DMONO

Attachment 1.
(FACE SHEET)

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date 28-10-97

Principal Investigator Rubhana Raqib

Application No. 97-021

Title of Study Pathogenesis and immune responses in patients with meningitis or pneumonia due to Haemophilus influenzae.

Trainee Investigator (if any) X

Supporting Agency (if Non-ICDDR,B) USAID

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

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

1. Source of Population:
   (a) Ill subjects (Yes) No
   (b) Non-ill subjects (Yes) No
   (c) Minors or persons under guardianship (Yes) No
2. Does the study involve:
   (a) Physical risks to the subjects (Yes) No
   (b) Social Risks Yes (No)
   (c) Psychological risks to subjects Yes (No)
   (d) Discomfort to subjects (Yes) No
   (e) Invasion of privacy Yes (No)
   (f) Disclosure of information damaging to subject or others Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) (Yes) No
   (b) Use of fetal tissue or abortus Yes (No)
   (c) Use of organs or body fluids (Yes) No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study (Yes) No
   (b) Procedures to be followed including alternatives used (Yes) No
   (c) Physical risks (Yes) No
   (d) Sensitive questions Yes (No)
   (e) Benefits to be derived (Yes) No
   (f) Right to refuse to participate or to withdraw from study (Yes) No
   (g) Confidential handling of data (Yes) No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure (Yes) No
5. Will signed consent form be required:
   (a) From subjects Yes (No)
   (b) From parent or guardian (if subjects are minors) (Yes) No
6. Will precautions be taken to protect anonymity of subjects (Yes) No
7. Check documents being submitted herewith to Committee:

   No Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   Yes Protocol (Required)
   Yes Abstract Summary (Required)
   Yes Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   No Informed consent form for subjects
   Yes Informed consent form for parent or guardian
   Yes Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

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

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

Rubhana
Principal Investigator

Trainee

TITLE OF THE PROJECT:	Pathogenesis and immune responses in patients with meningitis or pneumonia due to *Haemophilus Influenzae*.
PRINCIPAL INVESTIGATOR:	Rubhana Raqib, Ph. D., Laboratory Science Division (LSD), International Centre for Diarrhoeal Diseases Research, Bangladesh (ICDDR,B).
CO-INVESTIGATORS:	Samir K. Saha, Ph. D., Ruhul Amin, MBBS, FCPS; Dhaka Shishu (Children's) Hospital. Dilara Islam, Ph. D., M. John Albert, Ph. D., LSD; M. A. Salam, MBBS, Clinical Science Division, ICDDR,B.
LOCATION OF THE PROJECT:	LSD, ICDDR,B and Dhaka Shishu Hospital (DSH)
BUDGET:	US$ 206,542 (Direct Cost)
STARTING DATE:	When funds are available
DURATION:	Three years
SOURCE OF FUNDING:	USAID, Washington
APPROVAL OF THE DIVISION DIRECTOR, LSD:	.....

ABSTRACT

Acute lower respiratory tract infections (ALRI) in developing countries are the leading cause of childhood morbidity and mortality. *Haemophilus influenzae* causes a substantial proportion of severe ALRI (9) and is also the most common cause of bacterial meningitis. In developing countries, the case fatality rate for this disease is as high as 40% (6). In rural areas of Bangladesh, a child <5 years of age experiences 2 to 3 episodes of ARI each year (22). More than 95% of serious *H. influenzae* infections in childhood are caused by serotype b (Hib) organisms. Much needs to be learned about the microbial and host factors that determine successful colonization of respiratory tract by *H. influenzae* and the development of disease. Carriage of *H. influenzae* is also common among healthy individuals.

The aim of the protocol is to study the humoral and cellular immune responses in meningitis and pneumonia caused by *H. influenzae* in children and to investigate the role of immunoregulatory molecules in the pathogenesis of the disease. A group of children with *H. influenzae* type b infections with menigitis and pneumonia (age range 6m-5 yr; n=60) will be recruited as cases. Age, gender and socioeconomic status matched patients with *Streptococcus pneumoniae* and *H. influenzae* (n=60) will be recruited as controls (C-1). A representative population of healthy children living in the vicinity of the study hospital (within a radius of 5 miles) will be recruited as healthy controls (C-2). Blood, cerebrospinal fluid (CSF, where appropiate), and urine will be collected from patients on admission, and 3, 7, 15 and 30 days later. Plasma and urine will be collected from the healthy children once. Analysis of plasma will show the kinetics of humoral immune responses including *H. influenzae* type b specific antibody titers, cytokine profile and specific receptors during the course of the disease. *In vitro* analysis of peripheral blood mononuclear cells for cytokine production at the single cell level after stimulation with specific antigens (oligopolysaccharides) will be carried out to determine the effect of *in vitro* activation on the responding cells. The initial cytokine response is likely to play a critical role in determining the outcome of the interaction and the overall regulation of the ensuing, acquired response. Analysis of inflammatory mediators such as concentrations of C-reactive protein (CRP), complement (C3, C5a), endotoxin and proinflammatory cytokines in plasma and nitrate in urine may provide information on the clinical severity and may serve as predicators of pathophysiological events. Characterization of phenotypes of the mononuclear cells, cytokine profile and immunoglobulin levels may provide information on any defect in the T cell mediated immunity, differences in cytokine production known to influence B cell differentiation, as well as in the development of immune memory.

HYPOTHESES:

a) Children with ALRI and complications such as pneumonia or meningitis are likely to have impaired immune responses when compared with children with ALRI without pneumonia and meningitis, in terms of antigen-specific antibody response, cytokines and respective receptors and phenotypic characteristics of the peripheral mononuclear cells.

b) Exacerbation of host responses such as pro-inflammatory cytokines, complement, CRP and nitrate leads to complications in *H. influenzae* type b infection.

GENERAL AIM

To study the humoral and cellular immune responses in meningitis and pneumonia caused by *H. influenzae* in children and to investigate the role of immunoregulatory and proinflammatory molecules in the pathogenesis of the disease.

SPECIFIC AIMS

In order to identify risk factors involved in pneumonia or meningitis due to *H. influenzae* type b infection, characteristic immunological features of children with pneumonia or meningitis due to *H. influenzae* type b infection will be compared with those of children with ALRI due to *S. pneumoniae* and *H. influenzae* by:

1) elucidating the cytokine profile (IL-1β, IL-6, TNF-α, IL-4, IL-10 and IFN-γ) and the respective cytokine specific receptors (sIL-1ra, sIL-1R type I and II, sIL-6R, sTNFR typeI and II) in plasma and CSF.
2) studying cytokine production at the single cell level in peripheral blood mononuclear cells after stimulation with purified antigens (capsular polysaccharide and oligopolysaccharide).
3) determining the concentrations of inflammatory mediators or modulators such as complement (C5a, C3), CRP and endotoxin in plasma and CSF and nitrate in urine and correlate with clinical severity of the disease.
4) studying antigen (oligopolysaccharide)-specific antibody responses in plasma.
5) characterizing the phenotypes of peripheral blood mononuclear cells.

BACKGROUND

Acute lower respiratory tract infections (ALRI) in developing countries are the leading cause of childhood morbidity and mortality and *H. influenzae* causes a substantial proportion of severe ALRI (9). It is the most common cause of bacterial meningitis in many developing and developed countries. In developing countries, the case fatality rate for this disease is as high as 40% (6). In rural areas of Bangladesh, a child <5 years of age experiences 2 to 3 episodes of ALRI each year (22). *H. influenzae* is a constituent of normal respiratory flora in 60-90% of healthy children (18). 90% of all infections occur in children <5 yr of age. More than 95% of seroius *H. influenzae* infections in childhood

are caused by serotype b (Hib) organisms. The type b capsular polysaccharide is a major virulence determinant of Hib bacteria and anticapsular antibodies have been shown to prevent invasive Hib disease (4, 7). Other surface exposed antigens, e.g. some of the major outer membrane proteins (OMPs) and lipopolysaccharides (LPS) have also been associated with virulence of Hib and are important epidemiologic markers (11, 32).

H. influenzae infections are usually catagorized into invasive and noninvasive diseases. In invasive *H. influenzae* disease, bacteria which normally colonize the mucosal surface of the upper respiratory tract, invade the mucosal barrier, causing bacteremia. After reaching a significant concentration in the blood stream, *H. influenzae* colonizes various body sites such as meninges and choroid plexus, joints and soft tissues (9). Disease syndromes that result after the invasion are meningitis, septic arthritis, endophthalmitis, cellulitis, epiglotitis and sepsis. The non-invasive infections of the mucosal surfaces, such as otitis media, conjunctivtis, sinusitis and bronchitis are less severe and are more common. The organisms extend from their normel site of colonization to the eustachian tubes, through ostia or the bronchi. The strains of *H. influenzae* that colonize the upper airway are predominantly non-capsulated or non-type b and these diseases are not likely to be prevented by existing type b polysaccharide conjugate vaccines.

Much needs to be learned about the microbial and host factors that determine successful colonization of respiratory tract by *H. influenzae*. Since carriage of *H. influenzae* is common among healthy individuals, the carriage state may be considered as a dynamic co-existence of the microbe and the host. Disease devlopment on the other hand, is intimately associated with subtle changes in the host immune system as well as of microbial factors that permit its survival and propagation. Events that result in entry into the intravascular compartment by serotype b organisms are poorly understood (16). Type b strains resist intravascular clearence mechanisms more readily than do strains of other serotypes. Whether it is the type b capsule itself that confers the potential for invasive disease or there are other virulence factors are not clear. LPS from Hib is capable of activating alternate pathway of complements and eliciting antibody responses (33). LPS concentration in CSF of patients with Hib meningitis was found to correlate with clinical severity and neurological outcome (17). This antigen can induce meningeal inflammation in rabbits whereas the capsular polysaccharide cannot (31). However, LPS does not act alone during meningeal inflammation. Once the bacteria enter the subarachnoidal space, leukocytes, endothelial cells and other cells in the central nervous system are stimulated to produce proinflammatory cytokines and prostaglandins leading to acute inflammatory responses (10).

The susceptibility to invasive Hib disease was shown to be related to a lack of bactericidal power in serum. Several elements of host defense together reflect the bactericidal power of blood. One important component is antibody directed against the

type b capsular polysaccharide which is acquired in an age-related fashion (5). The mechanism of action of anti-capsular polysaccharide antibody is related in part to its opsonic activity. Both the classic and alternative complement pathways are important in opsonization of Hib (20, 33). The complement system plays a critical role in the host defense against *H. influenzae*. C3 has been found to be a critical opsonin for *H. influenzae*. Defects in complement mediated host defense may be common in systemic Hib infections (33). The macrophages of the reticuloendothelial system may help in intravascular clearance of *H. influenzae* by affecting intracellular killing after opsonization. Resistence of the organism to killing may be due to a defect in T cell dependent bactericidal activity of macrophages. Host factors such as cytokines (19, 23), platelet-activating factor (PAF) (3) and complements (14) have been shown to mediate inflammatory cascade in experimental Hib meningitis. Severity of bacterial meningitis has been correlated to increased concentration of proinflammatory cytokines and PAF. Increased production of nitric oxide in Hib meningitis in children may contribute to anaerobic glycolysis and neurologic damage (15). Serum CRP levels were shown to be good predicators of neurologic complications in meningitis due to Hib (2). The knowledge of the role of various proinflammatory and antiinflammatory mediators in the pathophysiology of bacterial meningitis and pneumonia may be important for understanding the mode of intervention with adjunctive therapy. A recent study on cell-mediated immune status of children with recurrent infection of the upper respiratory tract showed no significant difference in the phenotypes of mononuclear cells between patients and healthy children (12). The investigators, however, did not study the changes in the phenotypes of the cells during the course of the disease in repeated samples which might have revealed priming and development of immune memory.

Identification of host molecules/cells induced in response to natural infection, and *in vitro* antigen stimulation are important in our understanding of immunity, pathophysiology and vaccine development against infections with *H. influenzae*. The limited numbers of studies carried out on the immunological and immunopathological aspects of the diseases caused by *H. influenzae* have been in animal models. The magnitude of ARI caused by *H. influenzae* contributes to millions of death world wide each year and its incidence has dramatically increased in Bangladesh (27), overriding infections by *S. pneumoniae*. This emphasizes the need for better understanding of the epidemiology, pathogenesis and most importantly on immune mechanisms. We plan to undertake this study to obtain more insight into the pathophysiology of this infection and to determine if the outcome is influenced by the modulation of host responses.

STUDY POPULATION

Selection of cases and inclusion criteria.

Children eligible for the study will be those within 6 months to 5 years of age who attend

the Dhaka Shishu Hospital (DSH) with features of acute lower respiratory tract infections such as cough, fever, increased respiratory rate ≥ 50 / min for infants 2-12 months and > 40 / min for 1-5 years, chest retractions, wheezing, rales, stridor and cyanosis (lower respiratory tract infection is defined by a modification of the World Health Organization guidelines (1, 30) (Appendix I).

Pneumonia will be defined as:

Severe pneumonia	chest indrawing, wheezing
Pneumonia	no chest indrawing, fast breathing (≥ 50 / min for infants 2-12 months and > 40 / min for 1-5 years)
No pneumonia	cough and cold, breathing (< 50 / min for infants 2-12 months and < 40 / min for 1-5 years)

Bacterial meningitis will be diagnosed if clinically significant bacteria are isolated from cerebrospinal fluid or the fluid containing ≥ 10 white cells / mm^3, with either a predominance of polymorphonuclear cells or a glucose concentration of < 30 % (8).

After diagnosis, children with ALRI due to *H. influenzae* type b with meningitis and pneumonia will be the recruited as cases.

Exclusion criteria: Children with clinical evidence of epiglottitis, and children with ARI with concommitant diarrhea or other diseases will be excluded. Pediatric patients with upper respiratory tract infection, defined as the presence of any two symptoms (cough, sore throat, ear ache with or without discharge, or fever in absence of signs of lower respiratory tract infections as indicated above) will be excluded.

Selection of controls:

Children with ALRI due to *H. influenzae* type b and *Streptococcus pneumoniae* without meningitis and pneumonia will be enrolled as controls (C-1). A representative population of healthy children living in the vicinity of the study hospital (within a radius of 5 miles) will be recruited as healthy controls (C-2) to study baseline levels of various immunologic determinants. Children eligible for the study will have no history of illness or fever within the past 3 months. The study population will be matched for age, gender and socioeconomic factor.

A standardized medical history and physical examination will be recorded by the study physician for each subject.

SAMPLE SELECTION AND STUDY MATERIALS

Study children will be selected by the use of consecutive sampling technique to avoid seasonal variation of the disease. Based on logs for each case one control immediately following the recruitment of the case will be selected. Samples of venous blood (5.0 ml) and urine (5.0 ml) will be collected from study children on the day of admission and on

days 3, 7, 15 and 30 after admission. Please see the scheme for sample collection. CSF from patients with meningitis, obtained for routine test (culture, microscopy and protein and sugar concentrations) will be saved from the microbiology lab on the day of admission and 3 days after admission. No additional sampling will be carried out for the study purpose. Since CSF samples can not be obtained from healthy individuals, samples which do not show any abnormality i.e. show normal leukocyte count, protein and sugar concentration will be considered as control samples.

METHODOLOGY

I. Isolation of causative agent

Isolation of causative agents from blood, CSF and throat swab will be carried out by conventional methods. CSF specimens will be cultured on Chocolate and blood agar and incubated at 37°C for 48 h. Chocolate agar plates will be incubated in the candle jar. Isolates will be identified by the standard procedures. Serotyping of *H. influenzae* will be accomplished by slide agglutination with type-specific antisera (Muerex, UK). Latex particle agglutination assay (Muerex) kit will be used to detect serotype b capsular polysaccharide in serum, CSF and urine. The lysis direct plating/centrifugation technique (28) will also be used for isolation the causative agents.

Lysis direct plating/centrifugation: Blood cultures will be made using tubes containing a filter-sterilized solution of 0.8 mg of sodium polyanethol sulphonate (SPS) and 2 mg of saponin (Merck) in 2.0 ml of distilled water. Air will be drawn out using a suction pump. Two ml of patient's blood will be drawn in the tube and mixed with the reagents by gentle rotation of 2-3 times. Equal volumes of lysed blood will be inoculated directly on to blood agar and MacConkey agar plates within 20-30 min of collection with a loop. The blood agar plates will be incubated at 37°C in a candle extinction jar and the MacConkey agar plates will be incubated aerobically.

In the event of antibiotic therapy prior to study enrollment, the tubes with blood will be centrifuged (6000g, 30 min) and the supernatant will be removed and the precipitate will be cultured as above. The plates will be examined at 24h and 48 h and isolated organisms will be identified following standard procedures.

II. Sampling of blood, CSF and urine and preparation of samples

Peripheral blood will be obtained at venipuncture from median cubital vein. Whole blood will be used for isolation of causative agents. Peripheral blood mononuclear cells will be separated upon Ficoll/Hypaque centrifugation, will be counted and kept at numbers of 1-5x10^6 cell/ml in RPMI with 20% fetal calf serum and 10% dimethyl sulfoxide in liquid nitrogen (-196°C) until used. CSF will be obtained at lumbar puncture, centrifuged and

stored in -70°C until tested. Urine collected from patients will be centrifuged and supernatant will be collected and stored in -20° C.

III. Routine laboratory examinations

In blood, total and differential count, ESR, prealbumin and glucose will be determined as a routine for each patient. Tests for blood gas and oxygen saturation will be performed when necessary. CSF will be used for culture and tested for protein, glucose and cytology from meningitis cases only. These routine tests will be performed in DSH. At the Clinical Laboratory of ICDDR,B, CRP, total IgA, IgG and IgM content in blood will be determined for the study purpose. Glucose content will also be determined in urine. These tests will help in the clinical evaluation of each subject and determining the disease activity in patients.

IV. Quantification of secreted cytokines and specific soluble receptors in plasma and CSF.

Secreted cytokines and soluble cytokine receptors will be measured in plasma and CSF using commercial quantitative sandwich enzyme immunoassays for IL-1β, IL-6, IL-8, TNF-α, IL-10 and IFN-γ (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions (26). A microtiter plate precoated with a monoclonal antibody specific for the cytokine, cytokine standard, conjugate, substrate and assay diluent are provided in the kit. In brief, assay diluent will be added to each well, and 100 µl of standard or samples will be added per well. The plate will be covered with adhesive strip and incubated for 2-3 hours at room temperature. After washing with wash buffer and the conjugate will be added and the plate will be incubated for another 1-2 hours at room temperature. Following 3 washes, substrate will be added to each well and incubated for 20-30 minutes at room temperature. After incubation, stop solution will be added to each well and the optical density will be measured within 30 minutes, using a spectophotometer set at the desired wavelength. A standard graph is plotted and the concentrations of the unknown samples is determined from the graph. These assays identify both receptor-bound and unbound proteins. The principle of cytokine receptor ELISA (sIL-1ra, sIL-1R type I and II, sIL-6R, sTNFR type I and II) is the same as that of cytokines. Determination of secreted cytokines and soluble receptors will reveal the effects of *in vivo* activation of cells and the nature of modulation of the immune system during the natural course of ALRI.

V. Stimulation studies and determination of cytokine producing cells at the single cell level

The mononuclear cells (MNC) will be obtained from patients on days 0, 7 and 30 after admission and once from healthy controls. MNC will be washed and suspended in RPMI

1640 medium containing glutamine and supplemented with sodium pyruvate, 100 U/ml penicillin G, 100 μg/ml streptomycin and 10% fetal calf serum. The polymyxin B absorbed fetal calf serum will be used for the study. MNC will be aliquoted into 50-ml Falcon polypropylene conical tubes at $2x10^6$ cells/ml and will be cultured for 2, 4, 6, 12, 24, 48 and 72 hr with stimulation with purified capsular polysaccharide and oligopolysaccharide from Hib (American Type Culture Collection, Rockville, MD) (concentration to be standardized) or without any stimulation at 37°C in humidified atmosphere containing 7% CO_2. At each time interval, supernatant will be collected to determine cytokine concentration by ELISA. Cells from culture will be harvested after indicated time intervals, washed in fresh RPMI and used for immunostaining (24, 25).

For immunocytochemistry, MNC ($1x10^4$ cells per well) will be allowed to adhere electrostatically on Bio-Rad adhesion slides (Bio-Rad Laboratories, Hercules, CA) fixed in 4% paraformaldehyde (Sigma, St. Louis, Mo) and washed in RPMI 1640. Staining for cytokines by stimulated MNC will be performed as published previously (24, 25). In brief, endogenous peroxidase activity will be quenched by incubation in freshly prepared 1% H_2O_2 in balanced salt solution (BSS) for 15 min. After a brief rinse in BSS and saponin-BSS sequentially, the slides will be incubated for 2 hr in 37° C or overnight at room temperature with specific monoclonal antibodies at a concentration of 4 μg/ml. Sections will be incubated with normal goat serum to prevent non-specific binding followed by incubation with biotinylated goat anti-mouse immunoglobulin G and/or biotinylated goat anti rat antibody to be used as secondary antibodies for 30 min in room temperature. Incubation with avidin-biotin-horseradis peroxidase complex at 37° C for 30 min will be followed by rinsing in saponin-BSS and development with substrate chromagen. Cells will be counter stained in Mayer's hematoxylin and mounted in glycerin buffer. Cytokine producing cells will show accumulation of cytokine in the Golgi organelle. The cytokine-specific antibodies to be used are: IL-1β, IL-6, TNF-α, IL-4, IL-10 and IFN-γ (R&D Systems, Minneapolis, MN; Genzyme, Cambridge, MA; Pharmingen, San Diego, CA).

VI. Measurement of inflammatory mediators

Human complement C3, C5a: Plasma will be used for quantification of complements C3 and C5a by radioimmunoassays (Amersham Life Sciences, UK) using radioactive iodine [^{125}I] in conventional competitive assay formats.

CRP: CRP will be measured in plasma and CSF by immunoturbidometric method using monospecific antisera against CRP (21).

Endotoxin: The endotoxin content of plasma and CSF will be measured by Chromogenic Limulus Amoebocyte Lysate (LAL) assay (Whittaker Bioproducts Inc., Walkersville,

MD, USA). LPS from *Escherichia coli* will be used as standard (American Type Culture Collection, Rockville, MD).

VII. Measurement of antigen specific antibodies in serum: The WHO/CDC guidelines for standardization of ELISAs for Hib antibodies and the ELISA protocol of Dr. George Siber will be followed (29). Purified type specific OPS antigens will be obtained from either Denka, Tokyo, Japan or American Type Culture Collection, Rockvie, MD and used for ELISA. Standard pooled immune sera or reference standard will be obtained from Dr. Carl Frasch (Cheif, Laboratory of Bacterial Polysaccharides, HFM-428, Center for Biologics Evaluation and Research, 1401 Rockville Pike, Rockville, MD). Briefly, ELISA plates (Nunc MaxiSiorp plates, Nunc, Roskilde, Denmark) will be coated with oligopolysaccharide of *H. influenzae* type b (1 µg/ml, 100 µl/ well) with antigens in phospahte buffer saline (PBS) for 2 hours at 37°C. Diluted standard sample (1:100) and serum samples (1:1000) from patients and controls will be added to the plates. Serial dilutions of the standard will be done in the plate and incubated for 2 hours at 22°C. After washing with PBS-Tween, the conjugate anti-IgG (alkaline phosphatase) at a dilution of 1:1000, 100 µl/well is added. After incubation for 2 hours at room temperature the plates are washed again and substrate p-nitrophenyl phosphate (Sigma) will be added (1 mg/ml, 100 µl/well). The optical density (OD) will be measured after 30 minutes at 405 nm. The OD obtained with PBS-Tween will be subtracted from the OD of the test samples to avoid background level. Results will be expressed as relative titres which is defined as OD multiplied by the dilution factor of the test samples.

VIII. Immunophenotyping of peripheral blood mononuclear cells:
Characterization of phenotypes of cells in peripheral circulation (CD3, CD4, CD8, CD25, CD20, CD3, CD16, CD56, CD57, CD29, CD54, HLA-DR, CD45RA, CD45RO, CD58, CD11a, CD18, CD49c, CD49d, CD103, CD62L) will be carried out by flow cytometry (12, 13). Characterization of phenotypes of the mononuclear cells in repeated samples may provide information on priming, existence and development of immune memory during the course of pneumonia and meningitis due to *H. influenzae*. Specific monoclonal antibodies will be added to the peripheral blood mononuclear cells (MNC) in PBS with 2% heat inactivated pooled human AB serum and incubated at 4°C for 30 min. MNC will be washed and fixed in PBS coantaining 1% paraformaldehyde. Negative controls will also be included for staining. The flourescene analysis will be carried out with a FACSort flow cytometer (Beckton Dickenson, San Jose, California) in Huddinge Hospital, Stockholm, Sweden. The data will be analyzed with the Paint-A-Gate+ software (Becton Dickinson) using arbitrary linear units for flourescence intensity. Multiple samples from individual subjects will be run at a time.

SAMPLE SIZE CALCULATION AND DATA ANALYSIS

For this study five parameters will be studied to ascertain their association with the outcome variable. However, the exposure rate of such individual variable may vary and by large is unknown. Therefore, a conservative estimate of 50% would be assumed for each individual variable. Moreover, the observed odds ratio is assumed as smallest as possible with a view to get the maximum sample size that can detect significant association or is able to answer the research question.

Assumptions are:

Alpha	= 0.05 when two tailed	= 1.96
Beta	= 0.20 with power 80%	= 1.28
Exposure Rate	= 50%	= 0.50
Odds Ratio	= 2.0	= 2.0

$$n = 2\overline{P}\overline{Q}\left(Z_\alpha + Z_\beta\right)^2 / \left(P_1 - P_2\right)^2$$

where $p_1 = p_0 R / [1 + p_0(R-1)]$ and $\overline{p} = \frac{1}{2}(p_1 + p_0)$, $\overline{q} = 1 - \overline{p}$

and $q_1 = 1 - p_1$, $q_0 = 1 - p_0$

Thus, the calculated sample size is 60 in each group i.e. 60 cases, 60 as controls 1 (C-1) and 60 as controls 2 (C-2).

DATA ANALYSIS

All data will be validated by a series of logical and range checks. Finally a statistical package will be used for data processing and analysis. Data will be analyzed following the procedure for matched case-control design. Initially a descriptive analysis will be helpful. For that, data will be tabulated into 2x2 tables. Crude odds ratio will be computed initially for the association of each factor of interest with outcome variable. The final step of the analysis includes univariate and multivariate procedure where applicable. Repeated measure analysis of variance methods may be applied (multivariate procedure).

WORK PLAN FOR 3 YEARS

i) Procurement of supplies and standardization of methods - 4 months

ii) Recruitment of patients and controls in the study,
iii) Processing and storage of samples, iv) For some
assays, multiple samples from a single patient required

to be studied at a time will be analyzed after completion of sample collection	-	14 months
v) Analysis of specimens, completion of assays	-	10 months
vi) Compilation and analysis of data and reporting the outcome of the study in several articles.	-	8 months
Total time	-	36 months (3 yrs)

TASK OF INVESTIGATORS AND OTHER MEMBERS

Most of the work can be carried out in ICDDR,B, Dhaka, however, due to unavailability of a major equipment (flow cytometer) at ICDDR,B, phenotypic characterization (VIII, pg 10) needs to be carried out in Huddinge Hospital, Sweden.

Rubhana Raqib	Coordinate specimen collection from Dhaka Shishu Hospital (DSH), standardization of techniques, supervise work in the lab (I II, IV, V, VI, VII, pg 7-10), complilation of data and analysis, writing up for publications.
Dilara Islam	Supervise work in the lab (I, VII, VIII pg 7-8, 10) and contribute to analysis and interpretation of results.
M. A. Salam	Contribute to the analysis and interpretation of results.
Samir K. Saha	Provide clinical data of patients at Shishu Hospital and contribute to the analysis and interpretation of results.
Ruhul Amin	Recruitment, clinical management and treatment of the patients at Shishu Hospital and will provide clinical data of patients.
M. John Albert	Co-ordination, scientific and academic feedback
Research Assistant (1)	Patient and control enrollment, clinical management and follow-up at Shishu Hospital
Research Officer (1)	Carry out tests specified for the proposal involving both microbiological and immunological techniques at ICDDR,B

Lab attendent (1)	Specimen collection from DSH and processing, and other work in the lab at ICDDR,B

SIGNIFICANCE OF EXPECTED FINDINGS

Acute respiratory tract infections pose a major public health problem in Bangladesh. Several promising *H. influenzae* type b vaccines are now available with the potentials to significantly reduce the disease burden in infants (<1 yr of age) who are at greatest risk. Protein conjugate vaccines have only recently become widely used in the United States, and evaluation of its immunogenicity, safety and effectiveness have been done in some developed countries. However, the spectrum and incidence of invasive *H. influenzae* disease in developing countries has not been well characterized. Thus, the potential benefit of the new *H. influenzae* type b vaccines in such settings needs to be defined. In some population in the United States (Alaskan Natives and Apache Indians), the efficacy of the Hib conjugate vaccine was shown to be poor. Moreover, recently, the Food and Drug Administration Advisory Committee declared that the Pastuer Merieux Connaught Vaccine "TriHIBit" (Combination DPT / Hib) tested in 4300 infants failed to protect against Hib (Medical News Week 6-16-97; Lancet, 1997;349(9067):1). It is unethical to introduce a vaccine without having the knowledge of the baseline immune response in a population. When epidemiological and other questions have been satisfactorily investigated and if Hib vaccines have been shown to be effective, it will then be a strong case for introducing and combining the Hib vaccine in the EPI programmme in Bangladesh. Issues such as procurement costs and cost benefit, vaccine stability in the tropical climate needs to be addressed. It will take considerable time before these new vaccines can be applied as part of the EPI. Meanwhile, for better management, intervention and therapy, studies are required to understand the mechanisms of acquisition of immunity to Hib infection, the determinants of natural immunity and host determinants of immunologic responses.

We have access to patients with ARI both at ICDDR,B and at government hospitals, such as Shishu Hospital and the technical expertise. The study may help in better understanding of the mechanisms involved in host protective immunity, in pathogenesis of pneumonia and meningitis caused by Hib, improved therapy and management, as well as determining their correlation with safety and immunogenicity in the development of a protective vaccine. We expect to address and answer several important questions in this proposal: (i) the nature of the acquired systemic (both humoral and cellular) immune responses of the general peadiatric population naturally infected with *H. influenzae*. (ii) the initial cytokine response in patients with natural infection which is likely to play a role in determining the outcome of the disease. The cytokine and specific soluble receptor response can influence the overall regulation of the ensuing, acquired response. (iii) studies to characterize the immunomodulatory as well as

inflammatory mediators may provide important information for the general understanding of the early responses of the host to infection with *H. influenzae*. (iv) identifying the direct effects of bacterial products on host cells from the indirect effects of host-derived inflammatory modulators such as complement and cytokines.

Laboratory methods required to measure immune responses to natural infection would be used in future research studies. The study will help facilitate introduction of an effective *H. influenzae* vaccine into countries where the problems of mortality due to meningitis and pneumonia are very high and such a vaccine may play a significant role in reducing childhood deaths through preventing them.

DISSEMINATION OF RESEARCH FINDINGS

Research findings will be published in international journals to make them available to all researchers in the relevant feilds and will also be presented in international scientific forum.

ETHICAL CONSIDERATION

The proposed study involves management of pediatric patients of age group 6m-5yr with ARI and sampling of blood, CSF and urine. Permission will be obtained for the proposed study from Shishu Hospital and the Ethical Review Committee (ERC) of ICDDR,B. In accordance with the guidelines of the ERC, signed informed consents of the parents / or guardians will be obtained. Patients will receive clinical care and therapy free of charge. At the wish of the guardians, patients may discontinue their participation in the study at any time point. This decision would not have any influence on the clinical management or therapy of the patients. Each patient admitted in the hospital will be under close observation of the clinician for at least 5 days and will receive the standard treatment of this hospital. Compensation will be provided to the guardians for wage loss and travel costs for follow up appointments.

Patients will be recruited from the inpatient ward of DSH. Repeated sampling of blood, CSF (meningitis cases only) and urine will be performed from each patient (see scheme for sample collection) when the diagnosis is confirmed. Approximately 5 ml of venous blood (from median cubital vein) will be taken from children (5 times). CSF will be obtained upon lumbar puncture when done routinely for critical cases (twice). The CSF samples will be obtained passively from the microbiology lab and will not be done specifically for the study purposes. All sampling procedures will be done by well-trained experts in the procedures concerned. Sampling of blood (pneumonia and meningitis) and CSF (meningitis cases only) will be required for routine examination (culture, cell count, protein and glucose) once as part of the diagnostic program at admittance before antibiotic treatment and 4 additional sampling will have to be carried out for the study. Although

there will be slight inconvenience and local pain involved in sampling, no serious side effects associated with sampling are anticipated.

Anonymity of patients will be maintained and all information/data of this study will be kept confidential and will be provided to the guardian of patient upon request.

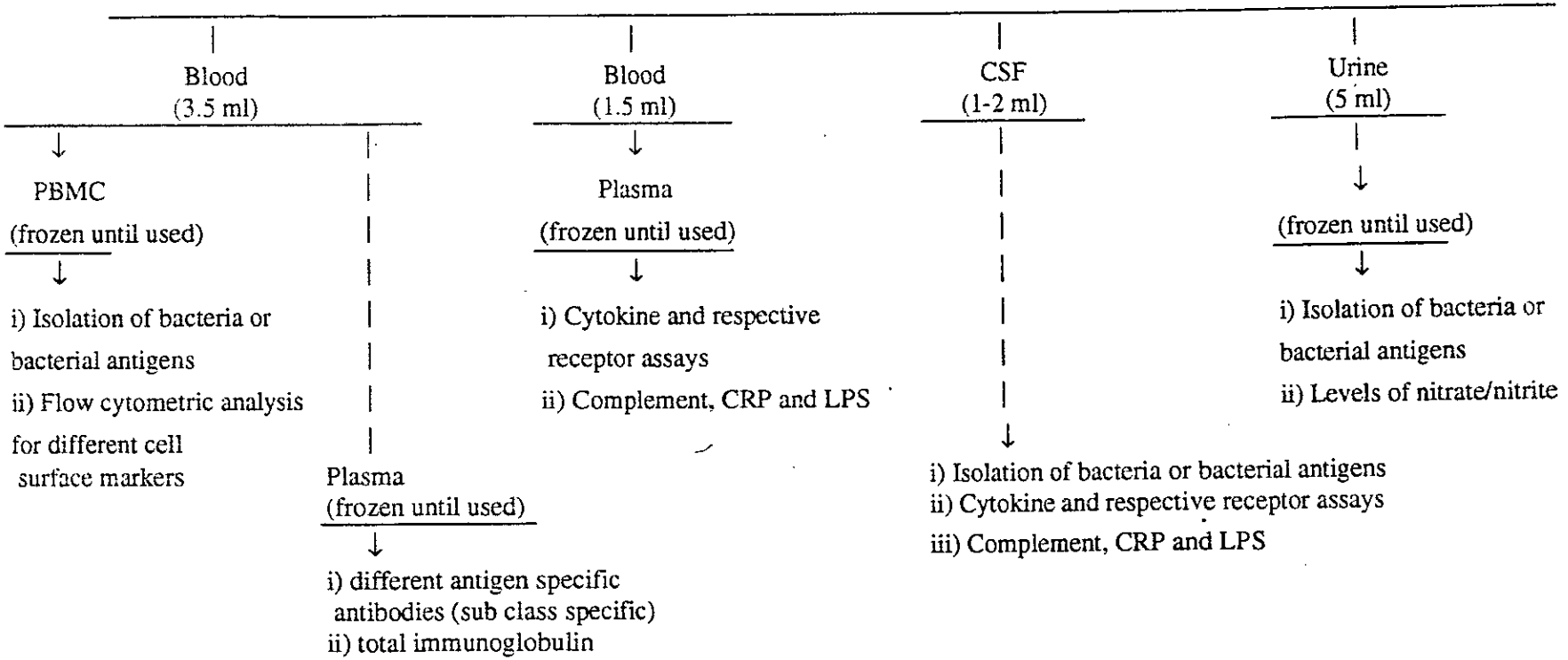
SCHEME FOR SAMPLE COLLECTION FROM PATIENTS AND HEALTHY CONTROLS

	Patients with meningitis or pneumonia due to *H. influenzae**			Controls (C-1)**		Healthy controls (C-2)¶	
Days from enrolment	Blood	CSF	Urine	Blood	Urine	Blood	Urine
D-0	+	+	+	+	+	+	+
D-3	+	+	+	+	+	-	-
D-7	+	-	+	+	+	-	-
D-15	+	-	+	+	+	-	-
D-30	+	-	+	+	+	-	-

*Patients with meningitis or pneumonia due to *Haemophilus influenzae* (n=60) infections will be recruited from Dhaka Shishu Hospital (DSH). **Controls (C-1) represent patients with *Streptococcus pneumonia* and *Haemophilus influenzae* infections without meningitis and pneumonia (n=60) at DSH. ¶ Healthy controls (C-2) (n=60) represent age, gender and socioeconomic factor matched healthy children from the vicinity of DSH (within a radius of 5 miles).

Flow Chart: Sample collection and sequence of work

Samples (Venous blood from median cubital vein, CSF* and urine) will be collected from children (6m-5yrs) with meningitis and pneumonia due to H. influenza type b (n=60) as the cases, children with H. influenzae and S. pneumonia without pneumonia and meningitis as controls (C-1), healthy controls as controls** (C-2).



*CSF will be obtained from patients with meningitis for routine analysis. CSF saved after the routine analysis will be used for the study purpose. **Age, gender and socioeconomic status matched healthy controls will be recruited from the vicinity of Dhaka Shishu hospital. Blood and urine will be collected from healthy controls once.

BUDGET FOR THREE YEARS (1998-2000):

		Amount in US$			
		Yearly budget			
		Ist year	2nd year	3rd year	TOTAL
PERSONNEL:	% of time				
Rubbana Raqib (PI)	50	5,800	6,090	6,395	18,285
Dilara Islam (Co-invest)	5	580	609	639	1,828
M. A. Salam (Co-invest)	5	955	1,003	1,053	3,011
John Albert (Co-invest)	5	6,135	6,442	6,764	19,341
Research Officer	100	4,500	4,725	4,961	14,186
Lab attendant	100	1,900	1,995	2,095	5,990
Study Physician (DSH)	100	2,500	2,625	2,756	7,881
Research Assistant (DSH)	100	2,100	2,205	2,315	6,620
TOTAL PERSONNEL COST		**24,470**	**25,694**	**26,978**	**77,142**
PATIENT COSTS:					
Hospitalisation cost ($30/day x 7 days per patient)		12,000	14,000	10,000	36,000
Wage loss for follow-up ($10/visit x 2 visit)		1,000	1,600	1,000	3,600
Medicines		500	800	100	1,400
TOTAL PATIENT COST		**13,500**	**16,400**	**11,100**	**41,000**
LABORATORY INVESTIGATIONS:					
Routine laboratory tests-					
(TC, DC, blood culture, CRP, Immunoglobulin types, sub-types,pre-albumin; Urine R/E, C/S)		3,000	2,500	1,000	6,500
Immunological assays-					
Cytokine and receptor assay kits		15,000	12,000	3,000	30,000
Cytokine antibodies, nitrate, complement assay kit		4,000	5,000	1,000	10,000
Antigen specific antibodies for ELISA		1,000	500	500	2,000
Immunophenotyping of PBL		0	8,000	0	8,000
Laboratory supplies:					
Plastic ware, glassware and office supplies		1,000	1,000	800	2,800
Chemicals and media		2,000	2,000	500	4,500
TOTAL LABORATORY COST		**26,000**	**31,000**	**6,800**	**63,800**
OTHER SERVICES:					
Printing and publication		0	2,000	3,000	5,000
Library charges and communication charges		200	300	200	700
Maintenance charge		100	200	300	600
SUB-TOTAL		**300**	**2,500**	**3,500**	**6,300**
CAPITAL EXPENDITURE:					
Equipment (Liquid Nitrogen tank)		10,000	0	0	10,000
Computer		2,000	0	0	2,000
SUB-TOTAL		**12,000**	0	0	**12,000**
TRAVEL:					
Local travel, folow-up, DSH		500	500	300	1,300
Presentation at International conference			5,000	0	5,000
TOTAL TRAVEL COST		**500**	**5,500**	**300**	**6,300**
GRAND TOTAL		**76,770**	**81,094**	**48,678**	**206,542**

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DHAKA SHISHU HOSPITAL

CONSENT FORM FOR PATIENTS

"PATHOGENESIS AND IMMUNE RESPONSES IN PATIENTS WITH MENINGITIS OR PNEUMONIA DUE TO *HAEMOPHILUS INFLUENZAE*"

Your child has acute lower respiratory tract infection which may be caused by pathogens such as *Haemophilus influenzae* and *Streptococcus pneumoniae*. Very little is known about the immunopathogenic mechanisms of this disease. In order to understand more about this illness and how to increase immunity against this disease, we are conducting a study. Results obtained from this study may help to understand protective immunity and provide information on better therapeutic interventions and management of this disease. For this purpose, we would like your permission to enroll your child in this study. Participation of your child in this study may help to save millions of lives due to this acute respiratory tract infections.

During the study period, your child will be admitted in the inpatients ward of this hospital until his/her condition permits discharge from the hospital. During hospitalization, he/she will receive the necessary treatment of this hospital. The hospital costs and therapy will be free of charge.

Tests that are routinely done for diagnosis at this hospital for such children will be carried out which include culture of blood and throat swab.

During the child's stay in the hospital, about 5 ml of venous blood (one tea-spoon full) will be collected from your child on the day of admission and on days 3, 7, 15 and 30 after enrolment (5 times). Urine will also be collected from your child on these days. Apart from the mild pain and discomfort associated with taking blood, drawing this amount of blood will not be harmful for your child. At the time of discharge, you will be requested to bring your child for follow-up visits on days 15 and 30 (after admission).

It is completely your decision whether your child should be enrolled in this study or not. After initial participation in the study, you have the right to withdraw your child from the study at any time point at your will. Your child will receive the standard care and treatment of this hospital whether he/she is enrolled in this study or not. We will compensate for any wage loss and travel costs that you may incur for each follow-up visit. All information/data of this study will be kept confidential and will be provided to you upon your request.

If you agree to let your child participate in this study, please sign or put your left thumb imprint at the specified space below.

Thank you for your co-operation.

Signature / left thumb impression of the guardian	Date
Signature of the investigator	Date
Signature of a witness	Date

DHAKA SHISHU HOSPITAL

CONSENT FORM FOR HEALTHY CONTROLS

"PATHOGENESIS AND IMMUNE RESPONSES IN PATIENTS WITH MENINGITIS OR PNEUMONIA DUE TO *HAEMOPHILUS INFLUENZAE*"

Children suffering from acute lower respiratory tract infection caused by pathogens such as *Haemophilus influenzae* and *Streptococcus pneumoniae* often have severe complications. Very little is known about the immunopathogenic mechanisms of this disease. In order to understand more about this illness and how to increase immunity against this disease, we are conducting a study. Results obtained from this study may help to understand protective immunity and provide information on better therapeutic interventions and management of this disease. Healthy subjects are needed to be examined in order to compare the findings in health to that in acute lower respiratory tract infections. Results obtained from this study may help to understand protective immunity and provide information on better therapeutic interventions and management of this disease. For this purpose, we would like your permission to enroll your child in this study. Participation of your child in this study may help to save millions of lives due to this disease.

For this purpose, your child will be examined by a qualified physician for a routine check up. For our study, we will collect blood and urine only once from your child. Approximately 5 ml (one tea-spoon full) of venous blood will be taken from your child. There will be slight pain and discomfort associated with this sampling. This will not be harmful to your child.

It is your decision to let your child participate in this study. All information/data of this study will be kept confidential and will be provided to you upon your request. We will compensate for any wage loss and travel costs that you may incur while your child participates in this study.

If you agree, please sign or put your left thumb imprint at the specified space below. Thank you for your co-operation.

_____ _____

Signature / left thumb impression of the guardian

Date

Signature of the investigator

Date

Signature of the witness

Date

CURRICULUM VITAE

NAME : Rubhana Raqib

ADDRESS : 24 Mitali Housing Society, East Kafrul, Dhaka Cantonment, Dhaka.

DATE OF BIRTH : October 19, 1961

ACADEMIC QUALIFICATION:

Degree	Year	Class / Division	University
PhD	1995	-	Karolinska Institute
M. Sc.	1988	First Class	Dhaka
B. Sc.	1985	First Class	Dhaka
H. S. C.	1979	First Division	Dhaka
S. S. C.	1977	First Division	Dhaka

RESEARCH EXPERIENCE:

1. For masters degree, research acitivities involved extraction, purification and study of the immunogenic properties of outermembrane proteins and lipopolysaccharides from *Shigella dysenteriae* type 1 and *Shigella flexneri* strains using immunoelectrophoresis, SDS-PAGE and Western blot.

2. For ph. D. dissertation, research activities were focussed on the study of the immunopathogenic mechanisms and immune responses in adult patients with shigellosis. Samples such as plasma, peripharal blood mononuclear cells, stools and rectal biopsies were collected from patients and healthy subjects and were analysed for cytokines (protein and mRNA), cytokine receptors and phenotypes of various cells and activation markers. The techniques used were immunohistochemistry, quantitative analysis of video microscopic images, ELISA, ELISPOT and *in situ* hybridization.

PUBLICATIONS

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MANUSCRIPT SUBMITTED

1. Raqib R, Ekberg C, et al. *Shigella dysenteriae* type 1 infection induces two distinct cell death pathways and alterations of apoptosis regulatory proteins. Submitted.

R1-I

EVALUATION OF CHILD HEALTH RESEARCH PROPOSAL

Reviewer #1

Reviewer's name:

Name of proposal: Pathogenesis and immune responses in patients with meningitis or pneumonia due to Haemophilus influenzae.

Name of proposed investigator: Rubhana Raqib

Date of review: June 25, 1997

The proposal is well-written and is designed to study humoral and cell-mediated responses to Haemophilus influenzae. The proposed study design is good. The principal investigator appears to be experienced in most, if not all, the techniques proposed. The Methods section fails to mention details of the cytokine ELISAs and what kits will be used to detect soluble cytokine receptors. The PI has described similar techniques in another proposal. The same concerns are issues herein, particularly with the time course of in vitro cytokine production upon antigen restimulation. All in all, an excellent proposal. Flow charts, timetables, and investigators responsibilities are nicely outlined; a good discussion of expected findings.

Review #2

Proposal #10

Pathogenesis and immune responses in patients with meningitis or pneumonia due to Haemophilus influenzae.

Rubhana Raqib

Goals The goal is to study immune responses to H. influenzae infection.

Design Inclusion criteria (page 5, bottom) indicate only children with pneumonia, yet patients with meningitis or pneumonia are discussed throughout the proposal.

Appropriateness An efficacious H. influenzae vaccine has been developed and proven to have remarkable effectiveness where distributed. The logic for conducting additional studies of this disease in light of the availability of effective vaccination is not well justified. What will we learn from this study that would improve the vaccination program or would lead to develop effective prevention measures for other disorders?

Timing and budget Timing: poor. Budget: no information.

Ethics Because enrollment criteria describe patients with pneumonia only, obtaining CSF samples cannot be justified. If the enrollment criteria were changed, this concern would disappear. Further, it is hard to justify five blood samples and the other intense evaluation of these patients when the argument for advance of knowledge in the Background portion of the proposal is weak.

Background Appropriate.

Other None.

K3-D1.1

Review # 3.

Proposal 10: "Pathogenesis and immune responses in patients with meningitis or pneumonia due to *Haemophilus influenzae*"

Principle Investigator: R. Raqib, PhD

1. Goals. The general aim is to study the humoral and cellular immune responses and immunoregulatory and proinflammatory mediators in *H. influenzae* (Hib) meningitis and pneumonia

2. Design. This is to be a descriptive study of 30 children 6 months to 5 years of age with Hib meningitis and pneumonia, compared to 15 patients with pneumococcal and 15 with *Branhamella catarrhalis* infections; an additional 15 patients will come from an ongoing study of isolation and PCR detection of Hib and pneumococcal infections by Dr. SK Saha. Sera from healthy patients in an immunogenicity study of pneumococcal conjugate vaccine will be used as additional controls. Diagnosis of Hib and pneumococcal infections will be made by blood cultures using a lysis direct plating/centrifugation method and by the experimental PCR methods. Seven interleukins and other cytokines will be measured in plasma and CSF. Secretion of cytokines by individual monocytes will be studied. Inflammatory mediators and modulators, including nitrate/nitrite, platelet activating factor, complement factors c3 and c5a, CRP, and endotoxin will also be studied. Immunophenotyping of peripheral blood mononuclear cells will be done by flow cytometry at the Karolinska Institute. Class- and subclass-specific antibodies to Hib will be measured by ELISA.

Mark Steinhoff, who is a consultant to Proposal 1, has recently written an eloquent editorial entitled, "*Haemophilus influenzae* type b infections are preventable everywhere" (Lancet 1997;349:1186). Emphasis now should be placed on disease surveillance and economical use of the vaccines as they become more readily available. In Gambia the Hib conjugate vaccines also reduced the frequency of Hib pneumonia. As much as I would like to better understand the pathogenesis of Hib infections, I am not convinced that the proposed studies will significantly improve patient care while we are waiting for Hib vaccines to become more readily available, as argued on page 14. Pneumococcal and meningococcal disease is in greater need of study by this argument, since vaccines are more problematic and much further in the future. Perhaps the investigators might consider restructuring the proposal to make pneumococcal disease the main subject, using patients with Hib infections as the controls. One area where the proposed studies that may have immediate utility is the diagnostic methods, which, as Steinhoff suggests, could facilitate surveillance, help elucidate the epidemiology of Hib disease in the investigators' locality, and lead to better targeted and more efficient deployment of the Hib vaccines.

It is unlikely that the extensive search for inflammatory modulator aberrations proposed will yield a quick fix for clinical Hib disease. A number of adjunctive therapies have been proposed, reviewed in the paper cited by Furth AV et al (Infect Immun 1996;64:4883), but so far only systemic steroids have made it--with ongoing controversy--into clinical practice. Nonsteroidal antiinflammatory agents (ibuprofen) reduce prostacyclin an thromboxane but do not affect the course of septic shock or ARDS. TNF inhibitors such as pentoxiphylline have been used in animal models for years but have not been used in humans. The few studies of nitric oxide synthase inhibitors suggest that they may ameliorate hypotension but do not affect mortality. One practical approach tested so far only in rats is the adjunctive treatment of Hib meningitis with low doses polymyxin, which binds endotoxin (Walterspeil JW, Pediatr Res 1989;20:237). This may deserve further consideration in humans.

2.1 Definitions of key concepts and variables. Adequate.

R-3-D, I

2.2 Study populations, sample size, and sampling strategy. The sample size calculation (p. 10) is arbitrary.

2.3 Clarity of analysis plans. A general description of analyses is given (pp. 10-11).

2.4 Feasibility of proposed methods. A major feasibility problem is the very large number of assays proposed. I think more focus is needed.

2.5 Adequacy of laboratory methods. As noted in critiques of Proposals 1 and 8, the investigators should try to follow the WHO/CDC guidelines for standardization of ELISAs for Hib and pneumococcal antibodies. The PCR detection methods for Hib and pneumococcus are experimental and yet unverified. See comments on this in Proposal 8.

2.6 Adequacy of record abstract forms (data collection). No information to review.

3. Appropriateness.

3.1 Potential for improving child health care. Over the past 20 years adjunctive therapies have had a significant but modest effect on the outcome of Hib infections. It would be more appropriate to focus on the diagnostic and epidemiologic features of Hib disease that could facilitate or target vaccine use. At this point in time every effort should be made to deploy the Hib vaccines.

3.2 Scientific significance.

4. Timing (and budget). Timing appears adequate and appropriate.

5. Ethics. No ethical objections.

6. Background. The background points out our continued ignorance of many features of Hib pathogenesis and disease but I think their efforts should be shifted to pathogens that are less amenable to prevention by vaccines.

7. Other.

Review #4 R1-M

EVALUATION OF CHILD HEALTH RESEARCH PROPOSAL

Name of proposal: Pathogenesis and immune responses in patients with meningitis or pneumonia due to *Haemophilus Influenzae*

Name of proposed investigator: Raqib et al.

Date of review: July 4, 1997

Review:

The design and selection of subjects for this study are very difficult to understand. It is not clear from the proposal whether the subjects selected from different studies/hospitals/populations are comparable. For example, on page 5, mention is made of serum from a study done by Saha that will be 'used in parallel'. More specific information is needed on how the subjects/samples will be collected and how representative they are of the population.

This study seems to be some hybrid of the case-control design, but the comparison groups and their origin are not clear. The authors should consult the literature on case-control studies to avoid biases in selecting their cases and controls. Three main points have to be considered in case-control studies. First, relative to the selection of subjects, both the cases and the controls must be selected from a representative population of cases and controls, respectively. Secondly, the cases and controls should be matched on factors that are important determinants of the outcomes studied (and could be confounding factors if not controlled for), but that are not among the risk factors of interest in the particular study. Finally, the analysis of the data must be 'matched', i.e. cases and controls must be paired in the analysis.

The formula used for sample size calculation is inappropriate for a case-control study and so are the analyses proposed.

Proposal #10

Review # 5

Title: Pathogenesis and immune responses in patients with meningitis or pneumonia due to Haemophilus influenzae (Hib)

P.I: R. Raqib

Goals: The goal of this study is to compare levels of cytokine, acute phase reactants, and immunoglobulins in children with Hib infection vs those with infections due to other bacterial pathogens.

Design: I have numerous concerns about this proposal. These are:

1. The hypothesis that the investigators wish to test is that "children with pneumonia or meningitis are likely to have impaired immune responses". The comparison group for these children and the specific meaning of "impaired immune responses" is unclear. If they believe that an intrinsic immune defect predisposed the child to pneumonia or meningitis, it would logically be tested either before the illness or well after recovery. The logical comparison group would be children exposed to Hib who did not develop these clinical syndromes, but these would be difficult to identify since it is impossible to determine if nasopharyngeal carriage is chronic or due to recent infection. Even if they are successful in identifying differences in the immune responses of children with pneumonia/meningitis vs. an appropriate control group, these data will probably have little clinical relevance because they are not linked to an identifiable clinical risk factor (e.g. malnutrition). The fact that an effective conjugate vaccine is available for Hib would suggest that limited resources might be better spent analyzing immune responses in Bangladeshi vaccine recipients rather than analyzing immune responses in children with clinical disease.

2. The rationale for selecting cases with pneumococcal or B. catarrhalis infection as controls and for expecting specific immune defects in children with Hib infection vs. those with other pathogens is not clear. Since Hib frequently causes CNS infection, whereas pneumococcus may commonly present with pneumonia alone and B. catarrhalis rarely produces meningitis, differences in the groups described may relate more to the severity of disease or presence of meningitis than to the specific pathogen. As patients with pneumonia and meningitis are lumped together without stratification, data generated will be difficult to interpret.

3. The selection criteria for bacteriologic confirmation of disease etiology also requires clarification. Blood or CSF isolation can be used to definitely prove the etiology of the illness, but throat swab isolation of bacterial pathogens frequently does not correlate with the cause of the pneumonic process. Well children may carry Hib in the throat for extended periods, and may also have Hib in the throat when they develop clinical disease due to another cause. Since

throat swab recovery appears to be used as verification of the etiologic agent, case selection criteria are not appropriate.

4. My greatest concern relates to the use of spinal taps in the study. As the age group runs from 6 months to 5 years, meningitis should be ruled out on clinical grounds without a LP in most cases. Although the ethical issues section says that LPs will only be done on meningitis patients, this is not clear in the protocol itself. The definition of a "meningitis patient", i.e. based on CSF results or based on clinical features, is not stated. CSF sampling in an older child without clinical signs of meningitis for study purposes is not appropriate and should raise ethical concerns. Even with clear meningitis, repeat LPs 3 days into treatment are rarely justified unless the child is not responding clinically. The protocol suggests that an LP on admission and 3 days later is routine (see Scheme for Sample Collection chart) for most if not all patients, which would not be justifiable. Clear criteria and justification for the use of spinal taps, weighing the benefits of this invasive procedure vs. the risks, must be provided.

5. Laboratory methods do not include standard isolation procedures for Hib, which is generally recovered from chocolate agar. Hib is not isolated from blood or MacConkey agar, which are the only media described in the protocol.

6. In addition to CSF sampling mentioned above, blood is sampled on 5 separate occasions in one month. The sample size calculations use 30 cases with vague reference to consideration of "the variance of different immunological parameters", which (as in other protocols) is stated without justification. Considering the large number of samples collected from each patient and the considerable potential for attrition, have the investigators determined criteria for an analyzable case or adjusted for attrition?

7. One flow chart refers to sputum collection. Sputum is rarely produced by children in this age group with pneumonia and would be virtually impossible to sample.

Appropriateness: Considering the numerous concerns mentioned above and the availability of the Hib vaccine (which does deserve testing in this population), the benefits from this study seem quite limited.

Timing: Time table is appropriate.

Ethical concerns: See #4 above

Background: Appropriate, although the investigators seem to focus entirely on the immunologic aspects and have little appreciation for the clinical aspects of the disease they plan to study.

#10

Review #6 R3-M

Child Health Research Proposals

Reviewer's name:

Name of Proposal: Pathogenesis and immune response in patients with meningitis...

Name of proposed investigator: Ragib

Date of review: 6/12/97

For CHR project staff only

Lumbar puncture is significant invasive procedure, & citeria for differential diagnosis of ALRI & meningitis is unclear — therefore criteria for performing lumbar puncture unclear. Concern re: possible unnecessary procedures.

Control pops are not justified based upon study aims & sample size given split between ALRI & meningitis is small.

Child Health Research Proposals

Name of Proposal: Pathogenesis + immune response in pdts w/ meningitis.

Name of proposed investigator: Ragib

Study has several major points requiring clarification. Clear delineation of pneumonia cases + meningitis cases not made.

1) Goals:

#2 hypothesis vague.

2) Design:

Basic premise sound but justification/rationale for control groups not clear? not indicated as part of any of specific arms of study. Sample size for Hib cases small — esp. since #/% of meningitis cases not described, + possible attrition a factor. Concern re: bias if some analyses are performed at two different sites, hosp + ICDDR/B. Also, are blood cases not performed on arterial blood?

3) Appropriateness

Kinetics of Hib infection are important, but it is not clear the proposed study is best method to address.

Name of Proposal: Pathogenesis + immune response in pts a/ meningitis

4) Timing ~~and Budget~~

Seems long given reported magnitude of problem.

5) Ethics

Strong concerns regading criteria for performance of CSF assays. How do you justify in controls at all, esp. since control pop rationale unclear.

6) Background

PIs appear to have expertise, but may need additional time to refine study aims + design.

7) Other:

Response to Reviewers' comments
on the proposal "Pathogenesis and immune responses in patients with meningitis or pneumonia due to *Haemophilus Influenzae*".

Reviewer # 1

1. Details of the ELISA technique to be used for meausrement of cytokine and cytokine specific receptor concentration have been included in the method section, as suggested by the reviewer.

2. It is not clear from the reviewer's comment as to what was the question and what was meant by "the time course of *in vitro* cytokine production upon antigen restimulation". Probably the experiment itself was not described adequately. For *in vitro* cytokine production assay, two time courses will be involved : (i) The samples used for stimulation studies will be peripheral blood mononuclear cells (MNC) from patients with either pneumonia or meningitis on day 0, day 7 and day 30 after admission (according to the Scheme for sample collection). (ii) Time of incubation with specific antigens and harvesting of MNC at definite intervals i.e. after 0, 2, 4, 6, 12 and 24 hrs for quantification of cytokine production at the single cell level.

Reviewer # 2

1. **Design:** As pointed out by the reviewer, inclusion criteria has been modified and patients with pneumonia and meningitis have been included.

2. **Appropiateness:** It is well known that several polyribosylribitol phosphate (PRP) conjugate vaccines have been developed that covalently link PRP to an immunogenic protein carrier. All these vaccines have been shown to be fairly immunogenic, specially in infants although the levels of PRP antibodies needed for protection have not been adequately defined (a wide range exists). The licensed PRP-diphtheria toxoid vaccine (PRP-D) (ProHibit, Connaught Laboratories, Swiftwater, Pa) has been shown to be immunogenic and protective in Finnish children and children in USA, however, immunogenicity and protective efficacy studies in Alaska native infants and Apache Indian infants have shown poor efficacy (Ward, J et al, 1990, New Eng. J. Med., 323:1393). The immunogenicity of PRP-D vaccine did not differ between Alaska Native and Finnish and other infants. Furthermore, in this population (Alaskan) the high levels of maternally acquired antibody before immunization did not influence the subsequent immune responses.

Factors that may influence the difference in efficacy are (i) differences between the populations in the degree of exposure to *H. influenzae* type b or differences in the susceptibiltiy to disease. It has been reported that intense and early exposure to *H. influenzae* type b contributes to increased risk of *H. influenzae* type b disease (Hall, DB et al. Am. J. Epidemiol. 1987, 126:1190). The risk of disease in Alaska Natives is 10 times higher than in other U.S. populations and is concentrated in the first few years of life (Ward JI et al, Lancet, 1981,1:1281). Low levels of immunity may have been sufficient to protect infants such as those in Finland who are less frequently and less intensely exposed and who tend to have disease at older ages, thus the level of immunity acheived after vaccination was insufficient for Alaska Native infants (ii) the microbiologic differences in *H. influenzae* type b strains in the two population. However, one would expect an effective vaccine to induce sufficient protective immunity for any natural exposure to *H. influenzae* type b and to work in all groups when considered for routine use in a heterogenous population world wide. (iii) The study in Alska Native has ruled out the genetic factors influencing immune response or susceptibiltiy to disease. (iv) the most probable reason for differences in efficacy could be an intrinsic limitation in the immunogenicity of the PRP-D vaccine in young infants (Ward, J et al, 1990, New Eng. J. Med., 323:1393).

Thus, a vaccine adequately effective in a developed country setting may not be equally effective in endemic, developing country settings. Therefore, before carrying out vaccine trials in developing countries, studies of the baseline immune status of children must be performed.

3. Timing

This comment is not clear. However, I suspect that the referee meant that the duration of the study was not adequate. According to a recent study conducted at Dhaka Shishu Hospital, 852 cases of meningitis were identified in a period of 8 years (1987-1994) (Saha. SK et al, Annals Trop. Peadtr.1997;17:5-8). There were 587 culture positive cases, *H. influenzae* accounted for 47% and *S. pneumonia* accounted for 32% of those. Analysis of culture negative specimens by antigen detection during the last 2 years revealed 71.4% cases positive for Hib. Ninety percent of the *H. influenzae* cases occured in the first two years of life. Thus, it is expected that at least 106 patients with meningitis attend the Dhaka Shishu Hospital per year. Therefore, recruitment of required number of patients within a period of 14 months is not improbable. While collecting patient samples, certain experiments can also be carried out. Another 10 months have been allocated for analysis of samples and completion of assays. The investigators are experienced in the techniques proposed. Most of these proposed methods have been standardized at ICDDR,B. Therefore, the time period suggested for the proposal appears appropriate.

4. Patients suffering either from pneumonia or meningitis will be enrolled for the study. CSF from patients with meningitis is routinely obtained for culture, cytology and other tests. A subsample of this specimen will only be used for the proposed study. Thus, no additional smapling will be carried out.

Reviewer # 3

Design:

(a) We already have responsded to this issue (Reviewer # 2, pg 1, # 2). Different racial groups may respond differently to any given vaccine. PRP-D vaccine was found to be highly effective in Finland (Eskola, J. *et al*, N Engl. J. Med., 1990; 323:1381-7) but poorly protective in Alaskan Eskimos (Ward, J. *et al*, N Engl J Med 1990;323:1393-401). An impaired response to the PRP-D conjugate vaccine could have contributed to the lower efficacy of this vaccine in Alaskan Eskimos than in Finnish infants. Apache children showed severly impaired antibody responses to T-cell independent antigens (*H. influenzae* type b polysaccharide vaccine) and less impaired response to T-cell dependent antigens (protein toxoids) as compared to white children. Differences in antibody responses to vaccines may contribute to differences in vaccine efficacy in different populations. The lower responses in Native American children may be an important factor contributing to their high risk of invasive *H. influenzae* type b infection. The same may be true for Bangladeshi children. The rate of infections due to *H. influenzae* type b is rising steadily in Bangladesh according to a recent report by S. K. Saha *et al.* and was found to be higher than infections due to *S. pneumoniae*. It is therefore, important to study the immune function of patients with pneumonia and meningitis and compare it with the immune status in healthy children living in endemic areas to see whether there is a humoral immune defect in these children. Recently, Food and Drug Administration advisory committee found that the Pastuer Mericux Connaught Vaccine "TriHIBit" (Combination DPT / Hib) might not protect fully against Hib since the Hib response tested in 4300 infants was not adequate (Medical News Week 6-16-97; Lancet, 1997;349(9067):1). It is unethical to introduce a vaccine without having the knowledge of the baseline immune response in a population. When epidemiological and other questions have been satisfactorily investigated and if Hib vaccines have been shown to be effective, then there would be a strong case for introducing and combining the Hib vaccine in the EPI programmme in Bangladesh

Separate studies involving standardization of new diagnostic methods (PCR, lysis-centrifugation plating method) have been undertaken by the co-investigators of the present study. However, as indicated the PCR technique will not be used for the proposed study. Our

study will serve two purposes: one disease surveillance in the locality, and two, better knowledge of the immune functions of children with complications due to *H. influenzae* type b infection in high risk areas.

(b) **Adjunctive therapy**- The reviewer is justified in saying that the present proposal aiming to search for inflammatory modulators during acute meningitis may not lead to a solution as to what adjunctive therapy may be used. However, the study probably will provide information on what specific modulators are detectable in meningitis and pneumonia in infants and at what concentrations, for how long while the antibiotic therapy or adjunctive therapy is being continued (at the Shishu Hospital, dexamethoasone is currently used either alone or in combination with antibiotics for the treatment of bacterial meningitis). Additional information on whether the severity of the disease correlates with the presence of high concentrations of these mediators as well as whether the levels of these mediators decrease during intervention may also be determined. Such studies of determination of various markers in a series of samples obtained on consecutive days after the onset of the disease have not been done previoulsy. Most studies on adjunct therapies are based on animal and cell culture models and have not reached the level of clinical research. Although there are a number of clinical prospective studies addressing the issue of adjunctive glucocorticosteroid therapy in the management of bacterial meningitis, a lot of questions remained unanswered such as: (i) how bactericidal should antibiotics be, (ii) should adjunctive corticosteroids be used (iii) how antibiotic resistant cases be treated ? The fact remains that further controlled clinical studies should be carried out in which adjunctive drugs should be used either alone or in combination with antibiotics before recommendations can be made.

Study populations, sample size and sampling strategy.

Children (6 months to 5 years of age) attending the Dhaka Shishu Hospital with meningitis and pneumonia due to *H. influenzae* type b will be recruited as cases. Children with ALRI due to *H. influenzae* type b and *Streptococcus pneumoniae* without meningitis and pneumonia will be enrolled as controls (C-1). A representative population of healthy children living in the vicinity of the study hospital (within a radius of 5 miles) will be recruited as healthy controls (C-2) who will not have any history of illness or fever within the past 4 months. The study population will be matched for age, gender and socioeconomic factor.

For this study five parameters will be studied to ascertain their association with the outcome variable. However, the exposure rate of such individual variable may vary and by large is unknown. Therefore, a conservative extimate of 50% would be assumed for each individual

variable. Moreover, the observed odds ratio is assumed as smallest as possible with a view to get the maximum sample size that can detect significant association or is able to answer the research question.

Assumptions are:

Alpha	= 0.05 when two tailed	= 1.96
Beta	= 0.20 with power 80%	= 1.28
Exposure Rate	= 50%	= 0.50
Odds Ratio	= 2.0	= 2.0

$$n = 2\bar{P}\bar{Q}\left(Z_\alpha + Z_\beta\right)^2 / \left(P_1 - P_2\right)^2$$

where $p_1 = p_0 R/[1 + p_0(R-1)]$ and $\bar{p} = \frac{1}{2}(p_1 + p_0)$, $\bar{q} = 1 - \bar{p}$

and $q_1 = 1 - p_1$, $q_0 = 1 - p_0$

Thus, the calculated sample size is 60 in each group i.e. 60 cases, 60 as controls 1 (C-1) and 60 as controls 2 (C-2).

Clarity of analysis plans.

All data will be validated by a series of logical and range checks. Finally a statistical package will be used for data processing and analysis. Data will be analyzed following the procedure for matched case-control design. Initially a descriptive analysis will be helpful. For that, data will be tabulated into 2x2 tables. Crude odds ratio will be computed initially for the association of each factor of interest with outcome variable. The final step of the analysis includes univariate and multivariate procedure where applicable. Repeated measure analysis of variance methods may be applied (multivariate procedure).

Feasibility of proposed methods.

The number of assays has been reduced such as, number of cytokine assays has been reduced, PAF assay has been taken out.

Adequacy of laboratory methods.

The investigators will follow the WHO/CDC guidelines for standardization of ELISAs for Hib antibodies as suggested by the referee (please see appendix II). One research officer has been

trained at Dr. George Siber's lab, Dana-Farber Cancer Institute, Harvard Medical School, Boston who will run the ELISA assays. He has learned to perform assays for serum IgG antibodies against *pneumococcus* type 6B and 19, *meningococcus* type A and antibodies against *H. influenzae* capsule, diphtheria and tetanus toxoids. The ELISA protocol of Dr. G. Siber will be followed (appendix II) (Siber, R. G. *et. al.* Pediatr. Infect. Dis. J. 1989;8:S84-91). Purified type specific PS antigens will be obtained from ATCC. Standard pooled immune sera or reference standard will be obtained from Dr. Carl Frasch (Chief, Laboratory of Bacterial Polysaccharides, HFM-428, Center for Biologics Evaluation and Research, 1401 Rockville Pike, Rockville, MD).

As suggested by the reviewer the PCR method will be omitted.

Potential for improving child health care.

At present, a proposal has been submitted by Dr. Robert E. Black from Dept. of International Health, John Hopkins, where an Hib vaccine will be given to children whose mothers will be supplemented with zinc is under consideration. If this study is approved, this will be the first ever trial of Hib vaccine in Bangladesh. Please see response to Reviewer # 2, # 2 and Reviewer # 3, # 1 for comments on the introduction of Hib vaccines in Bangladesh.

Background.

The two main pathogens frequently isolated from patients with ALRI in Bangladesh are *H. influenzae* and *S. pneumoniae*. Therefore, the proposal focussed on studying the immune responses against one of the pathogens.

Reviewer # 4

Selection of study population.

Children eligible for the study will be those within 6 months to 5 years of age who attend the Dhaka Shishu Hospital with features of acute lower respiratory tract infections such as cough, fever, increased respiratory rate ≥ 50 / min for infants 2-12 months and > 40 / min for 1-5 years, chest retractions, wheezing, rales, stridor and cyanosis (lower respiratory tract infection is defined by a modification of the World Health Organization guidelines (Appendix I). Pneumonia will be defined as:

Severe pneumonia	chest indrawing, wheezing
Pneumonia	no chest indrawing, fast breathing (≥ 50 / min for infants 2-12 months and > 40 / min for 1-5 years)

No pneumonia — cough and cold, breathing (< 50 / min for infants 2-12 months and < 40 / min for 1-5 years)

Bacterial meningitis will be diagnosed if clinically significant bacteria are isolated from cerebrospinal fluid or the fluid containing ≥ 10 white cells / mm^3, with either a predominance of polymorphonuclear cells or a glucose concentration of < 30 %.

Children (age range 6 m-5 yrs) with ALRI due to *H. influenzae* type b with meningitis and pneumonia will be the recruited as cases. Majority of these patients are of lower socioeconomic status. Children with ALRI due to *H. influenzae* type b and *Streptococcus pneumoniae* without meningitis and pneumonia will be enrolled as controls (C-1). A representative population of healthy children living in the vicinity of the study hospital (within a radius of 5 miles) will be recruited as healthy controls (C-2) to study baseline levels of various immunologic determinants. Both the study population, patients from the Dhaka Shishu Hospital and healthy infants will be matched for age, gender and socioeconomic status. Sample size calculation has been shown earlier (reviewer # 3).

Analyses: Rewritten. Please see comments to Reviewer # 3 under sample size calculations.

Reviewer # 5

Design

1. To test the hypothesis our comparison groups will be (a) patients with meningitis and pneumonia due to Hib; (b) the control groups are (i) patients with ALRI due to *H. influenzae* type b and *Streptococcus pneumoniae* without meningitis and pneumonia (C-1) and (ii) healthy children (C-2). The patient group with meningitis and pneumonia due to Hib and C-1 and C-2 groups are expected to show differences in the levels of immunoglobulin, cytokines, receptors, complement, as well as cellular responses. Both the patient and the control group (C-1) will be followed up to 1 month to see any changes in the parameters (please see scheme for sample collection). It has recently been reported that 24% of the patients discharged from the hospital after recieving treatment for meningitis died at home at a mean of 17 days (0-30 days) (Khan, NZ, Kalter, H. et al, Lancet, 1995;346:706). These patients had similar symptoms before death to those for which these children were initially admitted.

Sample size calculation has been given earlier (reviewer # 3). Please see justification against using Hib vaccine given in response to Reviewer # 3.

2. As suggested by the reviewer, the control group will be recruited from patients infected with Hib and *S. pneumonia* without meningitis and pneumonia for better interpretation. Infections with *B. catarrhalis* will not be studied. A healthy control group will also be included.

3. As suggested, throat swab culture will not be used as a selection criteria.

4. LP will not be done to serve the purpose of the study. Many patients are admitted to the Dhaka Shishu Hospital with fever and convulsion and in such cases LP is a must. Only then, CSF collected for microscopy will be used for the study purpose. Many of these CSF samples show normal range of leukocyte count, protein and sugar concentration. Those will serve as control CSF (please see pg 5-6).

5. Standard isolation procedure is included. When lysis centrifugation method is used, chocolate agar is not required, as the lysed patient's blood serves as the source of "V" factor (Saha SK, Khan, WA and Saha, S. Trans. Royal. Soc. Trop. Med. Hyg., 1992;86:554-556).

6. Sample size calculations have been given in answer to reviewer # 3.

7. Sputum collection is omitted.

Background:
Since it is an immunological study, we have been focussed on the immunological aspects in order to concise it. Even though, a paragraph on the clinical aspects of the disease has been added to the background section.

Reviewer # 6
Lumber puncture: Please see answer to reviewer # 5, point # 4.

Study population and sample size: Please see answer to reviewer # 3

Pneumonia and meningitis cases have been properly defined.

Goals: The hypotheses have been rephrased to show what aspects of the immunological parameters are going to be studied in order to reveal whether there is an impaired immune response or not in patients with meningitis and pneumonia. Determination of inflammatory mediators and modulators in a series of samples from patients may also show whether

exacerbation or remission of disease correlates with the concentrations of the mediators and whether there is a scope for adjunctive therapy.

Design: Sample size calculations shown in rebuttal to reviewer # 3. Attrition has been considered. To avoid bias, blinded tests will be performed at both the places (ICDDR,B and Hospital). Blood gases are performed in children with very critical conditions only.

Appropiateness: We are addressing only one aspect of the Hib kinetics i.e. the immunological kinetics.

Timing: Though the magnitude of the disease burden is huge, the study being a follow up study and informed consent from the parents / gurdians of the patients must be taken, it will take considerable time before we can reach the desired number of patients to study.

Ethics: LP will be performed by the clinician when necessary. CSF will be obtained passively from the microbiology lab. CSF samples can not possibly be obtained from healthy controls. Only those CSF samples will be considered as control, which will have normal cell count, protein and sugar concentrations.