CONSOLIDATION
        4=DIFFUSE INFILTRA
        5=ABNORMAL OTHER
-------------------------------------------------------------
Variable # 205 - HC ABD XRAY
Start column = 612    Number of columns = 1    Type = Alpha

        1=NOT DONE
        2=NORMAL
        3=SUSPECT ABNORMAL
-------------------------------------------------------------
Variable # 206 - HC IV RX
Start column = 613    Number of columns = 1    Type = Alpha

        1=NO
        2=YES
-------------------------------------------------------------
Variable # 207 - HC BLOOD TRANS
Start column = 614    Number of columns = 1    Type = Alpha

        1=NO
        2=YES
-------------------------------------------------------------
Variable # 208 - HC AMPI RX
Start column = 615    Number of columns = 1    Type = Alpha

        1=NO
        2=YES
-------------------------------------------------------------
```

Variable # 20? - HC NAL ACID RX
Start column = 616 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 21? - HC SXT RX
Start column = 617 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 211 - HC GENT RX
Start column = 618 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 212 - HC CHLORO RX
Start column = 619 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 213 - HC CLOX RX
Start column = 620 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 214 - HC TB RX
Start column = 621 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 215 - HC PEN RX
Start column = 622 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 216 - HC METRONI RX
Start column = 623 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 217 - HC MECILL RX
Start column = 624 Number of columns = 1 Type = Alpha

1=NO
2=YES

Variable # 218 - DAYS IN HOSP
Start column = 625 Number of columns = 2 Type = Numeric

Variable # 219 - OUTCOME
Start column = 627 Number of columns = 1 Type = Alpha

1=D/C,IMPROVED
2=DORB,IMPROVED
3=DORB,NO IMPROVE
4=DIED
5=TRANSFERRED

Variable # 220 - HC TEMP MIN
Start column = 628 Number of columns = 4 Type = Numeric

Variable # 221 - CAL INTAK DAY-1
Start column = 632 Number of columns = 4 Type = Numeric

Variable # 222 - CAL INTAK DAY-2
Start column = 636 Number of columns = 4 Type = Numeric

Variable # 223 - CAL INTAK DAY-3
Start column = 640 Number of columns = 4 Type = Numeric

Variable # 224 - CAL INTAK DAY-4
Start column = 644 Number of columns = 4 Type = Numeric

Variable # 225 - CAL INTAK DAY-5
Start column = 648 Number of columns = 4 Type = Numeric

Variable # 226 - CAL INTAK DAY-6
Start column = 652 Number of columns = 4 Type = Numeric

Variable # 227 - CAL INTAK DAY-7
Start column = 656 Number of columns = 4 Type = Numeric

Variable # 228 - MAX TEM DAY-1
Start column = 660 Number of columns = 4 Type = Numeric

Variable # 229 - MAX TEM DAY-2
Start column = 664 Number of columns = 4 Type = Numeric

Variable # 230 - MAX TEM DAY-3
Start column = 668 Number of columns = 4 Type = Numeric

Variable # 231 - MAX TEM DAY-4
Start column = 672 Number of columns = 4 Type = Numeric

Variable # 232 - MAX TEM DAY-5
Start column = 676 Number of columns = 4 Type = Numeric

Variable # 233 - MAX TEM DAY-6
Start column = 680 Number of columns = 4 Type = Numeric

Variable # 234 - MAX TEM DAY-7
Start column = 684 Number of columns = 4 Type = Numeric

ICDDR,B
1988 BUDGET PROPOSAL (IN US $) PAGE 1 OF 22

DIVISION NAME: CLINICAL SCIENCE DIVISION
PROTOCOL/BRANCH NAME: CYTOKINES IN SHIGELLOSIS
NAME OF P. I./BRANCH HEAD/DIVISION HEAD: MICHAEL BENNISH, M.D.
BUDGET CODE: STARTING DATE: BUDGET FOR THREE YEAR PROGRAM
PROTOCOL NUMBER: 88-001 COMPLETION DATE:
DONOR NAME: GRANT AMOUNT:

EXPENSE CATEGORY			Column A	Column B	Column C
A/C Code	Description	Refer to Page No.	Actual Jan.- June '87	Estim. Whole Yr 1987	Proposed 1988
3100	Local Salaries	02			141768
3200	Intl. Salaries	08			0
3300	Consultants	14			0
3500	Travel Local	15			3000
3600	Travel Intl.	16			0
3700	Supplies & Mat.	18			21710
4000	Other Costs	19			1000
4800	Inter Deptl. Ser.	21			36000
	Total Direct Operating Cost		0	0	203478
0300	Capital Expenditure (P.22)				15312
	TOTAL DIRECT COST		0	0	218790

Description	No. of Positions	No. of Man Months	$ Amount
A. Direct Project/Protocol/ Branch Staff at 01.01.1988 (Source: Page 3)	0	0	0
Add:			
B. New Recruitments (Source: Page 4)	7	252	141768
C. Staff allocated from other area (Source: Page 5)	0	0	0
(i) Sub Total	7	252	141768
Less:			
D. Separations (Source: Page 6)	0	0	0
E. Staff allocated to other area (Source: Page 7)	0	0	0
(ii) Sub Total	0	0	0
(i) - (ii) TOTAL	7	252	141768

NEW RECRUITMENT (LOCAL STAFF) - 1988

	A	B	C	D	E	F=(D x E)
Job Title	Level	Start Date	No. of positns	No. of Man Mnth	Rate Per Month	$ Amount
PROJECT CO-HEAD	NO-D	JUL 88	1	36	1251	45036
SR. MED OFFICER	NO-B	JUL 88	1	36	756	27216
SR. RES OFFICER	GS-6	JUL 88	1	36	518	18648
COMMUNITY HEALTH WORK	GS-4	JUL 88	2	72	263	18936
DATA PROCC ASSIST	GS-4	JUL 88	1	36	263	9468
PROJECT MANAGER	NO-A	JUL 88	1	36	624	22464
7.						0
8.						0
9.						0
10.						0
11.						0
12.						0
13.						0
14.						0
15.						0
16.						0
17.						0
18.						0
19.						0
20.						0
21.						0
22.						0
23.						0
24.						0
25.						0
26.						0
27.						0
28.						0
29.						0
TOTAL			7	252		141768

Job Title	Travel From	To-From	No.of Persn	Estim. Days of Travel Per Person	Person Days (A)	PerDiem Rate (B)	PerDiem Amount (C=AXB)	Transport Air (D)	Transport Ground (E)	Other Cost (F)	Total $ Amount G=(C+D+E+F)
COMMUNITY HEALTH WORKERS	TRTMENT CNTR		2	250			0		500		500
2.	TO HOMES						0				0
3.							0				0
PATIENTS	HOME TO			250			0		2500		2500
5.	TREATMENT CENTRE						0				0
6.							0				0
7.							0				0
8.							0				0
9.							0				0
10.							0				0
11.							0				0
12.							0				0
13.							0				0
14.							0				0
15.							0				0
16.							0				0
17.							0				0
18.							0				0
19.							0				0
20.							0				0
21.							0				0
22.							0				0
23.							0				0
24.							0				0
25.							0				0
26.							0				0
27.							0				0
28.							0				0
29.							0				0
TOTAL							0				3000

A/C Code	Item Description	$ Amount
3701	Drugs (used for medicationin the hospitals and field stations)	
3702	Glassware (bottle, beaker, cylinder, petridish, aluminium seal, slides stopper, tube etc.)	500
3703	Hospital Supplies (bandage, gauge blade, bowl, catheter, cotton, needle syringe, solution, leukoplast, towel etc.)	1000
3704	Stationery and Office Supplies (Battery, book register, binders, files, pencil, fastener, paper, ribbon, stapler etc.)	2000
3705	Chemicals and Media (Acid, reagent dextrose, sodium, bactoagar etc.)	1000
3706	Materials for Uniform (Cloth, button etc required for making uniforms)	
3707	Fuel, Oil and Lubricants (Diesel, mobil, petrol, kerosene etc.)	
3708	LaboratoSry Supplies (Aluminium foil, bag blade, brush, cap, container, X-ray etc.)	1000
3709	Housekeeping SUpplies (Aerosol, battery, wiping cloth, duster, lock and key etc.)	
3710	Janitorial Supplies (Bleaching powder, brush, detol, detergent, insecticide, soap etc.)	200
3711	Tools and Spares (Automobile spares, tyres, tubes, battery, stores required for maintenance services etc.)	
3712	Non-stock Supplies (Materials not normally kept in stock and purchased only against specific requisitions)	11000
	Sub Total	16700
3713	Freight and other charges (Add 30% to above sub total)	5010
	TOTAL	21710

A/C Code	Item Description	$ Amount
3800	Repairs and Maintenance (Maintenance and repairs of vehicles, equipments furniture and building)	
3900	Rent, communication and utilities (Postage, telephone, telegram, electricity etc.)	1000
4100	Bank charges	
4200	Legal and Professional Expenses (Professional membership fee, legal fee, audit fee etc.)	
4300	Printing and Publication (Printing of forms, books, journals, reprints etc.)	
4400	Hospitality and Doantion (Guest house accommodation, donations, hospital food, lunch, refreshment etc.)	
4500	Service Charges (porter, labour, washing, laundry and other misc. expenditure)	
4600	Staff Development and Training (Training course fee, training materials, stipend, scholarship, subsistance paid to the staff)	
	TOTAL	1000

INTER-DEPARTMENTAL SERVICES - 1988 PAGE 20 & 21 OF 22

A/C Code	Service Area	$ Amount
4801	Computer	
4802	Transport Dhaka	
4803	Transport Matlab	
4804	Water Transport Matlab	
4805	Transport Teknaf	
4806	Xerox and Mimeograph	1500
4807	Pathology	1500
4808	Microbiology Tests	2500
4809	Biochemistry	7000
4810	X-Ray	1500
4811	I.V. Fluid	
4812	Media	
4813	Patient Hospitalization study	21000
4814	Animal Research	
4815	Medical Illustration	500
4817	Telex	500
4818	Out Patient Care	
4819	Maintenance Charges	
4820	Vehicle Maintenance Charges	
4821	Library Service Charges	
4822	Staff Clinic Charges - Dhaka	
4823	Staff Clinic Charges - Matlab	
4824	Bacteriology Test	
4830	Transport Subsidy	
	TOTAL	36000

Item Description	Manufacturer	No.of Units	Cost+Freight $ Amount
MULTICHANNEL PIPET	TITERTEK	1	544
ELISA READER	MINIREADER II	1	5600
MINUS 80 FREEZER	REVCO 16860	1	6020
VOLTAGE SAFEGUARD	REVCO 13-990	1	570
ICE MAKER FLAKE TYPE	SOCTSMAN AF1AE-1D	1	2578
6.			
7.			
8.			
9.			
10.			
11.			
12.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			
TOTAL			15312