ICDDR,B: Centre for Health & Population Research RRC APPLICATION FORM

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RESEARCH PROTOCOL
Protocol No.: 2001-005

RRC Approval: Yes/ No Date:
ERC Approval: Yes/No Date:
AEEC Approval: Yes/No Date:

Project Title: Comparison of immune responses in children born with low birth weight and full term with normal birth weight: a pilot study.

Theme: ***(Check all that apply)***

- ☐ √ Nutrition
- ☐ Emerging and Re-emerging Infectious Diseases
- ☐ Population Dynamics
- ☐ Reproductive Health
- ☐ Vaccine evaluation
- ☐ Environmental Health
- ☐ Health Services
- ☐ √ Child Health
- ☐ Clinical Case Management
- ☐ Social and Behavioural Sciences

Key words: LBW, immunity

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Co-Investigator(s):
Dr George Fuchs, Dr Md Yunus

Population: Inclusion of special groups (*Check all that apply*):

Gender
- ☐ Pregnant Women
- ☐ Male ☐ Fetuses
- ☐ Females ☐ Prisoners
- ☐ Destitutes

Age
- ☐ √ 0 – 5 years ☐ Service providers
- ☐ √ 5 – 9 years ☐ Cognitively Impaired
- ☐ 10 – 19 years ☐ CSW
- ☐ 20 + ☐ Others (specify _____)
- ☐ > 65 ☐ Animal

Project / study Site (*Check all the apply*):
- ☐ Dhaka Hospital ☐ Mirsarai
- ☐ Matlab Hospital ☐ Patyia
- ☐ √ Matlab DSS area ☐ Other areas in Bangladesh _____
- ☐ Matlab non-DSS area ☐ Outside Bangladesh
- ☐ Mirzapur name of country: _____
- ☐ Dhaka Community ☐ Multi centre trial
- ☐ Chakaria(Name other countries involved)
- ☐ Abhoynagar_____

Revised on: 30 May 2000

Type of Study *(Check all that apply)*:

- ☐ Case Control study
- ☐ Community based trial / intervention
- ☐ Program Project (Umbrella)
- ☐ Secondary Data Analysis
- ☐ Clinical Trial (Hospital/Clinic)
- ☐ Family follow-up study
- ☐ Cross sectional survey
- ☐ √ Longitudinal Study (cohort or follow-up) ✓
- ☐ Record Review
- ☐ Prophylactic trial
- ☐ Surveillance / monitoring
- ☐ Others

Targeted Population *(Check all that apply)*:

- ☐ No ethnic selection (Bangladeshi)
- ☐ √ Bangalee
- ☐ Tribal groups
- ☐ Expatriates
- ☐ Immigrants
- ☐ Refugees

Consent Process *(Check all that apply)*:

- ☐ √ Written
- ☐ Oral
- ☐ None
- ☐ √ Bengali language
- ☐ √ English language

Proposed Sample size: Total sample size: 140 ☐

Sub-group 70x2 ☐

Determination of Risk: Does the Research Involve *(Check all that apply)*:

- ☐ Human exposure to radioactive agents?
- ☐ Fetal tissue or abortus?
- ☐ Investigational new device? (specify_____)
- ☐√ Existing data available from Co-investigator
- ☐ Observation of public behavior
- ☐ Human exposure to infectious agents?
- ☐ Investigational new drug
- ☐ Existing data available via public archives / source
- ☐ √ Pathological or diagnostic clinical specimen only
- ☐ New treatment regime

Yes/No

√ ☐ /☐ Is the information recorded in such a manner that subjects can be identified from information provided directly or through identifiers linked to the subjects?

☐ / √☐ Does the research deal with sensitive aspects of the subject's behavior; sexual behavior, alcohol use or illegal conduct such as drug use?

Could the information recorded about the individual if it became known outside of the research:

☐ / √ ☐ a. place the subject at risk of criminal or civil liability?

☐ / √ ☐ b. damage the subject's financial standing, reputation or employability; social rejection, lead to stigma, divorce etc.

Do you consider this research *(Check one)*:

- ☐ greater than minimal risk
- ☐ no risk
- ☐ √ no more than minimal risk
- ☐ only part of the diagnostic test

Minimal Risk is "a risk where the probability and magnitude of harm or discomfort anticipated in the proposed research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical, psychological examinations or tests. For example, the risk of drawing a small amount of blood from a healthy individual for research purposes is no greater than the risk of doing so as a part of routine physical examination".

RECEIVED 10 OCT 2004

Yes/No

√ ☐ / ☐ Is the proposal funded? If yes, sponsor Name: World Bank NCOE

Yes/No

☐ ☐ Is the proposal being submitted for funding ?
If yes, name of funding agency: _____

Do any of the participating investigators and/or their immediate families have an equity relationship (e.g. stockholder) with the sponsor of the project or manufacturer and/or owner of the test product or device to be studied or serve as a consultant to any of the above?

IF YES, submit a written statement of disclosure to the Director.

Dates of Proposed Period of Support *(Day, Month, Year - DD/MM/YY)*	**Cost Required for the Budget Period ($)** *a. Ist Year 2nd Year 3rd Year*
Beginning date 01-01-2001	20,000 $
End date 31-12-2001	*b. Direct Cost :* 20,000 *Total Cost :* 25,000 $

Approval of the Project by the Division Director of the Applicant

The above-mentioned project has been discussed and reviewed at the Division level as well by the external reviewers. The protocol has been revised according to the reviewer's comments and is approved.

Dr G. B. Nair
Name of the Division Director

Signature

04/02/2001
Date of Approval

Certification by the Principal Investigator
I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

Signature of PI:

Rubhana

Date: 04-02-2001

Name of Contact Person (if applicable)

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PROJECT SUMMARY:

Nutritional factors during early development not only have short-term effects on growth, body composition and body functions but also exert long-term effects on health, disease and mortality risks on adulthood. Several components of the human immune system mature early in fetal life, deficits in organ growth and development occurring in utero are more serious and long-lasting than those caused by later malnutrition. Adverse factors that impair fetal growth hinder immunological maturation as well. Maternal malnutrition has been observed to have greater effects on thymic and lymphoid tissue than on other organs. Pregnancy and the first few months of postnatal period are critical time periods for growth and development of the human nervous system, processes for which adequate substrate supplies are essential. Possible links between birth weights and atopy, and autoimmune thyroid disease have been described. There is evidence that low birth weight babies may have a sustained impairment of immune competence as infants and children.

The parent study entitled "Dietary fat and infection: effect on vitamin A status on mother and infant and dietary intake methodology" (PI: Dr Dewan Alam) was conducted in 1995-1997 in Matlab. In the study, children who were born singleton, had their length/size and birth weight measured within 72 hours. The population is fairly stable, and only a very few children are expected to have migrated from the study area. Study subjects will be selected from this cohort in Matlab for the new study. Current body size, dietary intakes, SES and parental factors (maternal height, educational level, socioeconomic status) will be measured. Dietary assessment including dietary pattern, single day dietary intake recall and nutrient intake calculated from food composition table will be made. Anthropometric measurements (weight, height and mid upper arm circumference) will be taken. All these measurements will be done for a parallel study (PI: Dr Dewan Alam) and the information will be utilized by the present study.

The objective of the present protocol is to delineate the differences in the development of immune responses in children born with normal birth weight, or with low birth weight (LBW) in the above birth cohort study. The children (age 4-6 years; n= 128) will be recruited from the ongoing birth cohort study. Blood and stool will be collected from each subject on a single day. Determination of serum total IgG levels that shows a linear relationship with birth weight in LBW will indicate whether the levels are different in the three groups. Diphtheria- or tetanus-specific IgG titers in serum will help to understand whether the development of specific responses in children is different in these groups. Complement concentrations in serum will reflect the competence of nonspecific immune responses in children in the different groups. Percentage of CD4+ cells and CD4+ to CD8+ ratio will be determined to examine whether the defense in children with a history of LBW is different from babies born with normal birth weight. Proliferation responses of T cell against mitogens will be determined by phenotyping the cells for CD25 and CD69, the earliest markers on activated lymphocytes. This property is believed diminished in LBW infants and is altered in infants with a history of IUGR. Study of the expression of adhesion molecules (CD11b/CD18) on mononuclear cells will aid in understanding whether LBW has an effect on the migration of intravascular monocytes that is essential during an infection. Results of the study may help us to better understand the effect of low birth weight on the development of immunity in children and in the long run may provide a basis for understanding the increased incidences of infection. The study may also provide a strong scientific basis for introduction of focused nutrition interventions especially during pregnancies to improve maternal nutritional status, to reduce the incidences of IUGR, to improve the birth weight of infant, thus improving the immune status of the infants and in the long run of the future adults.

DESCRIPTION OF THE RESEARCH PROJECT

Hypothesis to be tested:

Development of immune responses in children with LBW are impaired and/or delayed leading to increased morbidity and mortality compared to children born full term with normal body weight.

Compared to children born with normal birth weight, children with LBW will have-

1. Reduced concentrations of total IgG in serum.
2. Reduced neutralizing antibody responses against diptheria/tetanus.
3. Lower levels of complement C3 in serum.
4. Lower frequency of $CD69^+$ and $CD25^+$ cells in blood.
5. Increased CD4:CD8 ratio, and decreased percentage of NK cells.
6. Diminished expression of adhesion receptor (CD11b/CD18) on mononuclear cells.

Specific Aims:

To compare the development of immune responses in children with low birth weight, and children born full-term with normal birth weight by determining

1. Concentrations of total IgG in serum
2. Neutralizing antibody responses against diptheria/tetanus.
3. Concentrations of complement C3 in serum.
4. T lymphocyte proliferation response to mitogens (PMA plus ionomycin) using antibodies against activation markers CD 69 and CD25 on proliferating lymphocytes using flow cytometry.
5. The ratio of helper and cytotoxic T cells (CD^+4:CD^+8) and natural killer cells (CD^+16/CD^+56).
6. Expression of adhesion molecules (CD11b/CD18) on mononuclear cells.

Background of the Project

Previous study: The protocol entitled "Dietary fat and infection: effect of vitamin A status on mother and infant and dietary intake methodology" (PI: Dr Dewan Alam) was conducted from November 1995 to October 1997 in two clusters of villages, eight in each (separated by some intervening villages), in Matlab thana of Chandpur District, in Bangladesh. The area is located about 45 km southeast of Dhaka. The study was a population based longitudinal controlled intervention trial in which subjects (women) from one cluster of villages (randomly chosen) received the proposed intervention of 20 ml soybean oil (~18 g fat) daily during pregnancy and throughout the first six month of lactation and subjects from the other cluster of villages served as controls. The control subjects received neither any supplement nor any placebo and were on their usual diet. The aim of the study was that whether oil supplementation to pregnant women had any impact on the vitamin A status of infants born of these mothers or not. The usual diet consists of rice as the main staple with green leafy vegetables and small amount fish and very occasional consumption of meat and other animal products. Study subjects were enrolled at five to seven months of gestation, without any known chronic illness and willing to participate into the study. Trained Community Health Workers (CHWs) from the respective study villages conducted a door-to-door survey and

identified currently eligible subjects based on their menstrual history. A list was prepared of married women who were either at early pregnancy or at non-pregnant state. A field team consisting of a trained Health Assistant, a CHW and a logistical assistant visited eligible women at home and obtained their written informed consent. After selection, a detailed interview was conducted on socioeconomic, demographic and household characteristics. Anthropometric measurements were taken, dietary interviews were carried out, and the first blood sample was collected. A total of 341 and 335 subjects were recruited from the intervention and control villages, respectively. However, only data of women who delivered singleton babies and who were available during the postpartum follow-up periods were included in the analysis. About 248 intervention women and 251 controls met these general criteria. Each woman in the intervention villages was supplied with 140 ml of soybean oil weekly for daily consumption of 20 ml (~18 g fat) by the CHWs. CHWs collected information on compliance to the intervention. Study women were instructed to take 10 ml of oil twice a day with their main meals to ensure concurrent availability of oil with the food items. Total period of supplementation was between 8 and 10 months depending on stage of gestation at enrollment. Special care and motivational efforts were continued throughout the entire study period to maximize compliance to the intervention and to maintain study women on their usual diet. Each woman was trained to maintain a daily record of oil intake by putting a mark on easily understandable record-keeping sheet supplied at the time of the weekly oil delivery. CHWs collected the maternal records of oil intake during their next routine weekly visit, and also they checked and recorded the amount of unconsumed oil. In both intervention and control areas, CHWs collected morbidity data by weekly recalls. Anthropometric measurements, blood and breast milk samples were collected by a trained Health Assistant or by the trained study nurse. Dietary intake data was collected by 24-hour recalls and Simplified Dietary Assessment (SDA) questionnaires were administered for assessing risk for VAD. Maternal anthropometric measurements, blood and breast milk samples, and dietary interviews were scheduled at enrollment, and at one, three and six months postpartum. A single infant blood sample was collected between the age of 6-8 months. Plasma biochemical analyses were done by simultaneous determination of serum retinol, lutein and beta-carotene by HPLC. Retinol concentration in breast milk was also determined. Stool microscopy was done for any parasitic infestations. Results of the study indicated that there was a limited impact of the oil supplementation of the mothers on breast milk retinol concentration. However, there was no detectable difference in vitamin A status of the infants after oil supplementation of mothers.

Present study:

Nutritional factors during early development not only have short term effects on growth, body composition and body functions but may also exert long term effects on health, disease and mortality risks on adulthood, as well as development of neural functions and behavior, a phenomenon called metabolic programming. Early metabolic programming of a "thrifty phenotype" that is physiologically adapted to fetal nutrient depletion, has been proposed to be important in developing countries where gestational poverty is often followed by adult affluence (1, 2). Events in early life strongly influence the adult survival prospects. A possible explanation may be that malnutrition leaves a permanent effect on the development of the immune system during fetal growth. Studies by Andrew Prentice and group in rural Gambia, West Africa have shown that prenatal and early postnatal events affect the future health of rural Gambians in a manner first manifested around puberty and amplified by age (3, 4) and clearly parallels with the hypothesis of Barker (5). However, the study showed that mortality was dominated by infections and pregnancy related deaths with none being related to chronic degenerative diseases. Several components of the human immune system mature early in fetal life (6), deficits in organ growth and development occurring in utero are more

serious and long-lasting than those caused by later malnutrition (7). Maternal malnutrition has been observed to have greater effects on thymic and lymphoid tissue than on other organs (8, 9). Possible links between birth weights and atopy, and autoimmune thyroid disease have been described (10, 11). There is evidence that low birth weight babies may have a sustained impairment of immune competence as infants and children (12-14). In animal models the effects of fetal undernutrition (single nutrient deficiency) on immunological functions have been recorded in F2 and F3 offspring (13). Sophie *et al* presented evidence that risk of premature death from infectious diseases in young adulthood is programmed by early life events (4). The study suggested that nutritionally-mediated intrauterine growth retardation may permanently impair the development of immune function. Though it was not known whether the effect was confined to a subset of severely affected individuals or represented a graded premature immunosenescence.

The course of pregnancy, child-birth and lactation and the short- and long-term outcome of the child are influenced by the intake of foods and particularly micronutrients e.g. Fe, Zn, I and polyunsaturated fatty acids. The evaluation of dietary effects of on child growth requires epidemiological and field studies as well as evaluation of specific cell and tissue growth. There are several indications for beneficial effects of functional foods on the development of immune response e.g. induced by antioxidant vitamins, trace elements, fatty acids, nucleotides etc in infant food (6, 15). Pregnancy and the first few months of postnatal period are critical time periods for growth and development of the human nervous system, processes for which adequate substrate supplies are essential. The potential beneficial effects of a balanced supply of nutrients such as I, Fe, Zn and polyunsaturated fatty acids in early diet seems to have long-term effects on sensory and cognitive abilities as well as behavior (16).

Adverse factors that impair fetal growth hinder immunological maturation as well. The immunocompetance of LBW infants is compromised. Those who are small for gestation show persistent immunological impairment for several months, even years. Nutrient deficiencies impair immune responses and lead to frequent severe infections resulting in increased mortality (12-14). The use of nutrient supplements, singly or in combination, stimulates immune response and may result in fewer infections in LBW infants (17). The interactions between nutrition and the immune system are thus, of clinical, practical and public health importance. Chronic, mild postnatal malnutrition is associated with a variety of cognitive and behavioral deficits across the life span (18, 19). The role of prenatal malnutrition in this process is less clear. Some studies have indicated that during pregnancy, the Th2/Th0 immunity has a decisive impact on shaping of the Th1/Th2 T cell profile in the neonate (20-22). Preterm infants have increased incidences of infection primarily due to deficiencies in neonatal host defense mechanisms. Superoxide anion production was significantly diminished in cord blood from preterm babies compared to full term babies (28% vs 37%) (23). TNF-α production by LPS-stimulated monocytes from preterm babies was significantly decreased (35% decrease). Cell surface expression of the CD11b/CD18 adhesion receptor subunits was significantly decreased (60% and 52%) in monocytes from preterm babies (23). In response to inflammatory stimuli, monocytes migrate to the site of infection that is mediated by adherence via adhesion molecules such as CD11b/CD18 receptor on the surface of the monocytes and the intracellular adhesion molecule 1 on the endothelium. These activated monocytes transport additional CD11b/CD18 receptors from the intracellular compartment to the cell surface membrane to enhance adhesion. The intravascular monocytes then migrate from the endothelium to the extravascular site of infection and enhance antimicrobial functions. Diminished expression may be an important factor associated with reduced migration of intravascular mononuclear cells to the site of

infection and reduced bactericidal function in preterm babies. Small for gestational age (SGA) and LBW infants have an altered immunological profile. Lymphocyte percentage is low and CD4/CD8 ratio is abnormal. IgG levels are lower in SGA and LBW and are directly related to the birth weight of the neonates. IgA and IgM are not effected and complement C3 levels are significantly lower in the SGA neonates. (23-26). Neonates born with IUGR / SGA have significantly low levels of hemoglobin, serum albumin and absolute lymphocyte compared to full term babies (26). These preterm and SGA neonates showed low cord blood IgG levels and low neutralizing antibodies compared to full term babies that could be due to intrauterine undernutrition or other placental abnormalities. Prematurity may also influence the level of maternally acquired immunity in neonates. Maternal antibody transfer is significantly lower in preterm than in full term infants (27, 28). Percentage of CD4+ cells and CD4+ to CD8+ ratio were decreased in IUGR, CD8+ cells were increased (29). School children born preterm had significantly lower CD4+ T cell percentage and CD4:CD8 ratios ($P<0.05$) whereas natural killer cell percentages and serum eosinophil cationic protein values were significantly higher than controls thus indicating inflammatory basis for lung function abnormalities (30). In normal pregnancy, systemic immunological deviations occur toward suppression or decreased activity of the immunological response. In IUGR, there is a lack of suppression or increased T-cell activity that may have a primary pathogenic role in some women with complicated pregnancy (31).

Malnutrition and micronutrient deficiency were shown to have direct effect on the process of IUGR and LBW. Iron deficiency in pregnancy causes anemia as well as fetal growth retardation. In maternal anemia, all indices of fetal growth showed a linear relationship with maternal serum ferritin and hemoglobin (29, 32, 33). However, high levels of ferritin in IUGR mothers reflected sub clinical maternal infection. The growth retarding effect of maternal anemia was more on fetal birth weight and mid-arm circumference than on other anthropometric indices. In African-American women, adverse pregnancy outcome including IUGR was associated with elevated maternal serum alpha-fetoprotein and plasma. Mild maternal zinc depletion was strongly associated with IUGR, low Zn in maternal plasma and poor placental perfusion reduced the maternal-fetal transfer of zinc (32, 33). Though there are contradictory data also reporting that in Indian population, maternal zinc levels were not associated with IUGR (34).

Research Design and Methods

Study site: The study will be conducted in the 16 villages in Matlab Upazila under Chnadpur district in Bangladesh where the previous cohort study entitled "Dietary fat and infection: effect on vitamin A status on mother and infant and dietary intake methodology" (PI: Dr Dewan Alam) was conducted (1995-1997). In the study, children who were born singleton, had their length/size and birth weight measured within 72 hours. The population is fairly stable, and only a very few children are expected to have migrated from the study area. Study subjects will be selected from this cohort in Matlab for the new study. Current body size, dietary intakes, SES and parental factors (maternal height, educational level, socioeconomic status) will be measured. Dietary assessment including dietary pattern, single day dietary intake recall and nutrient intake calculated from food composition table will be made. Anthropometric measurements (weight, height and mid upper arm circumference) will be taken. All these measurements will be done for a parallel study (PI: Dr Dewan Alam) and the information will be utilized by the present study. Study subjects will be selected from this cohort in Matlab for the present study.

Study population: Children who were born singleton (n=128) and participated in the previous study mentioned above during their infancy, and had their size at birth measured within 72 hours will be invited to participate into the current study. The age of these children will be 4-6 years of age. Availability of the children will be initially confirmed from Matlab Health and Demographic Surveillance System records. Then a door-to-door survey will be conducted to confirm availability of the children and then from the list of available children, required number of children will be randomly selected for the study. In most developing countries, 80% of the LBW infants are IUGR (35). We will have two groups, one consisting of children born with LBW and the other with normal birth weight.

Inclusion criteria: Only those children will be included in the study who (1) are available for the study and are able to visit the Matlab Health Complex, (2) are born full term with normal birth weights, or low birth weight (<2.5 Kg) (3) were not born preterm.

Exclusion criteria: Children (1) who were born premature/preterm (2) with known chronic illness (3) who have had measles in the recent past.

Anthropometric measurements: The study children had their birth weights measured within 72 hours after birth with SECA beam balance accurate to 10 g. Length was measured with locally constructed length board to nearest 0.1 cm. For the present study, weight, height and mid upper arm circumference will be measured following standard guidelines (Gibson, 1990). Weight will be measured with portable SECA electronic scale to nearest 100 g. Height will be measured to nearest 0.1 cm with Accu-Hite Stadiometer with built-in leveling bubble.

Routine analyses: Blood sample will be collected by venipuncture and will be carried out in conjunction with the protocol # 2001-006 (PI: Dr D. S. Alam). Hemoglobin, total and differential count, and C-reactive protein (CRP), zinc and vitamin A will be measured in serum. Stool microscopy and culture will be performed for detection (if any) of intestinal parasites and pathogenic bacteria.

Blood: Blood will be collected in heparinized tubes (3 ml) for cell separation. Mononuclear cells (PBMC) will be separated from venous blood upon Ficoll-Hypaque separation and will be used for proliferation responses to mitogens (ionomycin and PMA) and for phenotyping of lymphocytes by flow cytometry. Lymphocytes will be frozen and carried in liquid nitrogen to the Karolinska Institutet at the Division of Infectious Diseases, Huddinge University Hospital where phenotying will be performed.

Serum immunoglobulin G: The concentrations of total IgG in plasma will be measured by nephlometry (BecKman Instruments, Inc, Galway, Ireland).

Neutralizing antibody responses against diptheria/tetanus: Red cells sensitized with tetanus toxoid agglutinate in a specific way in the presence of tetanus antibodies in serum. The method of Galazka A et al will be used (36).

T lymphocyte proliferation response: For the proliferative response of PBMC to mitogens, cells will be cultured with ionomycin plus PMA at 0.5 μM/ml and 1 ng/ml respectively and with medium alone (RPMI 1640 (GIBCO) with 10% fetal calf serum, 2 mM glutamine (Flow Laboratories, Rickmansworth, Herts, UK), 50 IU of penicillin, 50 μg of strepto,ycin per ml in 12 well Nunc plates (Nunc) and incubated in a humidified atmosphere containing 5% carbon dioxide for 24 hours. Cells will be scraped using a cell scraper (Nunc), collected in falcon tubes, centrifuged, frozen and stored in liquid nitrogen for determination of activation and

proliferation. Expression of early activation markers CD 69 and CD25 on proliferating lymphocytes will be studied by flow cytometry.

Phenotyping of mononuclear cells (CD4, CD8, CD16/56, CD11b/CD18, CD25, CD69): After separation from blood, lymphocytes will be frozen and carried in liquid nitrogen to the Karolinska Institutet at the Division of Infectious Diseases, Huddinge University Hospital where phenotying will be performed by flow cytometry. Frozen cells will be thawed at 37° C and immediately washed twice in RPMI 1640 and viability will be checked by trypan blue exclusion. Cells will be suspended in phosphate-buffered saline (PBS) containing 2% human AB serum (heat inactivated) and will be dispensed into tubes containing saturating concentrations of monoclonal antibodies. After a 30 minutes incubation period at 4° C, the cells will be washed in PBS containing AB serum and resuspended in PBS containing 1% paraformaldehyde and will be kept at 4° C until used for the procedure. Three-color fluorescence analysis will be performed with a FACSort flow cytometer (Becton-Dickenson). Data acquisition will be done by Lysys III softyware (Becton-Dickenson).

Sample size calculations and data analysis

Outcome variables	Expected levels in IUGR or LBW group	Expected levels in the control group	Sample size required in each group	References
Total leukocyte count (cells/mm^3)	10056 ± 4613	12336 ± 2652.8	58	24
Total lymphocyte count (cells/mm^3)	2949.4 ± 1402.3	4466.1 ± 1113.1	15	26
CD4 %	38.8 ± 4.2	46.6 ± 5.3	8	24
CD8 %	23.8 ± 2.9	21 ± 2.2	18	24
CD4:CD8	1.66 ± 0.31	2.17 ± 0.32	8	30
IgG mg/dl	752.6 ± 186.6	1053.5 ± 160.6	7	24
Complement 3 mg/dl	70.2 ± 17.4	97.8 ± 18.4	9	24

Sample size was calculated for major outcome variables to detect the hypothesized differences with a type I error of 5% and type II error of 10%. The estimated maximum sample size was 58 in each group. Considering a 20% dropout the required sample size is 70 per group. Thus, in total the sample size is 140.

Data analysis will be done using the software package JMP (SAS Institute Inc., Carey, NC, USA) and SPSS. Preliminary analysis of the data will be done by Student's t test to compare various parameters between the groups. Later results will be further adjusted for confounding variables such as current nutritional status, current illness as evident by CRP levels or stool culture etc. Correlation between birth weight and various immune parameters will be examined by Spearmen Rank Correlation test.

Facilities Available

Immunological assays will be carried out at ICDDR,B since the techniques have been standardized and the equipment needed for carrying out the assays are available in ICDDR,B. Since a flourescent associated cell sorter (FACS) is not available at ICDDR,B, frozen lymphocytes will have to be carried in dry ice (-70° C) to Sweden to perform phenotyping by flow cytometry. FACS will be available to the PI to perform flow cytometric assay (phenotyping) at the Division of Infectious Diseases, Karolinska Institutet, Sweden.

Ethical Assurance for Protection of Human Rights

The proposed study involves sampling of blood from children. Informed consents will be taken from the parents/guardians of the child. Approximately 3 ml of venous blood (from median cubital vein) will be taken from children. Age range of the children will be 4 to 6 years. There may be a momentary pain and a very small chance of bruising at the site of insertion of the needles. To minimize the chance of infection, aseptic precautions will be taken and disposable, sterile syringes and needles will be used for drawing blood.

Dissemination

Research findings will be published in international journals to make the results available to all researchers in the relevant fields and will be presented in international conferences.

Rationale

In developing countries like Bangladesh, pregnant mothers suffer from malnutrition including micronutrient deficiencies, energy deficiency. All these have direct effect on fetal growth and on the development of the immune system and the nervous system since several components of the human immune and nervous system mature early in fetal life. Deficits in organ (such as thymus and lymphoid tissue which are the most effected ones) growth and development occurring in utero are more serious and long lasting than those caused by later malnutrition. It is hypothesized that effect of malnutrition on the development of the immune system during fetal growth strongly influences the adult survival prospects and predisposes the children and adults to infections. Results obtained from this study may help in better defining possible links between birth weights, IUGR and sustained impairment of immune competence in infancy and childhood.

Thus, governments and international agencies will have a strong scientific basis to be much more active and innovative in the introduction of focused nutrition interventions especially during pregnancies to improve maternal nutritional status, to reduce the incidences of IUGR, to improve the birth weight of infant, reducing morbidity thus improving the immune status of the infants and in the long run of the future adults.

Literature Cited

Identify all cited references to published literature in the text by number in parentheses. List all cited references sequentially as they appear in the text. For unpublished references, provide complete information in the text and do not include them in the list of Literature Cited. There is no page limit for this section, however exercise judgment in assessing the "standard" length.

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spatial discrimination learning and retention in young rats. *Indian J Exp Biol 31(4):353.*

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Detailed Budget

	PERSONNEL	Position	Effort%	Salary	One Year
1	Rubhana Raqib, 7 months	NOB	35	970	2,380
2	Dewan S Alam, 7 months	NOB	10	-	-
3	Research Officer, 7 months	GS-5	100	351	2,460
4	Fellow/daily wager at Matlab				400
	Sub Total				**5,240**
	LABORATORY ANALYSIS				
5	Immunological Assays				5,000
6	Laboratory Supplies				3,000
7	Dry ice and freight charges				600
	Sub Total				**8,600**
	OTHER SERVICES				
7	Printing and Publication				500
8	Communication				200
9	Repair, maintenance				300
	Sub Total				**1,000**
	INTERDEPARTMENTAL SERVICES				
10	Pathological Tests				500
11	Microbiological tests				400
12	Biochemistry Tests				400
	Sub Total				**1,300**
	TRAVEL				
13	International Travel				3,500
14	Local transport to Matlab				360
	Sub Total				**3,860**
	TOTAL DIRECT COST				**20,000**
	Overhead 25%				5,000
	GRAND TOTAL				**25,000**

4.2.2001

Biography of the Investigator

NAME	Rubhana Raqib
DATE OF BIRTH	October 19, 1961
CITIZENSHIP	Bangladeshi
PRESENT POSITION	Assistant Scientist, LSD

ACADEMIC QUALIFICATION:

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

Research and Professional Experience

Present position

Assistant Scientist, Immunology Laboratory, Laboratory Sciences Division, ICDDR,B

Publications

1. Raqib R, Mia SMS., Qadri F, Alam TI, Alam NH, Chowdhury AK, Mathan MM, Andersson J. **2000**. Innate immune responses in children and adults with shigellosis. Infect Immun. 68(6):3620-3629.
2. Raqib R, Gustafsson A, Andersson, J. and Bakheit M. **1997**. A. A systemic downregulation of gamma interferon production is associated with acute shigellosis. Infect. Immun. 65:5338-5341.
3. Raqib R, Ljungdahl Å, Lindberg AA, et al. **1996.** Dissociation between cytokine mRNA expression and protein production in *Shigella dysenteriae* type 1 infection. Eur J Immunol 26:1130-1138.
4. Raqib R, Ljungdahl Å, Lindberg AA, et al. **1996**. Local entrapment of IFN-γ in the recovery of *Shigella dysenteriae* type 1 infection. GUT 38:328-336.
5. Raqib R, Wretlind B, Lindberg AA, Ljungdahl Å, Andersson J. **1996**. Pathogenesis and immune responses in shigellosis. Annals of the New York Academy of Sciences;797:299-301.
6. Raqib R, Wretlind B, Andersson J Lindberg AA. **1995**. Cytokine secretion in acute shigellosis is correlated to disease activity and directed more to stool than to plasma. J Infect Dis. 171:376-384.
7. Raqib R, Lindberg AA, Wretlind B, Bardhan PK, Andersson U and Andersson J. **1995**. Persistence of local cytokine production in shigellosis in acute and convalescent stages. Infect Immun. 63:289-296.
8. Raqib R, Lindberg AA, Björk L, Bardhan PK, Wretlind B, Andersson U and Andersson J. **1995**. Down-regulation of gamma interferon, tumor necrosis factor type I, interleukin 1 (IL-1) type 1, IL-3, IL-4 and transforming growth factor β type I receptors at the local site during the acute phase of *Shigella* Infection. Infect Immun. 63:3079-3087.
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11. Raqib R, Tzipori S, Islam M and Lindberg A. **1993**. Immune responses to *Shigella dysenteriae* type 1 and *Shigella flexneri* lipopolysaccharide and polysaccharide antigens in Bangladeshi patients with shigellosis. Serol Immun Infect Dis. 1:37-45.
12. Islam MM, Azad AK, Bardhan PK, Raqib R and Islam D. **1994**. Pathology of shigellosis and its complications. Histopathology. 24:65-71.
13. Qadri F, Raqib R, Hussain IA, Ciznar I. **1990**. Cell surface proteins from *Shigella dysenteriae* type 1. Zentralblatt für Bakteriologie Mikrobiologie und Hygiene, Series A. 273:1287-1299.

Protocol No: 2002-005

Project title: Comparison of immune responses in children born with low birth weight, having intrauterine growth retardation and full term normal birth weight: a pilot study.

PI: Dr. Rubhana Raqib

The objective of the study is to investigate whether intrauterine growth retardation (IUGR) has a negative impact on subsequent immune status in children 4-6 years of age. The study takes the advantage of a birth cohort whose birth weights were measured in a previous study. The same cohort will also be used concurrently by another study that is looking at the effect of IUGR on subsequent cardiovascular and metabolic disorders. As a result this will be a very efficient study. I have following comments:

This is not a case control study as mentioned on the second page of the RRC application form. Children will be selected on the basis of IUGR, the exposure. Cohorts of children with and without IUGR will be followed over time (birth to 4/6 years) retrospectively to assess the effect of IUGR on subsequent immune status, the outcome. Therefore, this is a cohort study.

The interval between birth and the measurement of birth weight has been mentioned inconsistently. In some places it has been mentioned as 48 hours (page 5, 9 & 10), where as in another place as 72 hours (page 10). The investigators should remove the inconsistency.

The 3rd inclusion criteria or the 2nd exclusion criteria may be dropped for following reasons.

- A recall period of 3 months is too long for diseases or conditions like diarrhoea, fever or pneumonia. A recall of more than seven days for diarrhoea and more than a month of pneumonia is extremely unreliable and is likely to introduce serious bias.
- Incidence of diarrhoea, fever and pneumonia in children in this area is quite high and if children are excluded on the basis of history these illnesses in the past three months, then I think a small number of children will remain for inclusion into the study.
- Most importantly, this is going to introduce a selection bias. The exclusion of children with illnesses might preferentially exclude children with IUGR who are likely to be immune deficient and thus underestimate the effect of IUGR.

Since the investigators will try to identify the presence of acute infections by performing clinical examination and several tests, they can exclude them during analysis. They may increase the sample size to take account of this exclusion. This will also allow them to compare prevalence of acute infections, which may themselves be important outcomes that could indicate a deficient immune status. They may, however, include history of measles in their exclusion criteria, as measles is associated with prolonged depression cell mediated immunity.

The investigators could consider using skin tests with recall antigens to evaluate cell mediated immune response.

The heading of the 2nd column of the table of sample size should read "Expected levels..." and not "Expected reduction..." The sample sizes on the last column in this table have been calculated with a type II error of 10% and not with 20% as has been mentioned.

Micronutrient status (e.g., zinc and vitamin A) could be an important confounding variable that investigators might take into account. Other potential confounding variables could be BCG, DPT and measles vaccination status.