Low birth weight is associated with altered immune function in rural Bangladeshi children: a birth cohort study[1–3]

Rubhana Raqib, Dewan S Alam, Protim Sarker, Shaikh Meshbahuddin Ahmad, Gul Ara, Mohammed Yunus, Sophie E Moore, and George Fuchs

ABSTRACT

Background: Low birth weight is generally an outcome of a fetal insult or nutritional insufficiency. Recent studies have shown that such exposure early in life may have long-term implications for later immunocompetence and susceptibility to infectious diseases.

Objective: We aimed to investigate the effect of birth weight on immune function in preschool-age children.

Design: A birth cohort cross-sectional study was conducted in children ($n = 132$) aged 60.8 ± 0.32 mo who were born in Matlab, a rural area of Bangladesh, and whose weight and length were measured within 72 h of birth. The outcome measures were thymopoiesis, T cell turnover, acute phase response, and percentage of lymphocytes.

Results: Children born with low birth weight (<2500 g; LBW group, $n = 66$) had significantly higher concentrations of T cell receptor excision circles in peripheral blood mononuclear cells—a biomarker for thymopoiesis—and significantly higher serum bactericidal activity and C-reactive protein concentrations than did children born with normal birth weight (≥2500 g; NBW group, $n = 66$) ($P < 0.05$ for both). The LBW group children had significantly lower concentrations of interleukin 7 in plasma ($P = 0.02$), shorter telomere length in peripheral blood mononuclear cells ($P = 0.02$), and a lower percentage of CD3 T cells ($P = 0.06$) than did the NBW group children.

Conclusions: Greater peripheral T cell turnover (shorter telomeres and lower CD3 concentrations) due to immune activation (elevated C-reactive protein concentrations and bactericidal activity) may have resulted in a greater need for replenishment from the thymus (higher T cell receptor excision circles); these events may cause lower immune functional reserve in preschool-age children born with LBW. Thus, LBW has implications for immunocompetence and increased vulnerability to infectious diseases in later life. *Am J Clin Nutr* 2007;85:845–52.

KEY WORDS Low birth weight, T cell receptor excision circles, TRECS, telomere, CD3 T cells, C-reactive protein

INTRODUCTION

Low birth weight (LBW) can be the product of prematurity or of intrauterine growth retardation (IUGR). Whereas IUGR has several different causes, it often is the result of poor nutrition during pregnancy (1). Morbidity and mortality of LBW infants due to infectious diseases are known to be high (1), which has led researchers to explore the effect of LBW on immune function. Although many of the early studies focused on the depressed immune responses of LBW infants (2–7), more recent research suggests that these defects may persist beyond infancy (8, 9).

In a series of studies, Barker et al (10) introduced the hypothesis of the developmental origin of health and disease. This hypothesis postulates that conditions that most probably are nutritional program the fetus for development of chronic noncommunicable diseases in adulthood. In parallel, evidence is accumulating to suggest that early events may also program immune function. Studies in The Gambia in West Africa have shown associations between birth during the nutritionally poor "hungry" season and a greater risk of premature adult mortality due to infectious diseases (9). Further prospective studies in that population showed seasonal effects on thymic size (11) and function (12). A longitudinal study of adolescents in the Philippines provided evidence that prenatal undernutrition is significantly associated with low thymopoietin production and less growth in length during the first year of life and is positively associated with adolescent thymopoietin production (13). These findings add to a growing body of evidence that events occurring in utero or early in life may permanently affect components of the immune system, especially the thymus.

The aim of the current study was to explore the hypothesis that LBW may impair long-term immune function and that—in a tropical setting with high infectious exposure that will lead to persistent activation of the immune system, increased peripheral infiltration of thymus-derived naive T cells, and premature lymphocyte senescence—the effect may not become apparent until the later years of life. To test this hypothesis, we have assessed thymic output, T cell turnover, and lymphocyte proliferation

[1] From the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B): Centre for Health and Population Research, Dhaka, Bangladesh (RR, DSA, PS, SMA, GA, and MY); the MRC International Nutrition Group, London School of Hygiene and Tropical Medicine, London, United Kingdom (SEN); and the Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR (GF).

[2] Supported by World Bank Development Grant Facility for ICDDR,B and the Swedish Agency for Research Cooperation with Developing Countries (Sida/SAREC Agreement support; grant 2002-2004).

[3] Reprints not available. Address correspondence to R Raqib, Immunology Laboratory, Laboratory Sciences Division, ICDDR,B: International Center for Health & Population Research, Mohakhali, Dhaka-1212, Bangladesh. E-mail: rubhana@icddrb.org.

Received September 20, 2006.

Accepted for publication November 9, 2006.

response in peripheral blood lymphocytes of children from rural Bangladesh born at either LBW or a normal birth weight (NBW).

SUBJECTS AND METHODS

Study design

The study was conducted at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) field research station in Matlab, a typical rural and riverine delta area of Bangladesh, located ≈45 km from the capital, Dhaka. The current study selected participants from a cohort of infants born in Matlab during 1995 or 1996, full details on whom can be found elsewhere (14, 15). Birth weight was measured within 72 h of delivery by using a pediatric balance-beam scale (Seca, Hamburg, Germany) that is accurate to 10 g. The infant's recumbent length was measured by using a regularly validated, locally constructed length board accurate to the nearest 0.1 cm. Infants' weight and length measurements were compared with those of the National Center for Health Statistics reference population and converted to age- and sex-standardized *z* scores with the use of ANTHRO software [version 1.02 (Y2K-compliant); developed jointly by the World Health Organization, Geneva, Switzerland, and the Centers for Disease Control and Prevention, Atlanta, GA).

Study subjects

The current study aimed to compare children born as singletons and at full term with NBW (≥2500 g) with those born at LBW (<2500 g). Children who had known chronic illness or recent episode of measles were excluded from the study. The availability of the children for the current study was initially confirmed from the Matlab Health and Demographic Surveillance System records. A door-to-door survey was conducted to confirm the availability of the children; then, from the list of available children (whose families had not migrated), the required number of children was selected for the study.

Initial screening in Matlab villages found 358 children who were available. For the study, sample size was calculated based on 5% type I error and 20% statistical power, with consideration of several outcome variables, such as total lymphocyte count, total leukocyte count, percentage of CD4 and CD8 T cells, the ratio of CD4 to CD8 cells, and the concentration of total immunoglobulin G (IgG). Because comparison of the immune variables—eg, T cell receptor excision circles (TRECs), telomeres, and C-reactive protein (CRP)—that are important for functional outcomes between LBW and NBW children has not previously been reported, it was of interest to explore these variables. A difference of 5% in the outcome variables between the 2 groups was considered to be important. We calculated that 66 subjects/group would be sufficient for the proposed study. From the cohort of 358 children, systematic random sampling was done in which every 2nd child was selected (the first child was chosen randomly), and a total of 179 children were selected (**Figure 1**). Thereafter, children were stratified into 2 groups: one consisting of children born with LBW (LBW group, $n = 75$) and the other with NBW (NWB group, $n = 104$). From each of these 2 groups, 66 children were randomly selected. Stratification into 2 groups, blinding, and decoding of the subjects was done by a person without relation to the study.

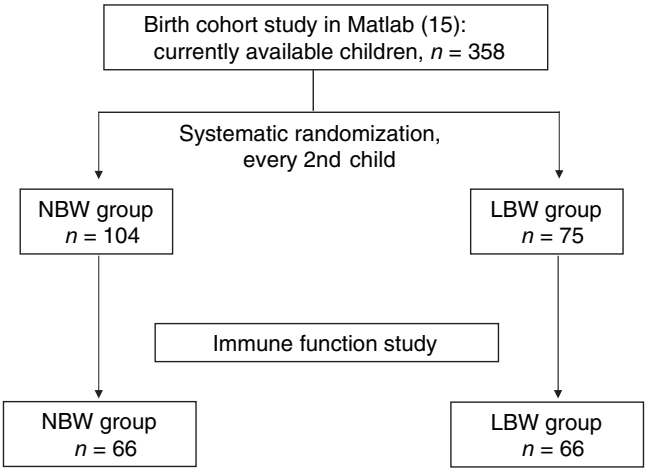

FIGURE 1. Profile showing the number of children in the birth cohort study (14, 15) who were assessed for availability for the immune function study and the numbers finally enrolled in the study.

Anthropometric measurements at follow-up [weight, height, and midupper arm circumference (MUAC), the mean of 2 measurements] were taken by trained field research assistants. Weight was measured with a portable electronic scale (SECA) to the nearest 100 g. Height was measured to the nearest 0.1 cm with a locally made, wooden height stick. Any history of diarrhea or acute respiratory infection (upper and lower respiratory tract infections including asthma) and skin infections within the past 2 wk or current infection were also recorded. All instruments were validated by the supervisor of the field research assistants before use. Physical examination was conducted, including body temperature, respiratory rate, heart rate, pulse, and blood pressure measurements. For anthropometric measurements at follow-up, standard guidelines were followed by using growth percentile curves from the National Center for Health Statistics.

Written informed consent was obtained from the guardian of each child. The study was approved by the ethics review committee of the ICDDR,B: Center for Health and Population Research.

Sample collection

Fasting peripheral venous blood samples were obtained from each child at enrollment in the Matlab hospital laboratory. Blood samples were collected in heparin-coated sterile vials (Vacutainer; Becton Dickinson, Rutherford, NJ) and were processed to isolate peripheral blood mononuclear cells (PBMCs) from blood on Ficoll-Paque (Pharmacia-Upjohn, Uppsala, Sweden) by density gradient centrifugation at 1800 rpm at 24 °C for 25 min (Sorvall Legend RT; Kendro Laboratory Products GmbH, Hanau, Germany). A portion of the PBMCs was used for the lymphocyte proliferation assay, and a portion was stored in liquid nitrogen until used for flow cytometry and DNA extraction. Plasma collected as supernatant from the gradient was stored at −70 °C until it was used. Serum samples were also collected, and they were stored at −70 °C.

C-reactive protein and interleukin 7

Plasma concentrations of the hepatically derived acute phase protein CRP were used to detect ongoing infection or inflammation. CRP concentrations were measured by immunoturbidometric assay with the use of a Hitachi 902 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Concentrations of

interleukin 7 (IL-7) in plasma were measured with a high-sensitivity colorimetric enzyme-linked immunosorbent assay (IL-7 immunoassay kit; R&D Systems QuantikineHS, Minneapolis, MN) according to the manufacturer's instructions. The lower limit of detection was 0.1 pg/mL. Plasma IL-7 concentrations were measured to assess the effect of the cytokine IL-7 on thymopoiesis or the T cell regeneration process.

Serum bactericidal response

Preterm and small-for-gestational-age (SGA) infants have low amounts of neutralizing antibodies (7). Because enteric diarrheal diseases are common in rural Bangladesh, the serum bactericidal response was evaluated against a nonpathogenic *Escherichia coli* K12 strain, which lacks the O surface antigens (16). The serum bactericidal assay similar to that used for *Shigella* was modified to optimize it for nonvirulent *E. coli* K12 (17). Bacteria grown in Mueller Hinton broth (MHB; Difco, Detroit, MI) at 37 °C for 3 h were suspended in saline to an absorption of 0.4 at 600 nm [1×10^8 colony-forming units (CFU)/mL], which was followed by serial dilution to a concentration of 1×10^5 CFU/mL. Guinea pig complement (1:5) was added to the bacterial suspension, and the mixture was applied to microtiter plates (Nunc, Roskilde, Denmark) containing serum samples, with a starting dilution of 1:10, that were complement-inactivated by heat treatment and serially diluted by 50%. The volume of 200 μL/well was adjusted with MHB, and the plate was incubated in a shaker incubator (200 rpm) at 37 °C for 16 h. The optical density of the plates was measured at 595 nm. The titer of the serum was defined as the reciprocal of the last dilution in which no growth was evident by visual inspection (17).

Lymphocyte proliferation response

The ability of lymphocytes to proliferate polyclonally on activation with mitogens provides a tool for the assessment of lymphocyte function. The assay was conducted in triplicate on microtiter plates as described previously (18). PBMC suspensions (3×10^5 cells/well) were incubated for 3 d in 37 °C and 5% CO_2 with the mitogen phytohemagglutinin [PHA (at 5 mg/L); Sigma, St Louis, MO) or without stimulant (RPMI 1640; Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol glutamine/L, 1 mmol Na-pyruvate/L, 100 U penicillin/mL, and 100 μg streptomycin/mL (Gibco). Cells were pulsed with [^{3}H]-thymidine and harvested on glass fiber filters 18 h later by using a multiwell cell harvester (Automesh 2000; Dynatech, Denkendorf, Germany). Thymidine incorporation into the fraction retained on the filter was measured by using liquid scintillation fluid (Ecolite+; MP Biomedicals, Solon, OH) in a β counter (LS 6500; Beckman Instruments, Irvine, CA) and expressed as counts per minute (cpm). The result per subject was expressed as the mean stimulation index (SI) of triplicates (cpm of stimulated cells/cpm of unstimulated cells).

Immunophenotyping

Quantifying lymphocyte subpopulations and measuring the extent of their activation by flow cytometry provide useful indicators of a person's immune status. T cell subsets were characterized by staining cells with fluorescent antibodies specific for various cell surface markers. Frozen PBMCs were thawed rapidly; after being washed, they were stimulated with phorbol 12-myristate 13-acetate (PMA) at 20 ng/mL in RPMI 1640 (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol glutamine/L, 1 mmol Na-pyruvate/L, 100 U penicillin/mL, and 100 μg streptomycin/mL (Gibco) for 4 h in 37 °C and 5% CO_2. After being washed in phosphate-buffered saline (PBS; pH 7.4), cells were suspended (1×10^5 cells/mL) in PBS containing 2% heat-inactivated pooled human AB serum and were stained with triple combinations of monoclonal antibodies for 30 min at 4 °C. Cells were washed and resuspended in PBS containing 1% paraformaldehyde and analyzed within 4 h. Negative controls were included in each experiment. Three-color fluorescent analysis was performed by using a fluorescent cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA). Fluorescein isothiocyanate– (FITC) labeled IgG2b and phycoerythrin-labeled IgG1 were used as isotype control. Before data acquisition, instrument settings were checked and optimized by using CaliBRITE beads (Becton Dickinson). Data acquisition and analysis were done with CELL QUEST software (version 3.3; Becton Dickinson). All samples were analyzed by setting appropriate forward- and side-scatter gates around the lymphocytes, and the percentage of positive cells was estimated.

Monoclonal antibodies

The following antibodies were used in the study for the phenotype analysis by 3-color flow cytometry: anti-CD45 (pan leukocyte), anti-CD14 (monocytes), IgG1, IgG2 (negative isotype control), anti-CD3 [pan T cells; FITC, peridinin chlorophyll protein (PerCP), and antigen-presenting cells (APC)], anti-CD4 (helper/inducer T cells and FITC), anti-CD8 (suppresser/cytotoxic T cells, phycoerythrin, and PerCP), anti-CD69 (early activation marker and PerCP), anti-CD45RA (naïve T cells and FITC), anti-CD45RO (memory T cells and phycoerythrin), anti-CD56 (neural cell adhesion molecules, pan-natural killer cells, and phycoerythrin), anti-CD16 (natural killer cells, Fcγ receptor III, and FITC). All antibodies were purchased from Becton Dickinson.

Quantification of signal-joint T cell receptor excision circles by real-time polymerase chain reaction

The thymus, a glandular organ, is the site of T cell differentiation and regeneration. Thymic T cell production can be assessed by measuring TRECs as a traceable molecular marker in newly produced naive T cells (19). Thus, the content of TRECs in peripheral T cells is an indicator of thymopoiesis or newly synthesized and exported naive T cells (19).

From the frozen whole-blood samples, DNA was isolated by using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Quantification of signal-joint (sj) TRECs was performed by using SYBR Green real-time quantitative PCR and an iCycler thermocycler (Bio-Rad version −3.021; Life Science Research Group, Hercules, CA). The following primers were used: [forward primer > 5' AAAGAGGGCAGCCCTCTCCAAGGCAAA 3′ and reverse primer < 5' AGGCTGATCTTGTCTGACATTTGCTCCG 3′]. Using natural 96-well plates that can be separated into 24- or 48-wells sections (semi-skirt; Nunc), a master mix was prepared that consisted of 12.5 μL Quantitect-SYBR Green (Qiagen), 0.5 μL forward and reverse primers each, 1.0 μL $MgCl_2$ (50 mmol/L), 1.0 μL of each primer pair, and 8.5 μL deionized water. Then, 23 μL of this master mix was added to the 96-well plate, which was followed by 2 μL of standards, samples, and

negative controls in corresponding tubes to obtain a 25-μL reaction volume. Real-time polymerase chain reaction (PCR) was then performed under the following conditions: denaturation (1 cycle) at 95 °C for 3 min; preamplification of 40–45 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 45 s; and a second amplification (final extension step) cycle at 72 °C for 5 min. A standard (donated by PT Ngom) was prepared by using serial dilutions of a known number of copies of a fragment of the sjTREC gene sequence and included in each iCylcler run to generate a standard curve (12). Serial dilutions of the cloned TREC-PCR product were used as a standard for absolute quantification of TREC concentrations. Samples were analyzed in duplicate. The number of copies of sjTRECs in the samples was automatically measured by reading off the standard curves generated and expressed as TREC content (ie, no. of copies) per 10^6 PBMCs.

Telomere length

Telomeres are specialized structures at the ends of chromosomes. The length of the telomeres was reduced progressively during each cell replication cycle, and, thus, telomere length was shown to reflect the replicative history of those cells (20). After extraction of DNA as above, DNA was rehydrated overnight at room temperature, quantified by absorbance spectrophotometry at 260-nm wave length, and stored at 4 °C. The mean length of the telomeric restriction fragment (TRF) was determined by using the TeloTAGGG Telomere Length Assay (Roche, Basel, Switzerland) and by Southern blot. After exposure of the blot to an X-ray film, the TRFs were visualized. For quantitative measurements of mean TRF length, the signal intensity was scanned and read by an imaging system with the use of QUANTITY ONE software (version 4.2.2; BioRad Laboratories). The mean TRF length was estimated as the midpoint of the smear on each lane relative to a molecular-weight standard that was run in each gel. Results are given as mean TRF length of the sum equation of the telomere signal distribution.

Statistical analysis

Statistical analyses were done by using SIGMASTAT statistical software (version 3.1; Jandel Scientific, San Rafael, CA) and SPSS for WINDOWS software (Release 10; SPSS Institute, Chicago, IL). Data were expressed as means ± SEs. Continuous variables were compared between groups by using the Student's *t* test, and categorical variables (ie, male-to-female ratio, concurrent diarrhea, fever, respiratory illness, breastfeeding status) were compared by chi-square test. $P < 0.05$ was considered significant. When a variable (eg, CRP, bactericidal antibody titers, proliferation response, or IL-7) was not normally distributed, an appropriate transformation was used to better achieve approximate normality. Analyses were performed on the transformed variables to meet the underlying assumptions of the statistical tests used. When the data could not be normalized, nonparametric analysis (a rank-sum test) was performed. Simple linear and multiple regressions (forward stepwise regression) were used to evaluate the relation between birth weight, nutritional status, and current immunologic variables. Only those variables were tested by the regression model, which showed significant differences between the 2 groups.

TABLE 1
Descriptive features at birth of study children who were born with normal (NBW) or low (LBW) birth weight[1]

Features	NBW group ($n = 66$)	LBW group ($n = 66$)	P
Gestational age (wk)[2]	39.55 ± 0.33[3]	39.33 ± 0.46	0.69
Weight-for-age (z score)	−1.18 ± 0.05	−2.29 ± 0.07	0.001
Height-for-age (z score)	−1.22 ± 0.07	−2.09 ± 0.1	0.001
Head circumference (cm)	32.97 ± 0.14	31.93 ± 0.15	0.001
Chest circumference (cm)	31.50 ± 0.16	29.50 ± 0.15	0.001
Length (cm)	47.70 ± 0.17	45.70 ± 0.22	0.001
Ponderal index (g/cm^3)[3]	17.34 ± 0.13	14.59 ± 0.154	0.001
Maternal weight (kg)[4]	45.60 ± 0.6	45.78 ± 0.7	0.80
Maternal MUAC (mm)[4]	228.30 ± 2	227.40 ± 2.4	0.80
EBF for 3 mo[4]	42/64[5]	48/66	0.49

[1] MUAC, midupper arm circumference; EBF, exclusive breastfeeding. Comparison between the 2 groups was done with Student's *t* test; $P < 0.05$ was considered significant.
[2] From mother's last menstrual period.
[3] Ponderal index = weight/(height^3).
[4] Measurements taken at 3 mo of pregnancy.
[5] Data on breastfeeding status of 2 children in the NBW group were missing.

RESULTS

Subjects

Descriptive features of the study children and their mothers at the time of the children's birth are shown in **Table 1**. No significant differences were found in gestational age at birth, maternal weight, and maternal MUAC at 3 mo of pregnancy. However, as expected, significant differences were obtained in z scores for weight-for-age and height-for-age, head circumference, chest circumference, length at birth, and ponderal index between the 2 groups at birth. The original birth cohort study showed that poor nutritional status in early pregnancy was the major determinant of LBW in rural Bangladesh (15). One limitation of the study is that, because infants were not followed from birth up to the age of 5 y, data on growth rate, morbidity, vaccination, nutritional status, and other factors were not available, and therefore the effect of those factors on current immune functions and association could not be studied (14). However, data on breastfeeding status were available. Current characteristics of children belonging to the 2 groups are given in **Table 2**. Proportions of males and females; current age; current weight-for-age, height-for-age, and weight-for-height z scores; body mass index (BMI; in kg/m^2); current diarrhea; current fever; and current respiratory illness did not differ significantly between the 2 groups. Because all children included in the study were immunized with vaccines (*Bacillus* Calmette-Guerin, diphtheria, pertussis, tetanus, oral polio vaccine, and measles) as part of the Expanded Program on Immunization (EPI), vaccination history could not be related to anthropometric measures or other immune functions. No significant differences in the current anthropometric measures and morbidity experiences were found between the exclusively breastfed and nonexclusively breastfed children (duration of exclusive breastfeeding: 3 mo or 6 mo). No differences were found in the current maternal nutritional status, height, or weight or annual family income. According to the growth reference of the National Center for Health Statistics, 58% of the LBW children and 57% of the NBW children were mildly (defined as <-1

TABLE 2
Current baseline features of children born with normal (NBW) or low (LBW) birth weight[1]

Features	NWB group ($n = 66$)	LBW group ($n = 66$)	P
Age (mo)	61.80 ± 0.40[2]	60.10 ± 0.48	0.85
Male/female ratio	32/34	41/25	0.16
MUAC (mm)	148.86 ± 1	151 ± 2.30	0.30
Weight-for-age (z score)	−2.20 ± 1	−2.30 ± 0.09	0.68
Height-for-age (z score)	−1.13 ± 0.10	−1.80 ± 0.60	0.24
BMI (kg/m^2)	12.60 ± 0.20	12.5 ± 0.10	0.85
Current diarrhea (n)[3]	9	14	0.35
Annual family income ($)	825 ± 104.50	759 ± 132.40	0.69
Maternal weight (kg)	43.31 ± 0.80	43.30 ± 0.90	0.90
Maternal height (cm)	155.86 ± 0.50	156 ± 1	0.90
Maternal MUAC (mm)	237.85 ± 2.50	236 ± 3	0.60

[1] MUAC, midupper arm circumference. Continuous variables were compared between the 2 groups by Student's t test; chi-square test was applied for categorical variables. No significant differences in the baseline features were found between the 2 groups. Current nutritional status in children was assessed through standard anthropometric measures by using the National Center for Health Statistics growth reference. Mild and moderate malnutrition in children was defined as <-1 but >-2 and <-2 but >-3 weight-for-height z scores, respectively. Ponderal index is weight/(height^3).

[2] $\bar{x} \pm$ SD (all such values).

[3] Mothers were asked at the time of the visit by trained field research assistants whether their children had had diarrhea within the past 2–3 d.

height-for-age z scores) to moderately (defined as <-2 but >-3 height-for-age z scores) malnourished.

C-reactive protein and bactericidal antibody titers

Because the variables (CRP and bactericidal antibody titers) were not normally distributed, analysis was performed on log-transformed data. Concentrations of CRP in plasma were significantly ($P = 0.02$) higher in the LBW than in the NBW group (**Table 3**). Regression analysis found no association between CRP concentrations and birth weight; birth length; current nutritional status as assessed by weight-for-age, height-for-age, and weight-for-height z scores; MUAC; and BMI.

The serum bactericidal response against nonpathogenic *E. coli* was significantly ($P = 0.03$) higher in the LBW group than in the NBW group (Table 3). However, no significant association was found between serum bactericidal response and CRP concentrations ($R = 0.13$, $P = 0.76$), nutritional status, or morbidity experiences.

Lymphocyte proliferation response

PBMCs from children were analyzed for the ability of lymphocytes to respond to mitogen (ie, PHA) by proliferation response (incorporation of tritiated thymidine). No significant difference between the 2 groups was found in the SI of lymphocytes to PHA (Table 3). No association was found between lymphocyte proliferation response and current nutritional status by regression analysis.

Proportion of pan T cells in low-birth-weight children

Flow cytometric analysis showed that the proportion of CD3 T cells in the peripheral blood of the LBW group children was not significantly lower than that in the NBW group children; however, the difference showed a trend ($P = 0.06$) (Table 3). No significant differences between the 2 groups were seen in the percentage of other phenotypic markers or activation markers studied, although a tendency was seen for the NK cell proportions to be lower in the LBW group than in the NBW (data not shown).

TABLE 3
Comparison of immune functions in 5-y-old children born at normal (NBW) or low (LBW) birth weight[1]

Features	NBW group	LBW group	P
C-reactive protein (mg/L)	0.10 (0.0–1.8)[2]	0.65 (0.1–2.6)	0.02
Bactericidal antibody titers	40 (20–40)	60 (40–160)	0.03
Proliferation (SI)[3]	15.8 (12.3–86.8)	100.9 (19–112)	0.80
CD3 lymphocytes (%)[4]	68.3 (64–75)	65.7 (58–69)	0.06
sjTRECs per 10^6 PBMCs	0.54 (0.25–0.76) × 10^5	0.95 (0.42–2.3) × 10^5	0.05
IL-7 (pg/mL)	215.7 (0–649.6)	48.4 (0–215.7)	0.02

[1] SI, stimulation index; sjTRECs, signal-joint T cell receptor excision circles; PBMCs, peripheral blood mononuclear cells; IL-7, interleukin 7. n in the NBW and LBW groups = 66 and 66, respectively, for C-reactive protein, bactericidal antibody titers, and IL-7; 49 and 51 for proliferation; 45 and 48 for CD3 lymphocytes; and 34 and 34 for sjTRECs. Student's t test was used for determination of P value. Differences were significant when $P < 0.05$. Because the variables C-reactive protein, bactericidal titers, SI, and IL-7 were not normally distributed, an appropriate transformation was used to better achieve approximate normality, and analyses were performed on the transformed variables. The bactericidal antibody titer of the serum was expressed as the reciprocal of the last dilution in which no growth was evident by visual inspection.

[2] Median; 25th and 75th percentiles in parentheses (all such values).

[3] SI is the ratio of counts per minute of phytohemagglutinin-stimulated cells to counts per minute of unstimulated cells.

[4] Percentage of pan T CD3 cells in gated lymphocyte cells.

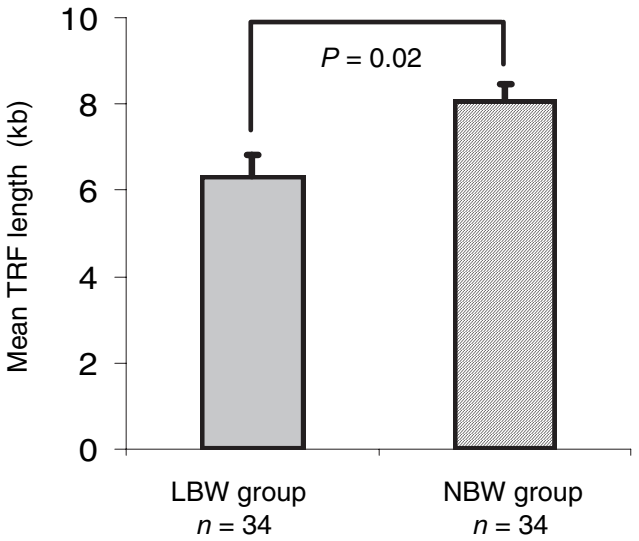

FIGURE 2. Mean (±SE) telomere restriction fragment (TRF) length in peripheral blood mononuclear cells (PBMCs) from low-birth-weight (LBW) and normal-birth-weight (NBW) children. The SE is expressed by error bars. Student's *t* test showed significantly shorter TRF lengths of the PBMCs in the LBW group than in the NBW group ($P = 0.021$).

Forward stepwise regression analysis was applied to predict CD3 from current nutritional status, birth weight, birth length, and values for IL-7 and TRECs. None of the variables significantly added to the ability of the equation to predict CD3, and they were not included in the final equation. Neither birth weight nor birth length was associated significantly with CD3 when these variables were forced into the model individually or in a linear combination.

Elevated T cell receptor excision circle concentrations in low-birth-weight children

Concentrations of TRECs were found to be significantly ($P = 0.05$) higher in the LBW group than in the NBW group (Table 3). Forward stepwise regression was applied to predict TRECs from birth weight, birth length, current nutritional status, and CD3 and IL-7 values. These variables did not add significantly to the ability of the equation to predict TRECs. Neither birth weight nor birth length was associated significantly with TRECs when these variables were forced into the model individually. TRECs values were measured from total PBMCs only, and, because of insufficient blood volumes, cells were not separated into CD4 and CD8 T cells or naïve and memory cells.

Shortened telomeric restriction fragment length in low-birth-weight children

The TRF length in PBMCs ranged from 3.8 to 9.4 kb; the mean length was 7.11 kb in all children. Significantly ($P = 0.02$) lower TRF values were seen in the LBW group than in the NBW group (**Figure 2**). Shortened telomere length in PBMCs indicated that cells have gone through more cell divisions in the LBW group. Because TRF was measured in total PBMCs, it was not possible to know whether the reduction in TRF involved only lymphocytes or both monocytes and lymphocytes. No association was found between the current nutritional status and the TRF length.

Lower plasma interleukin 7 concentrations in low-birth-weight children

Because the variable was not normally distributed, transformed data were used for statistical analysis. Plasma concentrations of IL-7 were significantly ($P = 0.02$) higher in the NBW group than in the LBW group (Table 3). A forward stepwise regression model was applied to predict IL-7 from birth weight, birth length, current nutritional status, and values for CD3 and TRECs. Birth weight was significantly associated with IL-7 when it was forced into the equation individually ($P = 0.008$), and IL-7 could be predicted significantly from a linear combination of birth weight ($P = 0.028$) and birth length ($P = 0.042$). In this model, birth weight was positively ($\beta = 0.528$) and birth length was negatively ($\beta = -0.318$) associated with IL-7, and the coefficient of determinants (*Rsqr*) obtained from the formula was 0.217. The variables current z scores for weight-for-age, height-for-age, and weight-for-height; MUAC; and TRECs did not significantly add to the ability of the equation to predict IL-7 and were not included in the final equation.

DISCUSSION

The data presented here suggest that LBW children of preschool age have a higher T cell turnover, as assessed by telomere length, TRECs concentrations, and T cell counts than do NBW children. We hypothesized that persistent immune activation in this setting with high levels of exposure to infectious diseases may have resulted in a greater need for thymic production of T cells, thereby generating a void in the T cell compartment.

Several studies have reported that infants with LBW or IUGR or who were SGA have a lower percentage of T or B lymphocytes and lower vaccine-specific IgG responses (2, 3, 21) than do newborn infants with NBW (6, 22–24). However, the persistence of these defects in older children has not been detected (25–27). In one study, schoolchildren born preterm had a significantly lower percentage of $CD4^+$T cells and lower CD4:CD8 ratios than did children born at term (28). We found that, at a mean age of 5 y, children who were born at full term but with LBW had a lower percentage of CD3 cells in peripheral blood than did NBW children. The lower percentage of $CD3^+$ cells in the LBW group could be a consequence of the limit of division having been met early in life through the accelerated apoptosis of lymphocytes (29). Increased apoptosis could cause increased proliferation in the periphery because lymphocytes strive to "fill the space" in the secondary lymphoid tissues. Indeed, a marked reduction in telomere length in PBMCs was observed in the LBW children; this reduction reflected higher T cell turnover, probably in response to a greater degree of apoptosis. Increased bystander T cell activation and proliferation can occur during HIV infections, and telomere shortening can result (30). Shorter telomeres have been seen in the gastric mucosa of patients with *Helicobactor pylori* infection than in the mucosa of healthy subjects or patients with gastric cancer (31). Premature telomeric loss also has been reported in rheumatoid arthritis (32). However, to our knowledge, this study is the first to show higher T cell turnover in apparently healthy children with LBW, irrespective of current nutritional status.

The role of the thymus as an important indicator of immunologic consequences of undernutrition has been brought into focus by several studies. Measurement of TRECs has been shown to be

a useful marker of thymic output or thymopoesis. Infants born during the "hungry" (ie, wet) season in rural Gambia were shown to have smaller thymuses and lower TREC concentrations than did infants born in the "harvest" season (11, 12). However, these infants also showed higher lymphocyte count and higher proportion of CD3 T cells. In the current study, the LBW group had higher TREC concentrations in PBMCs, lower percentages of CD3 T cell, and shorter TRF lengths than did the NBW group, albeit lymphocyte proliferation response was similar in the 2 groups. Higher thymic output could have resulted from the heightened demand for T cells in response to high burden of infections in the endemic setting of rural Bangladeshi villages. In HIV-infected subjects, chronic T cell turnover leads to abnormally shortened telomere length (30). The increased clonal exhaustion of cytolytic T cells and reduced cytolytic function in HIV infection have been attributed to replicative senescence. It is conceivable that higher TREC values and shorter telomere length in LBW children than in NBW children may be a consequence of a smaller initial pool of T cells in the thymus, which may lead to earlier exhaustion to maintain homeostatic T cell numbers for rapid replication of memory cells.

IL-7 plays an important role as a regulator of T cell homeostasis. Studies in The Gambia showed markedly higher TREC concentrations in breastfed infants whose mother's milk had higher IL-7 concentrations than in breastfed infants whose mother's milk had lower IL-7 concentrations (12). In HIV-infected patients, high IL-7 concentrations are produced in response to T cell depletion (33). The administration of exogenous IL-7 to mice increased the number of TREC-bearing cells (34), although exogenous IL-7 did not always enhance thymic function (35). We found that lower plasma IL-7 concentrations in the LBW children were concurrent with high TREC values. Low IL-7 concentrations in the LBW group may be related to the inability of the periphery to keep up homeostatic proliferation, which requires greater thymic output. An inverse relation between TREC and plasma IL-7 concentrations in HIV-infected children has been reported (36). A positive association of birth weight with plasma IL-7 concentrations also may partly explain low IL-7 concentrations in the LBW children.

In the current study, no association was observed between current nutritional status and any of the markers of immune function, which is consistent with the findings in the Gambian children (37). The current morbidity experiences of the 2 groups (eg, history of diarrhea, respiratory illness, and fever) were not significantly different. Concentrations of CRP found in LBW children were higher than those in NBW children; however, these concentrations were within the "normal" range—ie, they did not indicate the presence of acute inflammation. Numerous large-scale, prospective studies have found that low-grade inflammation, as represented by elevated concentrations of CRP in serum in the range traditionally accepted as normal (<6 mg/L), is an independent predictor of cardiovascular events, especially coronary heart disease (38). In addition, an inverse relation between birth weight and CRP was found: LBW contributed to elevated CRP concentrations in adult life (39) and predicted an elevated risk of cardiovascular events in adulthood (40). In the current study, no association was obtained between CRP concentrations and current nutritional status. However, birth weight had a marked effect on CRP concentrations ($P = 0.051$) when birth length was included in the multivariate analysis. In the LBW group, CRP concentrations tended to be higher in children with a history of ongoing infections ($P = 0.055$). Thus, elevated innate markers (ie, CRP and bactericidal activity) and telomere shortening could indicate greater stimulation of the immune system in the LBW group. Heightened innate immunity in the LBW group may be a counterbalance for altered cellular immunity.

A major limitation of the study is that the children were not followed from birth up to the current age, and data on growth rate, morbidity, vaccination and other factors that could potentially confound the results were not available; therefore, the effect of these factors on immune functions and the association could not be studied (14). However, despite these limitations, the findings from the current study suggest that small size at birth may result in altered immune function in later life, which provides further support for the hypothesis that events early in life or in utero may leave a permanent imprint on human immune function. The findings may also reflect a postnatal effect whereby LBW may be a marker for later exposures. It is plausible that the altered immunity may not be manifest in measurable morbidity outcomes unless the person is exposed to repeated infections; however, this validity of that possibility has yet to be seen.

We are indebted to the parents of the subjects for their cooperation and their permission for their children to participate in the study.

RR was responsible for the conception and design of study and for securing funding, implementing the study, supervising the laboratory experiments, compiling and analyzing the data, and drafting the manuscript. DSA (Principal Investigator of the birth cohort study in Matlab from which baseline data of current study subjects and respective mothers at birth were provided) contributed to the implementation of the study. PS processed specimens and performed flow cytometry–associated experiments and fluorescence-activated cell sorter FACS data analyses. SMA contributed to the statistical analysis of the data. GA processed specimens, performed laboratory experiments, and collected data; MY contributed to the implementation of the study and to recruitment of subjects in Matlab. SM contributed to the application of the T cell receptor excision circle method and analysis of the data. GF contributed to the study design and participated in securing funding. None of the authors had a personal or financial conflict of interest.

REFERENCES

1. Valero De Bernabe J, Soriano T, Albaladejo R, et al. Risk factors for low birth weight: a review. Eur J Obstet Gynecol Reprod Biol 2004;116:3–15.
2. Chatrath R, Saili A, Jain M, Dutta AK. Immune status of full-term small-for-gestational age neonates in India. J Trop Pediatr 1997;43:345–8.
3. Das S, Sanyal S, Banerjee U, Basu K. Humoral immunity status in neonates born to pre-eclamptic toxaemia mothers. J Indian Med Assoc 1998;96:77–9.
4. Ferguson AC. Prolonged impairment of cellular immunity in children with intrauterine growth retardation. J Pediatr 1978;93:52–6.
5. Hasselbalch H, Jeppesen DL, Ersboll AK, Nielsen MB. Thymus size in preterm infants evaluated by ultrasound. A preliminary report. Acta Radiol 1999;40:37–40.
6. Moscatelli P, Bricarelli FD, Piccinini A, Tomatis C, Dufour MA. Defective immunocompetence in foetal undernutrition. Helv Paediatr Acta 1976;31:241–7.
7. Saha K, Kaur P, Srivastava G, Chaudhury DS. A six-months' follow-up study of growth, morbidity and functional immunity in low birth weight neonates with special reference to intrauterine growth retardation in small-for-gestational-age infants. J Trop Pediatr 1983;29:278–82.
8. McDade TW, Beck MA, Kuzawa C, Adair LS. Prenatal undernutrition, postnatal environments, and antibody response to vaccination in adolescence. Am J Clin Nutr 2001;74:543–8.
9. Moore SE, Cole TJ, Collinson AC, Poskitt EM, McGregor IA, Prentice AM. Prenatal or early postnatal events predict infectious deaths in young adulthood in rural Africa. Int J Epidemiol 1999;28:1088–95.

10. Barker DJ. In utero programming of chronic disease. Clin Sci (Lond) 1998;95:115–28.
11. Collinson AC, Moore SE, Cole TJ, Prentice AM. Birth season and environmental influences on patterns of thymic growth in rural Gambian infants. Acta Paediatr 2003;92:1014–20.
12. Ngom PT, Collinson AC, Pido-Lopez J, Henson SM, Prentice AM, Aspinall R. Improved thymic function in exclusively breastfed infants is associated with higher interleukin 7 concentrations in their mothers' breast milk. Am J Clin Nutr 2004;80:722–8.
13. McDade TW, Beck MA, Kuzawa CW, Adair LS. Prenatal undernutrition and postnatal growth are associated with adolescent thymic function. J Nutr 2001;131:1225–31.
14. Alam DS. Maternal and child nutrition in rural Bangladesh: special reference to effect of dietary fat supplementation on vitamin A status. Wageningen, Netherlands: Division of Human Nutrition and Epidemiology, Wageningen University, 2001.
15. Alam DS, Yunus M, Aziz KMA, et al. Birth weight and its association with maternal nutrition and socioeconomic variable in rural Bangladesh. J Diarrhoeal Dis Res 1998;16:21–57.
16. Lederberg J. *E. coli* K-12. Microbiol Today 2004;31:116.
17. Rahman MJ, Sarker P, Roy SK, et al Effects of zinc supplementation as adjunct therapy on the systemic immune responses in shigellosis. Am J Clin Nutr 2005;81:495–502.
18. Raqib R, Roy SK, Rahman MJ, et al. Effect of zinc supplementation on immune and inflammatory responses in pediatric patients with shigellosis. Am J Clin Nutr 2004;79:444–50.
19. Douek DC, McFarland RD, Keiser PH, et al. Changes in thymic function with age and during the treatment of HIV infection. Nature 1998;396:690–5.
20. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990;345:458–60.
21. Golebiowska M, Kardas-Sobantka D, Chlebna-Sokol D, Sabanty W. Hepatitis B vaccination in preterm infants. Eur J Pediatr 1999;158:293–7.
22. Arinola OG, Obisesan KA, Afolabi K, Salimonu LS. Complement levels and leucocyte phagocytosis in newborn babies. Afr J Med Med Sci 2003;32:401–4.
23. Kiss S, Walcz E, Revesz T, Bors Z, Schuler D. Lymphocyte subpopulations in peripheral blood of small-for-gestational age and appropriate-for-gestational age preterm neonates. Acta Paediatr Hung 1984;25:291–7.
24. Singh M, Manerikar S, Malaviya AN, Premawathi, Gopalan R, Kumar R. Immune status of low birth weight babies. Indian Pediatr 1978;15:563–7.**s**
25. Belloni C, Chirico G, Pistorio A, Orsolini P, Tinelli C, Rondini G. Immunogenicity of hepatitis B vaccine in term and preterm infants. Acta Paediatr 1998;87:336–8.
26. Khalak R, Pichichero ME, D'Angio CT. Three-year follow-up of vaccine response in extremely preterm infants. Pediatrics 1998;101:597–603.
27. Moore SE, Collinson AC, Prentice AM. Immune function in rural Gambian children is not related to season of birth, birth size, or maternal supplementation status. Am J Clin Nutr 2001;74:840–7.
28. Pelkonen AS, Suomalainen H, Hallman M, Turpeinen M. Peripheral blood lymphocyte subpopulations in schoolchildren born very preterm. Arch Dis Child Fetal Neonatal Ed 1999;81:F188–93.
29. Barg E, Gasiorowski K, Brokos B, Swiedrych A, Skorkowska K. A high frequency of apoptosis was found in cultures of lymphocytes isolated from the venous blood of children born with a low birth weight. Cell Mol Biol Lett 2004;9:135–43.
30. Dagarag M, Ng H, Lubong R, Effros RB, Yang OO. Differential impairment of lytic and cytokine functions in senescent human immunodeficiency virus type 1-specific cytotoxic T lymphocytes. J Virol 2003;77:3077–83.
31. Kuniyasu H, Kitadai Y, Mieno H, Yasui W. *Helicobactor pylori* infection is closely associated with telomere reduction in gastric mucosa. Oncology 2003;65:275–82.
32. Schonland SO, Lopez C, Widmann T, et al. Premature telomeric loss in rheumatoid arthritis is genetically determined and involves both myeloid and lymphoid cell lineages. Proc Natl Acad Sci U S A 2003;100:13471–6.
33. Napolitano LA, Grant RM, Deeks SG, et al. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat Med 2001;7:73–9.
34. Okamoto Y, Douek DC, McFarland RD, Koup RA. Effects of exogenous interleukin-7 on human thymus function. Blood 2002;99:2851–8.
35. Chu YW, Memon SA, Sharrow SO, et al. Exogenous IL-7 increases recent thymic emigrants in peripheral lymphoid tissue without enhanced thymic function. Blood 2004;104:1110–9.
36. Resino S, Correa R, Bellon JM, Munoz-Fernandez MA. Preserved immune system in long-term asymptomatic vertically HIV-1 infected children. Clin Exp Immunol 2003;132:105–12.
37. Moore SE, Morgan G, Collinson AC, Swain JA, O'Connell MA, Prentice AM. Leptin, malnutrition, and immune response in rural Gambian children. Arch Dis Child 2002;87:192–7.
38. Arroyo-Espliguero R, Avanzas P, Cosin-Sales J, Aldama G, Pizzi C, Kaski JC. C-reactive protein elevation and disease activity in patients with coronary artery disease. Eur Heart J 2004;25:401–8.
39. Sattar N, McConnachie A, O'Reilly D, et al. Inverse association between birth weight and C-reactive protein concentrations in the MIDSPAN Family Study. Arterioscler Thromb Vasc Biol 2004;24:583–7.
40. Barker DJ. Fetal origins of cardiovascular disease. Ann Med 1999;31(suppl):3–6.