Development of Immune response in different age groups of children from Birth to 2 years of age

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To whom it may concern

This is to certify that the research work embodying the results reported in this thesis entitled " Development of Immune response in different age groups of children from Birth to 2 years of age" submitted by **Nowrin Nowshaba**, has been carried out under co-supervision in the Immunology Laboratory of the Centre for Vaccine Sciences at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.

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DEDICATED TO

MY BELOVED FAMILY

ABSTRACT

Human neonates are markedly more susceptible to infection than are older children or adults. This increased susceptibility is generally believed to be due to immaturity of the immune system to combat pathogens in both quantitative and qualitative terms. Therefore, it is important to understand the pattern of immune response present at early age in children. This study was conducted to observe the development of the immune system in newborns and infants of different ages. For this purpose, 50 participants were selected and divided into five groups as newborns, 6, 12 , 18 and 24 months of age (for each group, the sample size was n=10). The T cell proliferation response to tetanus toxoid (TT) was considerably low in newborns than in children of other age groups whereas differences in PHA response were not significantly different. The distribution of T-cell and B-cell specific surface proteins clusters of differentiation (CD) markers on lymphocytes of participant newborns and infants were analyzed, The percentage of $CD3^+$ T cells were significantly less in newborns than in children 6 months ($P< 0.0001$),12 months ($P< 0.0001$),18 months ($P= 0.001$) and 24 months ($P< 0.0001$) old children while age-dependent gradual decrease was seen in % of $CD3^+CD4^+$ (T helper cells) and % of $CD3^+CD8^+$ (cytotoxic T cells) and their ratio ($CD4^+/CD8^+$) where % of $CD19^+$ B cells were much elevated in older age groups than in newborns. Cholera toxin (CT) of *Vibrio cholerae* specific plasma IgA response was found notably low in newborns than in 6 months ($P= 0.0003$), 12 months ($P= 0.0001$), 18 months ($P= 0.0001$), and 24 months ($P= 0.0001$) aged children. When compared with 6 month old infants with those that were 12 months ($P= 0.02$), 18 months ($P= 0.029$), and 24 months of age ($P= 0.007$) the CT- IgG response was elevated in 24 months children than newborns ($P< 0.0001$) and 6 months infants ($P= 0.03$). Similarly the IgA and IgG response was seen against the heat labile toxin (LT) of *Escherichia coli*. Significantly high tetanus toxoid (TT) specific IgG response was seen in newborns than in those 6 months ($P= 0.04$), 12 months ($P= 0.02$), 18 months ($P= 0.007$) and 24 months ($P= 0.02$) of age. In contrast, measles virus specific IgG was distinctly high in 6 months ($P= 0.001$), 12 months ($P= 0.03$), 18 months ($P= 0.009$) and 24 months ($P= 0.015$) old children when compared to newborns using cord blood analyses. A base line level vibriocidal antibody response was observed in the study children. The fecal IgA response was comparable among the infant' groups where

heat-labile toxin (LT) fecal IgA was found to be lower in the 6 and 18 months old children, although it was significantly elevated in those 12 and 24 months old children (P= 0.021). This study demonstrated that the maturation of the immune system is mirrored in cellular and humoral immune responses to specific antigens and minimizes the developmental limitations with growing age during infancy.

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ABS	**Human AB blood group serum**
BSA	Bovine serum albumin
BD	Becton Dickinson
BHIB	Brain-heart infusion broth
CBMC	Cord blood mononuclear cell
CD	Cluster of differentiation
CT	Cholera toxin
CTB	Cholera toxin B subunit
ELISA	Enzyme linked Immunosorbant assay
FACS	Fluorescent activated cell sorting
GM1	Monosialosyl ganglioside
HRP	Horse Radish Peroxidase
Ig	Immunoglobulin
LT	Heat labile toxin
LTB	Heat labile toxin B subunit
ml	Mililiter
nm	Nanometer
OD	Optical Density
OPD	Orthophenylene diamine
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered Saline
PHA	Phytohemaglutinin

rpm	Revolution per minute
TT	Tetanus toxoid
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background

For the newborn, birth is a major physiological event, with its transition from a state of intrauterine environment to one of *ex utero* independence. At this time, the immune system faces the challenge of transferring from a sterile environment, in which the responsibility is on avoiding responsiveness, to a world of microbes where protection is vital.

The neonate, whether premature or of normal gestational age, is a unique host from an immunologic perspective. Many components of the immune system function less well in neonates compared with adults, giving rise to the concept of an "immunodeficiency of immaturity"{Schelonka, 1998 #290}, although it has recently been confirmed that the neonatal immune system not to be immature but rather specifically adapted for early stages of life {Wood, 2011 #291}.

Considering the importance of neonatal immune capability in influencing predisposition to infection, till date, little work has been done on adaptation of the immune system to these changed circumstances.

However, several factors are conspiring to focus attention on neonatal immunology. **First**, the belief that neonatal exposure to antigen results in tolerance rather than immunity has been challenged {Ridge, 1996 #160}. **Second**, in reducing infant morbidity and mortality, development of effective neonatal vaccines is likely to be extremely important {Marshall-Clarke, 2000 #7}. **Third**, recent evidence suggests that exposure to high levels of antigen during early life might contribute to the increasing prevalence of various diseases in adulthood.

The greatest burden of morbidity and mortality from infectious diseases occurs in children under 5 years of age. Of the 8.2 million under-five child deaths per year globally, about 3.3 million occur during the neonatal period, in the first four weeks of life, with the highest rates occurring in resource-poor countries. It is especially in Africa and South Asia that the least progress in reducing neonatal deaths has been made {Imtiaz Jehan, 2009 #206}.

As Millennium Development Goals (MGDs) adopted by the United Nations (UN) in 2000, aim to decrease by two-thirds child deaths worldwide by 2015, therefore, efforts are focused on reducing neonatal deaths in high-mortality countries to achieve the goal.

Furthermore, vaccines designed/optimized for early life immunization may reduce infant mortality and morbidity. Efforts should be made to promote its use at a global level.

1.2 Immune System - An Overview

The immune system is a complex array of defense mechanism that relies mainly on the ability to distinguish between the host and foreign elements. It is composed of specific cells, soluble mediators and specialized organs to protect our body from foreign pathogens. The immune response initially recognizes the type of pathogen and secondarily must mount a protective immune reaction to eliminate the pathogen {Roitt I, 2001 #154} {Adkins B, 2004 #16}.

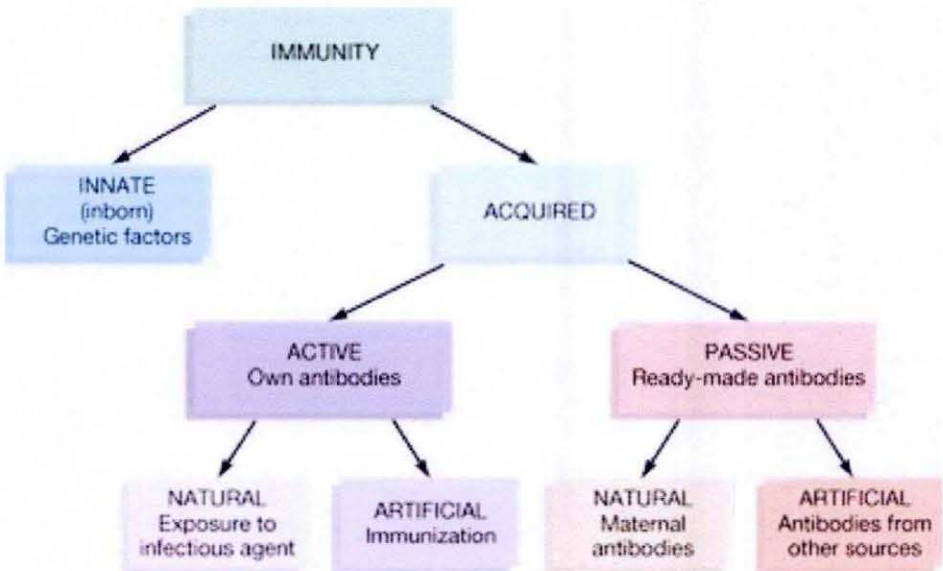

Figure 1.1: Divisions of immunity- Innate and Adaptive

[http://www.biology.arizona.edu/immunology/tutorials/immunology/page3.ht

Immune system composed of mainly two types of immunity

- Innate or non specific immunity
- Adaptive or specific immunity

1.2.1 Innate immunity

The innate immune system is made up of many cells, humoral factors, and the surface barrier, and it is a universal and evolutionarily ancient form of host defenses{Trinchieri, 1995 #95}{Underhill, 2002 #97}{Gantner, 2003 #98}{Moynagh, 2005 #99}{Chirico, 2005 #163}. However, most cells of the innate immunity contribute to the antigen processing and presentation {La Pine TR, 2004 #165} {Zaghouani, 2009 #171}.

Innate immune responses are triggered by bacteria, viruses, protozoa, and fungi, as non-self, and involve nonspecific activation of different cells and complement {Marodi, 2006 #11} (Fig-1.2).

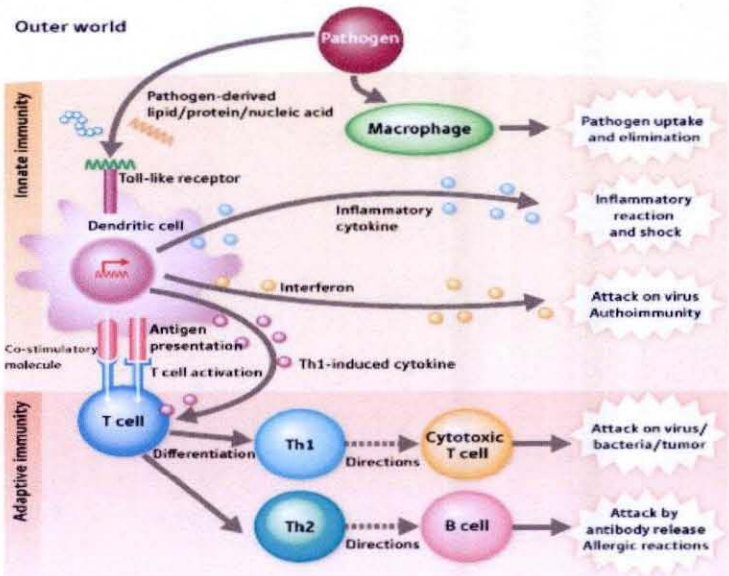

Figure 1.2: Distinct though mutually coordinated Innate and adaptive immunity http://www.rikenresearch.riken.jp/eng/frontline/5028

1.2.2 Adaptive immunity

The adaptive immune system has evolved to protect humans from a continuous onslaught of infectious microbes, and the system must respond to an enormous diversity of foreign antigens while avoiding accidental responses against self antigens. T and B lymphocytes, antibodies, natural killer cells, colony stimulating factors, T cell derived effector and immunoregulatory cytokines, and Interferon-γ, and are the main components of the adaptive immunity {Chirico, 2005 #163}. However, innate recognition of pathogens is the first step in inducing adaptive immunity {Janeway, 2002 #112} and producing memory which ensures both stronger and more rapid immune response to the antigen during re-exposure even years after the initial encounter {David A. Randolph, 2005 #211}.

1.3 Development of Immune Response

The first stage of human fetal haemopoiesis occurs in the mesoderm of the yolk sac and the extra embryonic mesenchymal tissue {Holt, 2000 #83}. After birth, there is an age-dependent maturation of the immune response. Of note, prenatal and postnatal exposure to environmental microbial products that can activate innate immunity might accelerate this maturation process, particularly if the exposure occurs repeatedly over time {Ng, 2006 #148}. Defects of neonatal adaptive immunity which requires experience with antigens, defects in innate immunity are more elusive as, in contrast to adaptive immunity, the innate immune system does not require immunologic experience to function well {Marodi, 2006 #2}.However, more recent experiments have proven that, under the right conditions, the neonatal immune system generates responses similar in strength and quality to those of adults{David A. Randolph, 2005 #211}.

Table 1.1: Some functional development during immune system development {Paul A. Offit, 2006 #138}

Function	Difference during infancy	Implication
Non Specific immunity	Phagocytes cannot migrate towards infectious sites, although their bactericidal activity is normal.	Slow response to infection
Cytokine production	Poor production of cytokines, in particular Th1 cytokines such IFNγ by T-cells.	Impaired responses of other cell populations that rely on their functions such as natural killer cells.
Natural killer T cell cytotoxicity (killing)	Is incomplete, probably caused by immaturity in cytokine production of T cells and monocytes	Inefficient killing of viruses
Complement system	Develops progressively during the first year of life	Inefficient phagocytosis
Specific immunity (T-cells and B-cells)	Naïve T and B cells first appear in key organs from an early point in fetal development	Repeated antigenic stimulation leads to the complete maturation of specific immunity during the first few years of life.
Immunoglobulin (Ig or antibody) production	Impaired production of some isotypes. Low serum IgM, IgA and IgE. IgG mostly of maternal origin.	Inability to respond to polysaccharide encapsulated bacteria until about 2 years of age.
Maternal antibody protection from placenta	IgG against some infectious organisms crosses the placenta. Wanes during first year of life.	Gives protection against some infections that mother exposed to or immunized against including measles and meningococcal disease. Can interfere with vaccines such as MMR.
Maternal protection from breast milk	Mostly IgA	Provides additional protection against gut microbes, less effective against respiratory infections

1.4 Neonatal Immune System

The infants' immune system is intact but immature at birth. The fetal and neonatal immune systems are associated with physiological demands that are three-fold {Levy, 2007 #15}.

First, protection against infection, including viral and bacterial pathogens at the maternal–fetal interface {Klein, 2001 #140}{McDonagh, 2004 #139};

Second avoidance of potentially harmful pro-inflammatory/T helper 1 (TH1)-cell polarizing responses that can induce alloimmune reactions between mother and fetus {Makhseed, 2001 #141} and

Third mediation of the transition between the normally sterile intra-uterine environment to the foreign antigen-rich environment of the outside world, including primary colonization of the skin{Marchini, 2005 #142} and intestinal tract{Karlsson, 2002 #143}by microorganisms.

The neonates are markedly more susceptible to infection than are older children or adults {Lewis DB, 1995 #120}{ME, 1978 #121}. Hence, a number of hypotheses have been proposed to explain this phenomenon including:

- antigenic inexperience {Chirico, 2005 #163},
- a lack of pre-existing antibody; {Trivedi, 1997 #119},
- decreased complement levels {Lewis DB, 1995 #120}, {Baker, 1976 #117} deficient phagocytic cell function {Weston, 1977 #123},
- immature T cell function {Lewis DB, 1995 #120} {Zola, 1995 #124},
- decreased numbers of CD45RO1 cells {Clement, 1988 #127} {Pirenne, 1992 #128} and
- the prevalence of suppression factors {Chirico, 2005 #163}.

1.4.1 Neonatal Innate Immune Response

Given the limited exposure to antigens in utero and the well-described defects in neonatal adaptive immunity{Adkins B, 2004 #16}, newborns must rely on their innate immune systems for protection to a significant extent{Krishnan, 2003 #144}{Firth, 2005 #145}. Biasing fetal immunity toward Th2 polarization than Th1 {Firth, 2005 #145} {Marodi, 2006 #2} appears to be an evolutionary adaptation orchestrated via production of cytokines and other regulatory molecules to protect placental and fetal damage through spontaneous abortion. {Nicolaos Vitoratos, 2006 #146}{Roth, 1996 #103}{Szekeres-Bartho, 2002 #106}{Piccinni, 1995 #104}. In contrast, predominant production of Th2 cytokines in fetal and neonatal life plays a key role in damping the newborn's innate immune responses. {Wegmann, 1993 #101}.

1.4.2 Neonatal Adaptive Immune Response

As the innate immune system can instruct the adaptive immune response {Janeway, 2002 #112}, distinct functional expression of neonatal innate immunity, including a bias against Th1-cell-polarizing cytokines, contributes to a distinct pattern of neonatal adaptive immune responses {Levy, 2007 #15}. Passively acquired antibodies can alter the humoral and antibody-dependant (B cell mediated) response to immunogens for up to 18 months in infants of infected or immunized mothers; in contrast, cellular immune responses (T cell mediated) appear unaffected by maternal antibody {Siegrist, 1998 #39} {Siegrist, 1998 #20}.

Adaptive immune response is generally mediated by two distinct mechanisms in neonates like the adults .They are;

- ✓ Cellular or T cell mediated adaptive immune response
- ✓ Humoral or B cell mediated adaptive immune response

1.4.2.1Cellular or T cell mediated neonatal adaptive immune response

The neonatal T cells are generally naïve. The fetal-neonatal predominant functionally immature naïve phenotype is likely to be related to the absence of antigenic stimulation in the germ free intrauterine environment{Gasparoni, 2003 #170}{Clement, 1988 #127}{Pirenne, 1992 #128} Neonatal T cells are easily induced to proliferate by homeostatic signals, but they appear to be more difficult to activate fully through the TCR due to reduced density of surface TCR complexes, CD40-ligand expression{DB., 2004 #167} and lack of APC interaction molecules{Schonland, 2003 #213}{Harris, 1992 #173} resulting in delayed differentiation of naïve T cell into Th1 effector cells and poor cytokine production{Bryson, 1980 #92}. Moreover, induction of cytotoxic T lymphocyte (CTL) response is age-dependant and is impaired in infants {Chiba, 1989 #132}, though maturation appears to occur within the first year of life {Siegrist, 2001 #1} (Fig 1.3).

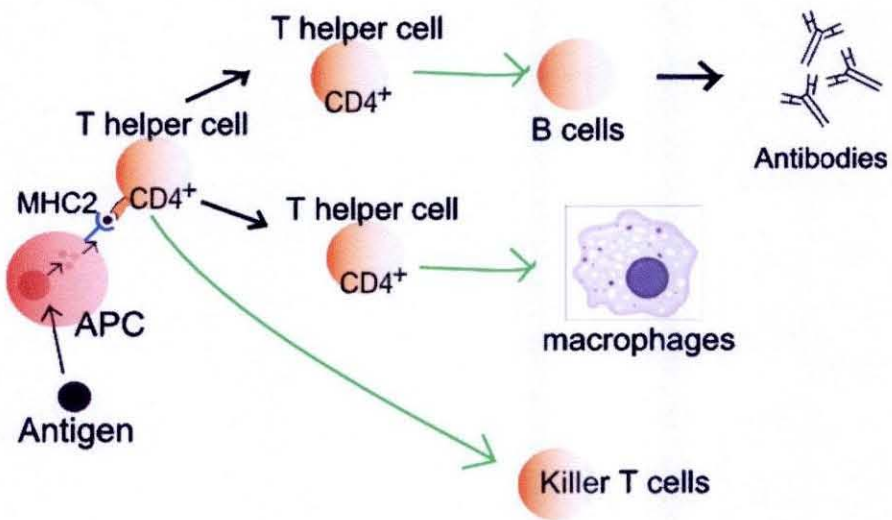

Figure 1.3: Mechanisms of T cell mediated cellular and B cell mediated humoral immune response [http://en.wikipedia.org/wiki/File:Lymphocyte_activation_simple.png]

1.4.2.2 Humoral or B cell mediated neonatal adaptive Immune response

In the neonatal period, humoral or B cell mediated adaptive immune response i.e antibody response is relatively weak as infants largely depend on transplacentally acquired maternal IgG during gestation and IgA from breast milk {Marodi, 2010 #12} (Fig 1.3). However, three potential problems are consistently found that may contribute to the overall poor antibody responses;

1) T-cell help may be inadequate due to down regulated CD40 ligand leading to impaired immunoglobulin class switching {Jullien, 2003 #218}{Han, 2004 #219}{Stavnezer, 1996 #179}.

2) Neonates have relatively low levels of complement, and B cells complement receptors {Siegrist, 2001 #1}.

3) The lymphoid structures necessary to optimize B-cell function may not be operational in the neonatal period {David A. Randolph, 2005 #211} resulting in low avidity antibodies.

In contrast, there are some potential evolutionary factors that limit plasma-cell differentiation in early life {Siegrist, 2009 #26}. These are as follows:

• Antibodies of maternal origin decrease the antigen load and provide protection, so there is no drive for plasma-cell differentiation

• Preventing plasma-cell differentiation until memory B cells have been primed and/or reactivated could limit potentially harmful cross-reactive responses to self polysaccharides and glycosylated proteins during the vulnerable periods of fetal and postnatal development

• As a mechanism to limit responses against prevalent but harmless environmental antigens (resource sparing)

• As a mechanism to prepare the post-weaning period for prompt reactivation in case of repeat exposure.

1.5 Maturation of Immune System with Age

Neonatal age is characterized by a delicate process of adaptation from intra- to extra-uterine life. The immune system is particularly subject to problems of adaptation to the new environment. Neonates differ from adults at virtually every step toward an effective immune responses.

The most important difference between neonatal responses and adult responses is the inability to mount rapid {Holt, 2000 #83}, strong memory responses {David A. Randolph, 2005 #211}because neonates are immunologically naı¨ve having fewer antigen-specific T-cell precursors, hard to stimulate with immature APCs {Jaspan, 2006 #135} {Hassan, 1996 #24} {Shearer, 2003 #25}and once activated, neonatal T cells produce fewer cytokines than an adult and are predisposed toward Th2 responses than robust Th1 response producing various Th1 cytokines {Marodi, 2006 #2} usually found in adults {Adkins, 1999 #177}.

In contrast to mature, adult B cells, immature B cells are negatively signalled by ligation of the B-cell receptor (BCR) and fail to upregulate co-stimulatory molecules (such as CD86), lead to CD40 ligand (CD40L) induction {Brugnoni, 1994 #162} different adhesion molecules {Tasker, 2003 #220}and MHC class II molecules that are essential for effective interaction with T cells through T-cell receptors (TCR){King, 1999 #151} {Marshall-Clarke, 2000 #152} {Benschop, 2001 #153} (Fig 1.4).

Many studies have shown that the primary T-cell-dependent antibody responses induced in the neonatal period differ from adult responses {Marshall-Clarke, 2000 #7}{Siegrist, 2001 #1}. Neonatal antibody responses are delayed at onset, reach lower peak levels, are of shorter duration, lower distribution of IgG2 isotype and are of lower average affinity {Schallert, 2002 #150}{Janeway CJr, 1999 #134}and reduced heterogeneity than that of adults.

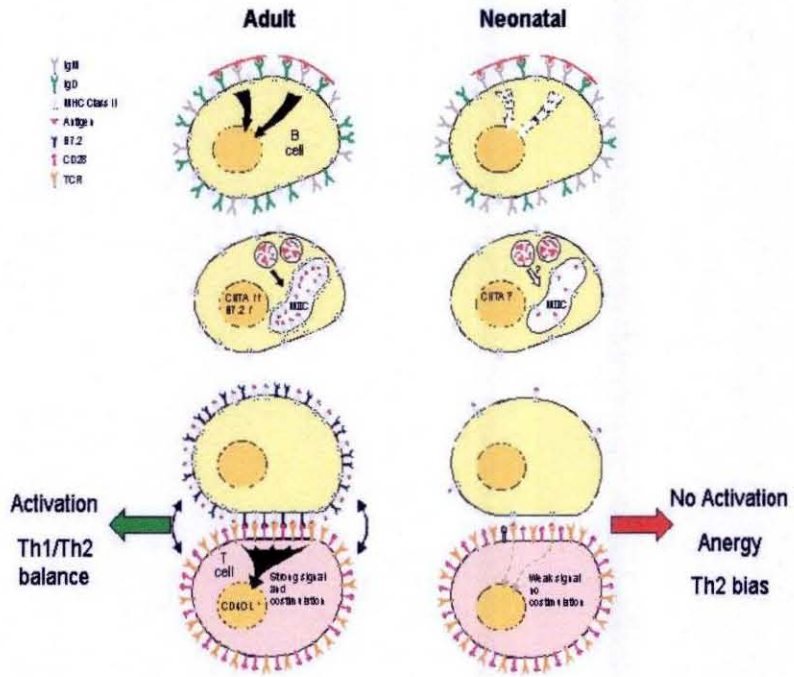

Figure 1.4: A model of the possible effects of differences in signalling between adult and neonatal B cells on the outcome of T–B interactions.

In addition to quantitative differences in antibody production during early life versus adulthood, there are also qualitative differences. B lymphocytes from newborns can be made to synthesize IgM at adult levels, but not IgG and IgA. Within 24 months from birth the secretion of IgG has reached adult capacity whereas IgA formation is still diminished {Andersson, 1981 #185} {Janeway CJr, 1999 #134}.

1.6 Neonatal Vaccine Response

The aim of vaccination is to rapidly elicit protective immunity and generate memory cells, which respond rapidly and strongly to subsequent antigen exposure {Bjarnarson, 2005 #3}. The ideal vaccine would be a single dose given at birth, preferably avoiding injections, and providing immediate and long-lasting protection against multiple diseases {Wood, 2011 #291}.

The longer-term effectiveness of a vaccine is determined by a combination of

- persistence of antibody {Snape, 2008 #31},
- immunological or B cell memory (here defined as the ability to make a secondary response to re-exposure to the antigen) {Kelly, 2005 #32} and
- persistence of herd immunity, which reduces transmission of the organism in the population{Maiden, 2008 #33} {Pollard, 2009 #34}.

Most children in the world receive their primary immunizations in the Expanded Programme on Immunization (EPI) schedule at 6, 10 and 14 weeks of age because infectious diseases cause the highest morbidity and mortality rates in early life {Pollard, 2009 #34}.

Infant vaccine studies worldwide have shown that there is an age-dependent stepwise increase in the rates of seroconversion and the magnitude of antibody responses, regardless of whether repeat immunization is administered{Halsey, 1985 #27}{Einhorn, 1986 #28}.

At birth, immune responses to protein antigens are much greater than responses to glycoprotein and polysaccharide antigens {WP, 2001 #19}. Responses gradually improve by 6-9 months of age for glycoproteins and by 12-24 months for polysaccharides {WP, 2001 #19}.

Optimization of vaccine-induced protection of infants against pathogens to which they are exposed within the first weeks and months of life requires one to avoid limitations linked to

(i) the induction of weaker, shorter or biased immune responses in infancy and **(ii)** the presence of preexisting antibodies of maternal origin to protect the newborn from various infections. In most circumstances, similar specific antibody titres to vaccine antigens, like tetanus toxoid (TT) and measles are observed in mothers and newborns which have been linked to failure of infant responses to vaccines, resulting in a window of susceptibility to infectious disease {Osborn, 1952 #244}.

The immunogenicity of many vaccines in infancy is increased when;

- ✓ immunization starts later (less interference from maternal antibody){O'Brien, 2007 #55} {Tiru, 2000 #30}
- ✓ more doses are given{Booy, 1992 #29} and
- ✓ there is a greater length of time between doses {Taranger, 1999 #56} {Giammanco, 1998 #57} {Carlsson, 1998 #58}.

Although, neonatal immunization does not generally lead to rapid antibody responses; however, it may result in efficient immunologic priming which can act as a basis for future responses {Chirico, 2005 #163}.

Current vaccines mediate their protective efficacy through the generation of neutralizing antibodies {Siegrist, 2009 #26}. But one of the limitations of current vaccine mechanisms is due to short duration of antibody responses elicited before 12 months of age. Most vaccinated infants have low or undetectable vaccine antibody concentrations as early as 6–9 months after completion of the primary immunisation series, requiring administration of a booster dose in the second year of life {Siegrist, 2001 #1}. More recently, certain vaccine delivery systems with adjuvants have been found to provide more sustained protection against various diseases {BP, 2001 #21}.

1.7 Vaccine Specific Immune Response

1.7.1 Measles

Measles, also known as Rubeola, is an infection of the respiratory system caused by a virus, specifically a paramyxovirus of the genus *Morbillivirus*. Measles is spread through respiration (contact with fluids from an infected person's nose and mouth, either directly or through aerosol transmission), and is highly contagious - 90% of people without immunity sharing living space with an infected person will catch it. The infection has an average incubation period of 14 days (range 6–19 days) and infectivity lasts from 2–4 days prior, until 2–5 days following the onset of the rash.

1.7.1.1 Epidemiology of measles

According to the World Health Organization (WHO), measles is a leading cause of vaccine-preventable childhood mortality. Worldwide, the fatality rate has been significantly reduced by a vaccination campaign led by partners in the Measles Initiative: the American Red Cross, the United States Centers for Disease Control and Prevention (CDC), the United Nations Children's Fund (UNICEF) and the World Health Organization (WHO). Globally, measles fell 60% from an estimated 873,000 deaths in 1999 to 345,000 in 2005 {Helfand, 2008 #61}. Estimates for 2008 indicate deaths fell further to 164,000 globally, with 77% of the remaining measles deaths in 2008 occurring within the South-East Asian {WHO, 4th December 2009 #62}.

In Bangladesh, Measles is the fifth leading cause of death among children under five years of age. Annually, an estimated 20,000 children die from measles in this country which has a population of around 160 million.

1.7.1.2 Measles vaccine: The MMR vaccine

The Measles Mumps Rubella (MMR) vaccine is an injection that prevents us from catching measles, mumps and rubella. Although people usually recover from these illnesses, each one can be unpleasant and have serious consequences.

MMR is a live attenuated vaccine. This means that it contains the living viruses, which have been altered in such a way as to prevent the vaccine from actually causing the diseases. However, the body's immune system still responds to it, providing future protection against infection.

Recommendations for measles immunisation have accordingly traditionally been based on age of MatAb disappearance for the majority of infants {Williams, 1995 #37}.

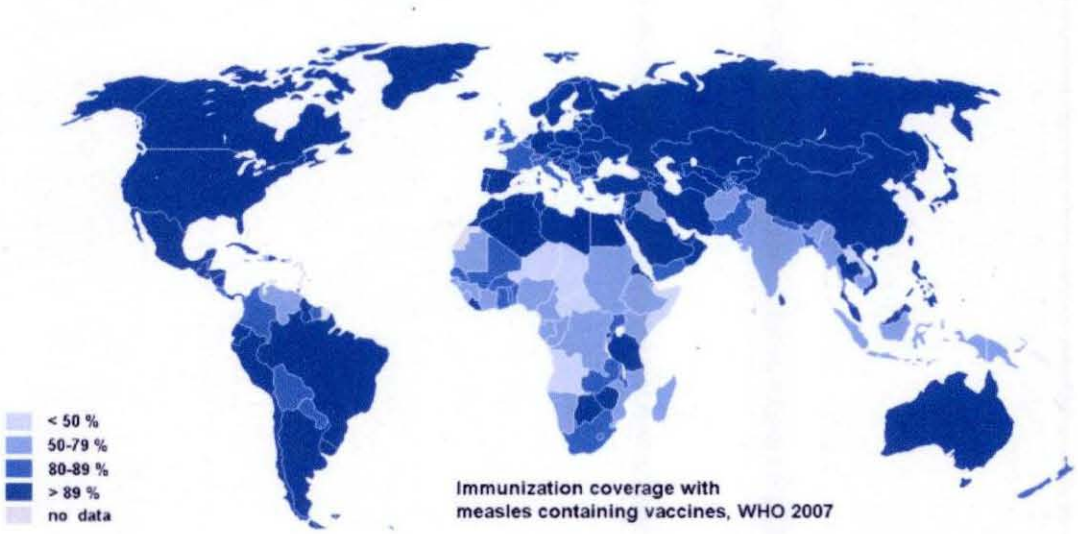

Figure 1.5: Immunization coverage with measles containing vaccines in infants, WHO 2007 [http://en.wikipedia.org/wiki/File:Measles_vaccination_worldwide.png]

1.7.1.3 MMR vaccine timetable

The MMR vaccine is usually given to children, who have the first injection when they are about 10 (9-15) months old. A second dose is given between the ages of three and five years to cover any children who haven't responded to the first one*.

Table 1.2: The recommended immunization schedule for Measles

Age	Vaccine	Dose
9-15 months	Measles, mumps and rubella (MMR)	One injection (Trivomax®)
3 years – 5 years*	Measles, mumps and rubella (MMR)	One injection (Trivomax®)

1.7.1.4 Measles Vaccine Response

The early priming is likely to explain the reduced measles morbidity and mortality observed in vaccinated infants as an age-dependent stepwise increase is observed in rate of seroconversion and antibody concentrations following measles immunization at 6, 9, 12 or 15 months of age {Johnson, 1994 #182}{Gans, 1998 #52}{Cherry, 1973 #210}{Siegrist, 1998 #39}{Albrecht, 1977 #42}. In contrast it was recently suggested that immunization <12 months of age may be associated with waning immunity against measles in West Africa {Siegrist, 1998 #20}.

Presence of very high neutralizing MatAb/antigen ratio could eventually result in complete neutralization of MMR vaccine load, preventing in-vivo replication and thus reducing the effective immunizing dose below the immunogenicity threshold required for induction of antibody responses without disturbing T cell response{Siegrist, 2001 #1}{Gans, 1998 #52}{Gans, 1999 #53} as measles immunization of 6-month-old infants in presence of MatAb led to positive antibody responses in only 36.7%, whereas positive T-cell responses were measured in 86.8% {Pabst, 1999 #54}.

1.7.2.1 Tetanus

Tetanus is an acute and often fatal disease caused by tetanospasmin, an extremely potent neurotoxin produced by *Clostridium tetani*. The bacterial spores are deposited in tissue, usually by traumatic injury, retained placenta or endometrial injury and under anaerobic conditions vegetate. The muscle spasms cause a stiff gait, rigid posture, extension or elevation of the tail, protrusion of the third eyelid and trismus. Spasms of facial muscles cause abnormally erect ears and retraction of the lips that resembles the 'risus sardonicus' seen in humans with tetanus. Stimulation precipitates generalized muscle contractions and tetanic spasms or convulsions. The disease can be prevented by immunization with tetanus toxoid or the use of antitoxin.

1.7.2.2 Epidemiology of Tetanus

Tetanus is a vaccine-preventable disease that yearly causes a total of 309,000 deaths {Vandelaer, 2003 #229} as *C. tetani* spores are ubiquitous. The disease occurs almost exclusively in persons who are unvaccinated or inadequately immunized {Wells CL, 1996 #62}. Tetanus – particularly the neonatal form – remains a significant public health problem in non-industrialized countries. As about 1.4 million neonates die yearly in the first 4 weeks after birth {WHO,2000 #228}.The World Health Organization (WHO) estimates that 59,000 newborns worldwide died in 2008 as a result of neonatal tetanus {WHO, 2010 #64}.

In Bangladesh, one of the world's least developed countries, over 80% of women give birth without any help from a skilled birth attendant. In the mid-1980s, Bangladesh had one of the highest rates of neonatal tetanus in the world: 41 cases for every 1000 live births {Hlady, 1992 #65}. Only 5% of women of childbearing age were immunized with tetanus toxoid and only 5% of pregnant women had access to a clean birth. Not surprisingly, neonatal tetanus accounted for one in four infant deaths.

Today, Bangladesh has succeeded in reducing death rates from 41 for every 1000 live births in 1986 to only 4 per 1000 by 1998. And in a final push to reach the WHO global target for elimination of neonatal tetanus is less than one neonatal tetanus death per 1000 live births in every district in every country{Group, 1994 #230}.

1.7.2.3 Tetanus vaccine

Tetanus vaccine (Tetanus toxoid) is a vaccine used against *Clostridium tetani*, the agent that causes tetanus. It is a component of the DPT vaccine. This vaccine is for intramuscular or subcutaneous use; is a sterile solution of toxoid in isotonic sodium chloride solution. The tetanus vaccine is required again after 10 years if the individual is exposed to possible infection.

Tetanus vaccine was first deployed in the 1930s, and it is now well established that at least five doses of this vaccine should be administered to provide long-term protection {Pollard, 2009 #34}).

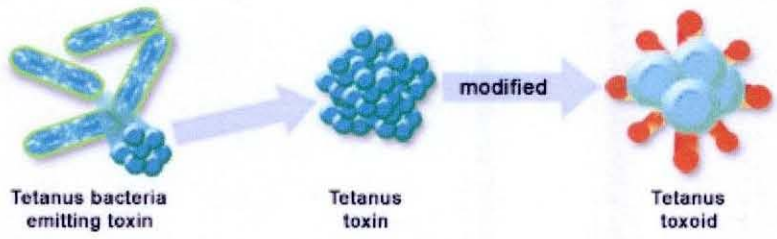

Fig 1.6: Production of Tetanus toxoid vaccine from emitted toxin of *Clostridium tetani* by chemical modification [Source: HealthSoft, Inc, USA]

Tetanus toxoid, given as Tetanus vaccine is chemical conjugation of the polysaccharide to a protein carrier —directs processing of the protein carrier by polysaccharide-specific

B cells and presentation of the resulting peptides to carrier-peptide-specific T cells in association with MHC class II molecules. So, a conjugate polysaccharide vaccine induces a T-cell-dependent response from early infancy and induces an anamnestic (memory) response to a booster dose of the vaccine ({Kelly, 2006 #36}.

1.7.2.4 TT vaccine timetable

The TT vaccine is usually given to children, who have the first injection when they are 6 weeks old. Two more doses are given between the ages of two and six month and a booster dose ig given at 12 month of age.

Table 1.3: The recommended immunization schedule for Tetanus

Age	Vaccine	Dose
6 weeks, 10 weeks, 14 weeks	DPT (Diptheria, Pertussis, Tetanus)	One injection each time
15 months (12 Months after last dose)	DPT (Diptheria, Pertussis, Tetanus)	One injection

1.7.2.5 TT vaccine response

Tetanus toxoid (TT), a protein antigen is able to elicit antibody response from neonates {Day, 2004 #107} {Ku, 2005 #108} {Orange, 2004 #109}.Tetanus toxoid (TT) is known to induce a strong and long lasting humoral immune response in humans after vaccination {Wellhorner, 1981 #221}{Excler, 1998 #4}. After TT vaccination generates induction of clonal T cell response {Adams, 1991 #224}{Geha, 1973 #225}{Kabilan, 1990 #226}. It has been demonstrated that TT-specific T cells are mainly CD4+ cells secreting Th1 cytokines such as IFN-γ {Parronchi, 1991 #227}{Mayer, 2002 #5}.

Administration of a birth dose of tetanus toxoids (TT) vaccines usually fail to induce significant antibody responses to the first neonatal dose due to age-dependent limitation of infant antibody response. {Kurikka, 1995 #186}{Lieberman, 1995 #187} {Dengrove, 1986 #192} and presence of maternal antibodies {Sarvas, 1992 #47}.

Like other infectious disease antigens, TT stimulates a very robust T cell immune response that can easily be detected using a variety of assays {Goodell V., 2007 #246}, like lymphocyte proliferation assay (LPA).

1.8 Mucosal Immune Response

Mucosal immunity is characterized by a specific maturational pattern initiated in the intrauterine fetal development and continued during the neonatal period, and in infancy and childhood, dynamically leading to a highly specialized immune response as intestinal mucosa is in intimate contact with microbiota, the symbiotic ecosystem of more than 450 species of mutualist and commensal microorganisms {Szczawinska-Poplonyk, 2011 #68}. The fundamental challenge of mucosal immune response is to prevent effectively the entry of invading pathogens, the development and the disseminating of infection whereas simultaneously its exposition to the external environment and to a high antigenic load that elicits immune tolerance {Szczawinska-Poplonyk, 2011 #68}.

Mucosal immune response is generated to

(i) protect extensively the mucous membranes against colonization and invasion by potentially dangerous microbes that may be encountered,

(ii) prevent uptake of and

(iii) prevent the development of potentially harmful immune responses to these antigens if they do reach the body interior {Holmgren, 2005 #79}.

IgA is the most important immunoglubulin in the intestine and other mucosal surfaces. The induction of IgA against mucosal pathogens and soluble protein antigens is dependent on T helper cells {Lycke, 1987 #80} {Hornquist, 1995 #81}, although IgA immunity to commensal flora may be thymus independent and of low affinity {Stoel, 2005 #82}.

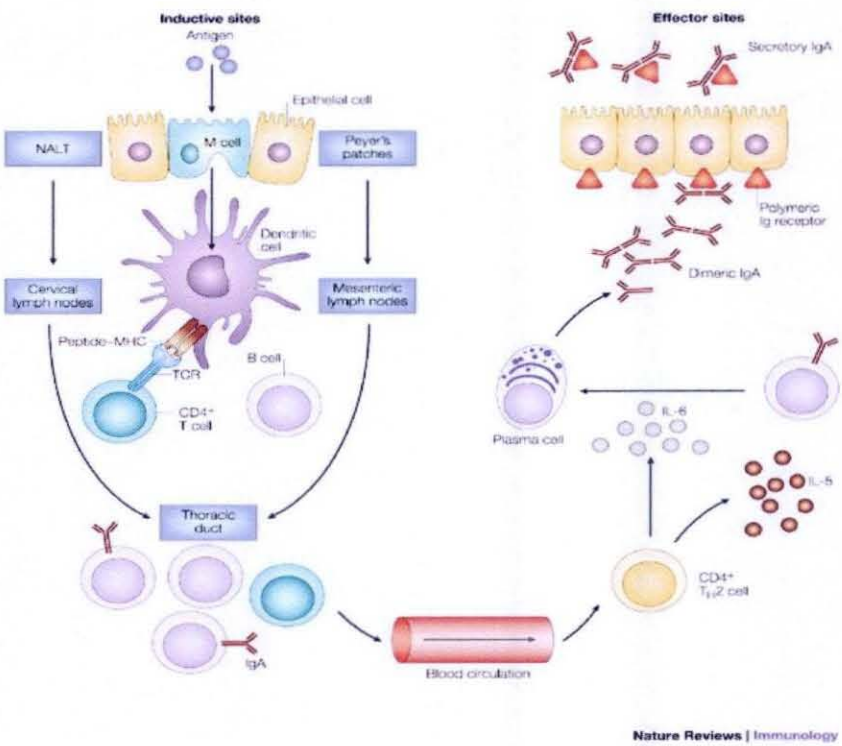

Figure 1.7: Mechanism of Mucosal innate immune response
[http://www.nature.com/nri/journal /images/nri1439-f1.jpg]

1.9 Neonatal Mucosal Immune Response

A functioning mucosal immune system is essential for survival in infancy and beyond {Holt, 2000 #83} {Thrane, 1991 #74}. At birth this system is quite immature, putting the newborn mammal at risk for a variety of infectious and noninfectious diseases. The mucosal surface is exposed to a huge antigenic challenge and the immature mucosal

immune system must learn to distinguish when a tolerogenic versus an immunogenic response is most appropriate {Vancikova, 2002 #133}.

Despite the anatomical and functional immaturity of the mucosal immune system and crosstalk between innate and adaptive immune responses, infants and young children are capable of mounting effective immune defense mechanisms {Szczawinska-Poplonyk, 2011 #68}.

1.10 Maturing Mucosal Immune Response with Age

The development of mucosal immunity in children is a time-dependent process initiated in the intrauterine growth and is continuous during the postnatal period {Szczawinska-Poplonyk, 2011 #68}. With age and antigenic stimulation, maturation occurs and intestinal host defense then functions quite effectively to protect the host from ingested noxious substances and microorganisms {Mannick, 1996 #77}.

The structures of the mucosal immune system are fully developed by the 28 gestational week and thus premature infants older than 28 weeks of gestation are capable of mounting an effective mucosal immune response {Gleeson, 2004 #71}. Mucosal permeability is rapidly reduced within the first 48 hours after birth. In the oral mucosa disappearance of maternally-derived IgG reflects this postnatal mucous membrane closure {Gleeson, 1982 #70}. The rapid increase of innate defense factors, such as salivary lysozyme, lactoferrin and amylase during the first six postnatal months may provide the infant necessary protection during the period when specific adaptive immunity at mucosal sites is not fully developed {Thrane, 1991 #74}.

IgA-producing immunocytes, though increase in number during neonatal period and reach an initial peak about 4-6 postnatal weeks, they approach the low normal adult level at about 18 months of age, subsequently with small increase throughout early childhood {Maheshwari A, 2006 #73} and disease {Stoltenberg, 1993 #156}.

1.10 Aim of the Study

The aim of the study was to analyze the development of immune response in newborns, infants and children at different time point from birth to two years of age. The present study was conducted with the following specific objectives;

- To observe the T cell proliferation response to tetanus toxoid (TT) and phytohemagglutinin (PHA) in study infants.
- To differentiate the distribution of T and B cell specific cluster of differentiation (CD) markers on lymphocyte of infants of different ages by flow cytometry (FACS).
- To compare plasma IgA and IgG antibody response to cholera toxin (CT) of *Vibrio cholerae* among the children.
- To estimate heat labile toxin (LT) of *Escherichia coli* specific plasma IgA and IgG antibody responses.
- To analyze vibriocidal antibody response in plasma.
- To evaluate vaccine specific antibody responses to tetanus.
- To assess vaccine specific antibody responses to measles.
- To determine fecal antibody (IgA) response of the study infants.
- To measure heat labile toxin (LT) of *Escherichia coli* specific fecal antibody (IgA) responses in enrolled participants.

CHAPTER 2

METHODS AND MATERIALS

2.1 Study Location

This study was carried out in the Immunology Laboratory of the Centre for Vaccine Sciences at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b).

2.2 Study Area and Subjects

The study area of this investigation was in Mirpur in Dhaka city. Specimens were obtained from forty infants from newborn and four other age groups (6 month, 12 month, 18 month and 24 month; ten in each group). Ten cord blood specimens from newborns during delivery were also collected to serve as 'newborn specimen" for this study. All forty infants were healthy (without any symptoms of diarrhea or acute respiratory infection). Stools were tested to confirm the healthy status of the children.

2.3 Study Design

The study participants were divided into the following categories based on their age

Group	**I**	**II**	**III**	**IV**	**V**
Age (months)	0 (Cord Blood specimens)	6	12	18	24
Number of infants	10	10	10	10	10

All the children of the above four categories (except newborns from whom cord blood was collected) were vaccinated under the Expanded Program of Immunization (EPI) schedule which also included vaccines for Tetanus and Measles.

The study groups were compared in the following parameters:

- T-cell Proliferation response to tetanus toxoid (TT) and phytohemaglutinin (PHA)
- Distribution of T-cell and B-cell specific Surface proteins clusters of differentiation (CD) markers on lymphocytes
- Cholera Toxin (CT) specific plasma Immunoglobulin G (IgG) and Immunoglobulin A (IgA) responses
- Heat labile toxin (LT) of *Escherichia coli* specific plasma IgG and IgA responses
- Vaccine specific plasma IgG immune response to Tetanus
- Vaccine specific plasma IgG immune response to Measles
- Plasma Vibriocidal Antibody response
- Fecal total IgA profile
- Heat labile toxin (LT) of *Escherichia coli* specific fecal IgA response

The design of the study plan for immunological analyses is shown in Fig 2.1

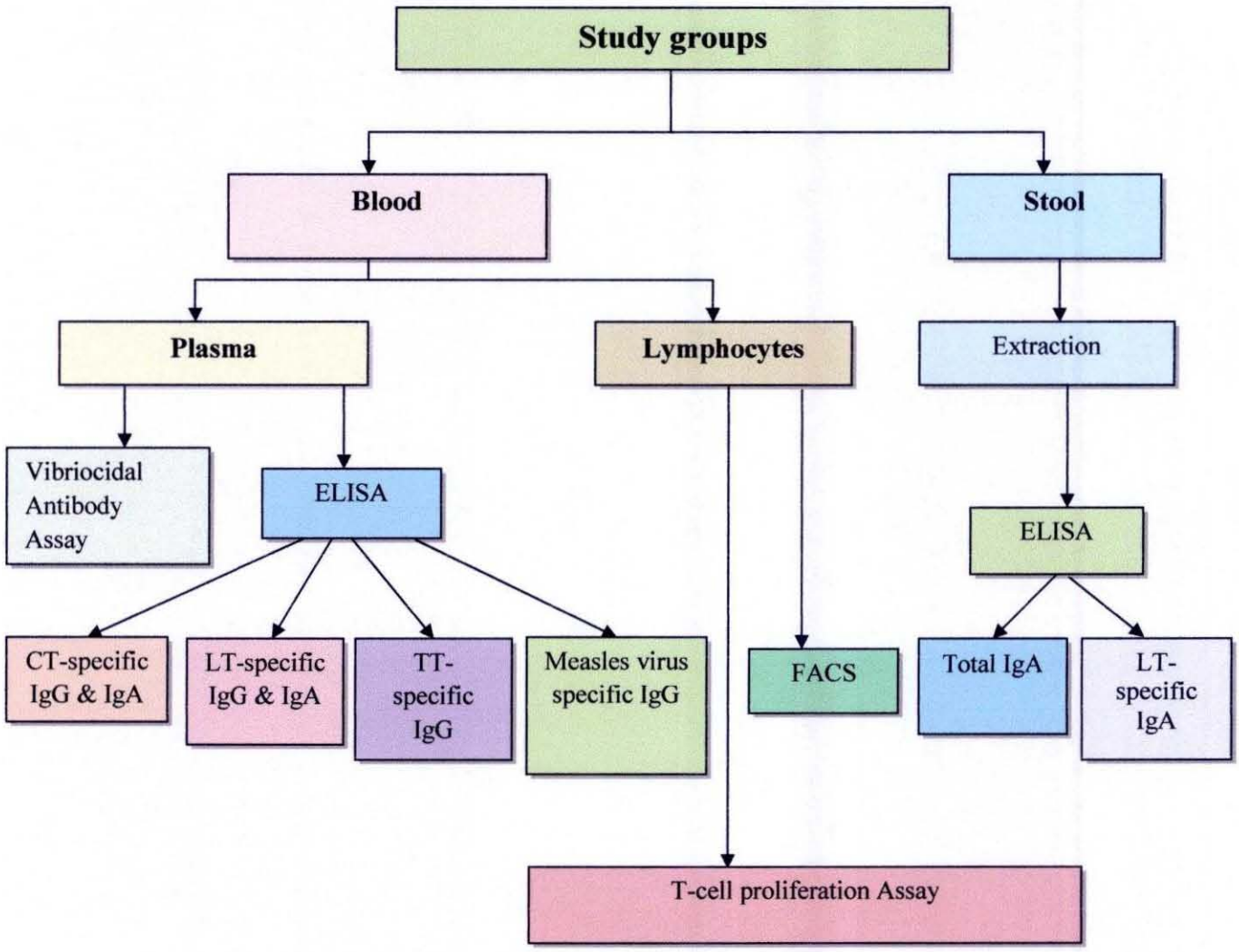

Fig 2.1: The design of the study plan

2.4 Collection of Specimen

About 3ml venous blood for each child was collected in sterile, rubber capped vacutainer (*Beckton Dickinson,* BD, USA). The stool samples were collected in sterile plastic containers and stored at -70 °C for further analyses.

2.5 Laboratory Methods

2.5.1 Isolation of cord blood mononuclear cells (CBMC) and Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood by density gradient centrifugation on Ficoll Isopaque (GE Healthcare, Uppsala, Sweden). As the density of red blood cells (RBC) is higher than PBMC, RBC is precipitated. The plasma remains in the upper portion. Isolated PBMCs were incubated in 96-well tissue culture plates at 37°C in 5% CO_2. The T-cells sensitized with antigens secrete cytokines during the culture period. The culture supernatants were preserved for the detection of various inflammatory and pro-inflammatory cytokines as well as other immunomodulators.

Procedure

a) Heparinized cord/venous blood was diluted with equal volume of Phosphate Buffered Saline (PBS; 10 mM, pH 7.2) in Falcon tubes (Beckton Dickinson, BD).
b) Diluted blood was carefully added onto half the volume of Ficoll-Isopaque (GE Healthcare, Uppsala, Sweden) without disturbing the Ficoll layer. Hence, two distinct layers were maintained.
c) The tube was centrifuged at 772g for 25 minute at 20°C (Sorvall RT 6000B Refrigerated Centrifuge).

d) After centrifugation, PBMCs remained at the interface of plasma and Ficoll. RBCs and other cell debris were precipitated at the bottom of the tube. The mononuclear cells were then collected from the top of the Ficoll layer carefully.

e) The PBMCs were washed once in PBS at 953g for 10 minute at 20°C.

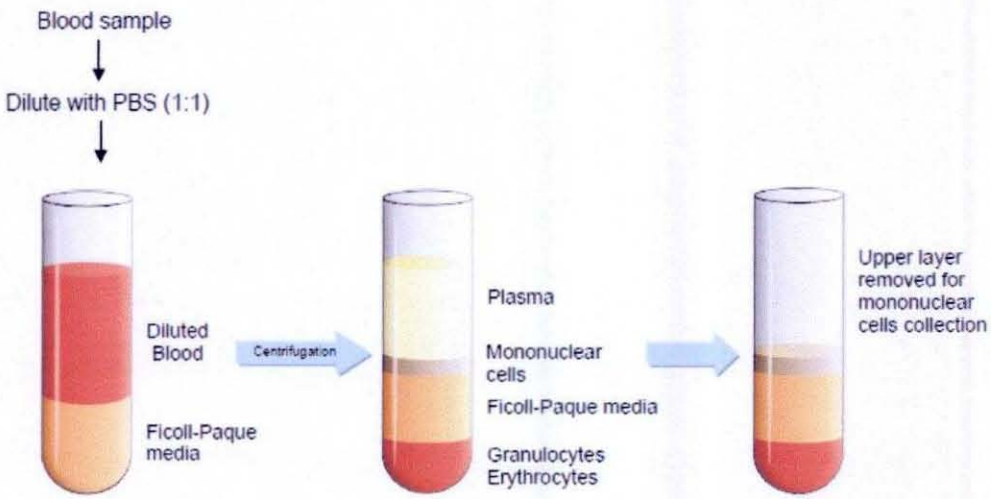

Figure 2.2: Isolation of PBMC by density gradient centrifugation on Ficoll-Isopaque.

f) The PBMCs were resuspended in 10 ml of PBS and cells were counted using a haemocytometer.

g) After the second wash, the cells were resuspended in appropriate medium (appendix) to a required concentration to be used for further analysis.

2.5.2 Tritiated Thymidine (^{3H}T) T-cell Proliferation Assay

Isolated PBMCs were incubated in 96-well tissue culture plates at 37°C in 5% CO_2 for T-cell proliferation. The T-cells sensitized with antigens proliferate in response to those antigens and secrete cytokines during the culture period. The extent of T-cell proliferation can be measured by incorporation of radiolabelled Tritiated Thymidine (^{3H}T) into the cells during cell divisions. More the incorporation of Thymidine in the T-cell during proliferation indicates more the response to the antigens, which can be measured by liquid scintillation counter (LS 6500 Multipurpose Scintillation Counter, Beckman, USA).

Table 2.1: Concentration of antigens used to stimulate cells

Name of antigen	Stock concentration	Working concentration
TT	1.90 mg/ml	5 µg/ml
PHA	200 µg/ml	1 µg/ml

Procedure

1. Separated PBMCs from blood [section 2.6.1] were resuspended in DMEM-ABS medium to a concentration of 10^7cells/ml.
2. 100 µL/well cell suspension was added at triplicate in 96well cell culture plate (U bottom plate, cat# 163320, Nunclon, Denmark).

3. 100 µL stimulants [PHA (1µg/ml); TT (5µg/ml)], diluted in DMEM-ABS, were added to the appropriate wells and 100 µl DMEM-ABS medium to the negative control wells.
4. The plate was incubated at 37^0C plus 5% CO_2 incubator for 2 days.
5. After incubation, 100 µL culture supernatant was aspirated from each well gently and store at -70^0C.
6. Then 100 µL DMEM-ABS medium was added to each well.
7. The plate was incubated at 37^0C plus 5% CO_2 incubator for 3 more days.
8. After incubation, 25 µl Tritium (^{3}H Thymidine) was added per well at a dilution of 1:20 in DMEM-ABS medium. The Plate was incubated for at least 6 hours at 37^0C plus 5% CO_2 incubator.
9. The plate was harvested in automash.
10. The paper was dried by 1 day at room temperature.
11. Then radioactivity of the dried paper was counted by Scintillation counter (LS 6500-Multipurpose Scintillation Counter, Beckman, USA) after adding 2ml of scintillation fluid in each tube individually.

2.5.3 Determination of T-cell and naïve B-cells' percentage in Cord blood and peripheral blood by Flow cytometry (FACS)

The basis of a **FACS analysis** is a labeled (colored) suspensions of individual cells which passes a focused laser-beam. Capillary forces cause the cells to pass the **flow-cell**, where the labels are stimulated by the laser light. The emitted florescent light from the flurophores, which are coupled to the antibodies, and the scattered-light are detected separately.

Table 2.2: Antibodies and their dilutions used in flow cytometry

Antibody	Dilution	Company
anti-CD3-allophycocyanine (anti-CD3-APC), Cat#347543	1:100	BD
anti-CD4-peridinin chlorophyll protein (anti-CD3-PerCP), Cat#347324	1:10	BD
anti-CD8-fluorescein isothyocyanate (anti-CD8-FITC), Cat#340692	1:5	BD
anti-CD19-phycoerythrin (anti-CD19-PE), Cat#120457	1:5	BD

2.5.3.1 Procedure for staining lymphocyte surface antigens by antibody mixture

1. 5x 10^4 PBMCs/well [collected by Ficoll procedure described in section 2.6.1] were taken on a V bottom-96 well plate (V96 MicroWell™ Plates, cat# 442587, Nunc) and resuspended in 200 µl of PBS.
2. The cell suspension was centrifuged at 2000 rpm for 5 minutes at 4°C temperature.

3. The pooled FACS antibody mixture was prepared [anti-CD3 APC (1:100), anti-CD4 PreP (1:10), anti-CD8 FITC (1:5), anti-CD19 PE (1:5)] (Table 2.2)
4. Fifteen µl of antibody mixture was added in each well.
5. The plate was incubated at 4°C for 30 minutes.
6. 100 µl/well FACS buffer was added and the plate was centrifuged at 2000 rpm for 5 minutes at 4°C temperature.
7. The supernatant was discarded ant the cells were resuspended with 100 µl/well cell fix (BD™ Stabilizing Fixation, BD Bioscience, San Jose, USA), ready for acquisition.

2.5.3.2 Cell acquisition and analysis by Flow cytometry

2.5.3.2.1 Cell Acquisition

Cells were suspended with cell fix (BD™ Stabilizing Fixation, BD Bioscience, San Jose, USA) to stabilize the cells until acquisition. For acquisition of four-color fluorescence (PerCP, FITC, PE, APC) tagged cells, acquisition was performed on a FACS Calibur instrument (BD, San Jose, CA) and CELLQUEST multipurpose software (version 3.3; Becton Dickinson) was used. Before data acquisition, instrument settings were checked to get a desired position of the cells according to the tagged fluorochrome.

During cell acquisition, cells were recorded according to the antibody mixture. For every sample one tube was prepared and acquired for more than 20,000 cells. One negative control was used to compare with every stimulated sample. All samples were analyzed by setting appropriate forward and side-scatter gates targeting the round shaped lymphocytes population and thus the percentage of positive cells were estimated from gate.

2.5.3.2.2 Gating strategies and analysis

[a] Gating principles:

The data generated by flow-cytometer can be plotted in a single dimension, to produce a histogram, or in two-dimensional dot plots or even in three dimensions. The regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates.

It is an important principle of flow cytometry data analysis is to selectively visualize the cells of interest while eliminating results from unwanted particles e.g. dead cells and debris. This procedure is called gating. Cells have traditionally been gated according to physical characteristics. For instance, subcellular debris and clumps can be distinguished from single cells by size, estimated by forward scatter. Also, dead cells have lower forward scatter and higher side scatter than living cells. These same principles can be used to distinguish different cell populations.

[b] Strategies used in the analysis:

The Flow cytometry (FCM) data were analyzed with FLOWJO (version 8.5.3) software (Tree Star Inc.). Lymphocytes were gated via forward and side scatter properties (Fig 2.3 A). Mononuclear cells were first gated based on forward and side scattering light, then different subpopulations were gated according to different staining patterns and results expressed as frequency of expression (Fig-2.3). T cells were identified based on their expression of the $CD3^+$ cell surface marker (Fig 2.3 B). Among the CD3 cells, $CD4^+$ and $CD8^+$ cells were gated (Fig-2.3 C). In the same way, B cells were gated from lymphocytes based on their expression of $CD19^+$ marker (Fig 2.3 D) on lymphocyte population.

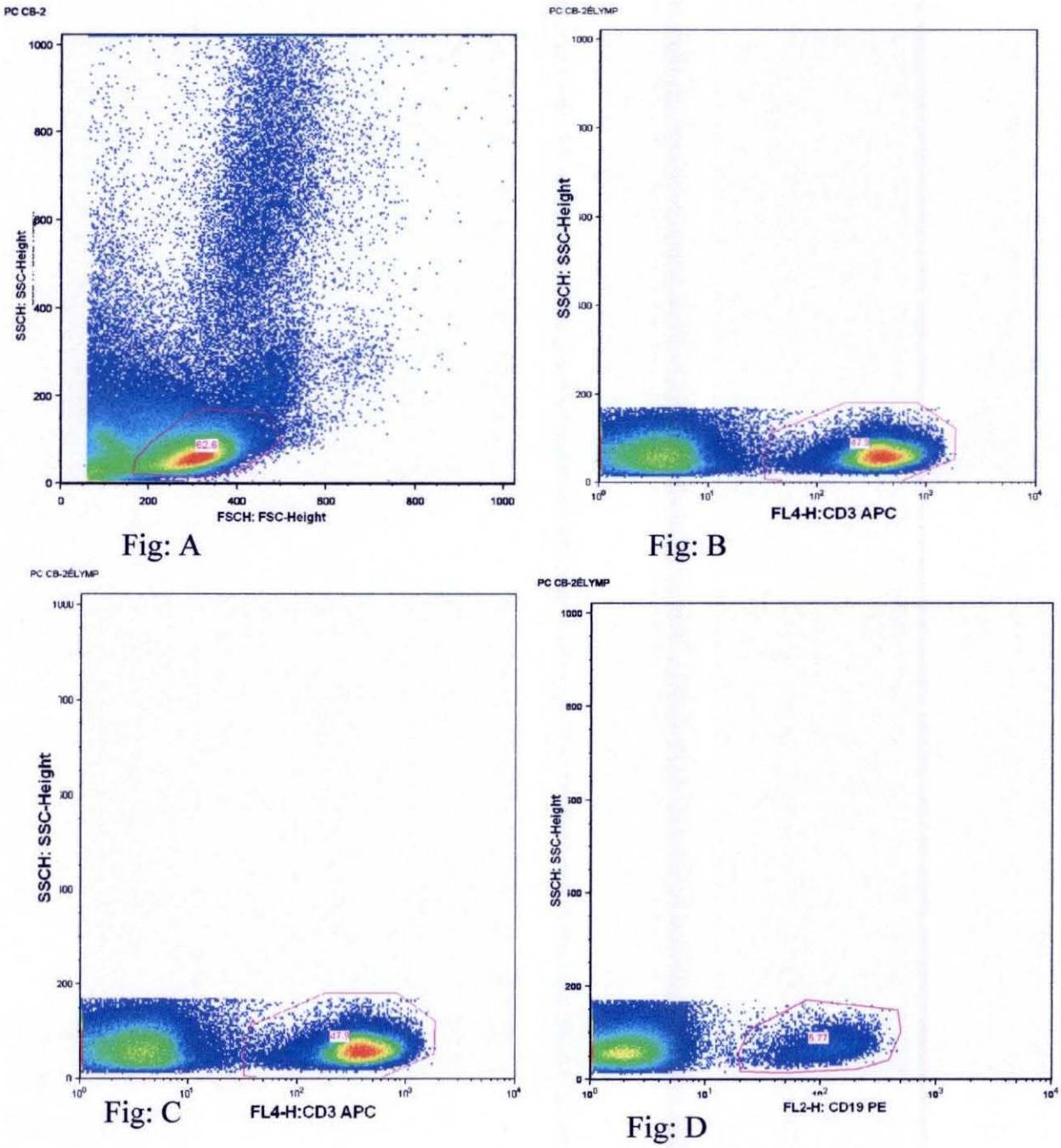

Figure 2.3: Demonstration of FACS Plot gating strategy following stimulation with antigens: **A)** Cells are separated according to FSC versus SSC. **B)** T-cells are separated by surface marker $CD3^+$ T-cells **C)** $CD4^+$ and $CD8^+$ cells are separated from $CD3^+$ cells and **D)** B cell as $CD19^+$ positive from total lymphocytes.

2.5.4 Enzyme Linked Immunosorbant Assay (ELISA)

Enzyme Linked Immunosorbant Assay (ELISA) is a qualitative and quantitative immunoassay in which antibodies or antigens are detected by the binding of an enzyme coupled to either anti-Ig antibody or antibody specific for the antigen.

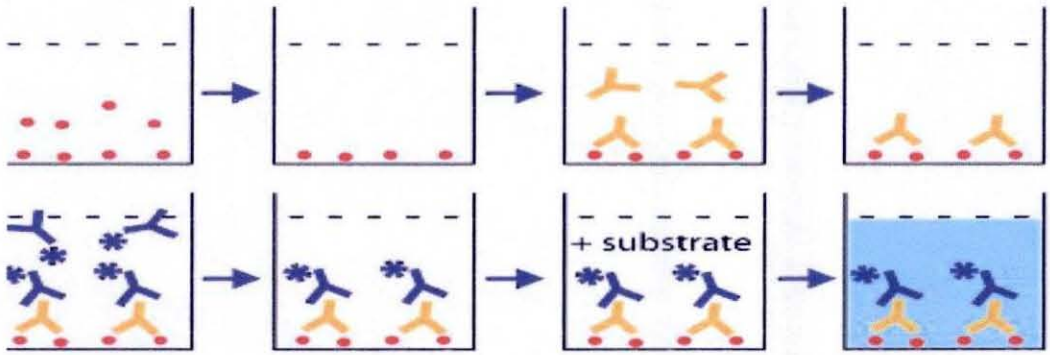

Figure 2.4: Indirect ELISA to detect presence of antibody. (http://www.al3malka.com)

Plate is coated with specific antigen. Serum sample is added next. Antigen-specific antibodies will bind to the antigens. Secondary antibodies conjugated to an enzyme are added thereafter to allow its binding with the Fc portion of the bound antibodies. Finally a chromogenic substrate is added that will form color upon reacting with the enzyme. (Fig 2.4)

2.5.4.1 Detection of IgA and IgG antibodies against B subunit of cholera toxin (CT) in plasma samples using GM1-ELISA

Plate coating:

1. The antigen GM1 (Sigma, cat#G-7641) was diluted with Phosphate buffer saline (PBS) at concentration of 120nmol/ml.
2. ELISA plates (*Nunc F, Denmark*) were coated with 100 µl/well of antigen suspension.
3. The plates were kept at 4°C overnight. Following overnight incubation, the ELISA plates could be stored at 4°C up to 2 weeks for further use

Blocking:

4. The plates were washed thrice with PBS
5. The plates were blocked with 0.1% Bovine Serum Albumin in PBS (BSA-PBS); 200 µl/well. The plates were then incubated at 37°C for 30 minutes.

Sample loading:

6. The plates were washed thrice with PBS-0.05% Tween and once with PBS only.
7. The purified rCTB (0.5µg/ml in PBS) diluted in 0.1% BSA-PBS were added, 100µL per well and incubated at Room Temperature (RT) for 60 min at 37°C.
8. The plates were washed 3 times with PBS - Tween (0.05 %) and once with PBS.
9. The plasma samples (initial dilution 1:10 for IgA & 1:100 for IgG) diluted in 0.1% BSA-PBS containing 0.05% Tween were placed 150 µl/well and were serially diluted three fold in microtiter plates with the final volume of 100 µl/well and
10. The plates were incubated at Room Temperature (RT) for 90 minutes

Addition of Conjugate:

11. The plates were washed thrice with PBS-0.05%Tween and once with PBS only.
12. The conjugates (rabbit anti-human IgG conjugated to horse radish peroxidase (HRP), Jackson Immune Research Laboratories Inc.) were diluted (1:1000) in 0.1% BSA-PBS containing 0.05% Tween, and added to the wells (100 µl/well).
    I. Anti-human IgA HRP (e.g. Jackson 309035011, 1/1000)
    II. Anti-human IgG HRP (e.g. Jackson 309035006, 1/1000)
13. The plates were incubated in room temperature for 90 minutes.

Addition of Substrate plate developing:

14. The plates were washed thrice with PBS-0.05%Tween and once with PBS only.
15. The plates were developed by adding the substrate H_2O_2-OPD (Orthophenylenediamine, *Sigma*), prepared by dissolving 10 mg OPD in 10 ml of 0.1M sodium citrate buffer (pH 4.5), to which 30% H_2O_2 was added immediately before use, 100 µl/well.
16. The reaction was stopped by adding 25µL H_2SO_4 after 20 minutes.
17. The optical density (O.D.) was measured at 492 nm by the ELISA reader in end point mode.

2.5.4.2 Detection of IgA and IgG antibodies against Heat Labile Toxin (LT) of *Escherichia coli* (*E.coli*) in Plasma samples using GM1-ELISA

Plate coating:

1. The antigen GM1 (Sigma, cat#G-7641) was diluted with Phosphate buffer saline (PBS) at concentration of 120nmol/ml.
2. ELISA plates (*Nunc F, Denmark*) were coated with 100 µl/well of antigen suspension.
3. The plates were kept at 4°C overnight. Following overnight incubation, the ELISA plates could be stored at 4°C up to 2 weeks for further use

Blocking:

4. The plates were washed thrice with PBS
5. The plates were blocked with 0.1% Bovine Serum Albumin in PBS (BSA-PBS); 200 µl/well. The plates were then incubated at 37°C for 30 minutes.

Sample loading:

6. The plates were washed thrice with PBS-0.05% Tween and once with PBS only.
7. The purified LTB (0.5µg/ml in PBS) diluted in 0.1% BSA-PBS were added, 100µL per well and incubated at Room Temperature (RT) for 60 min at 37°C.
8. The plates were washed 3 times with PBS - Tween (0.05 %) and once with PBS.
9. The plasma samples (initial dilution 1:10 for IgA & 1:100 for IgG) diluted in 0.1% BSA-PBS containing 0.05% Tween were placed 150 µl/well and were serially diluted three fold in microtiter plates with the final volume of 100 µl/well.

10. The plates were incubated at Room Temperature (RT) for 90 minutes

Addition of Conjugate:

11. The plates were washed thrice with PBS-0.05%Tween and once with PBS only.
12. The conjugates (rabbit anti-human IgG and IgA, conjugated to horse radish peroxidase (HRP), Jackson Immune Research Laboratories Inc.) were diluted (1:1000) in 0.1% BSA-PBS containing 0.05% Tween and added to the wells (100 µl/well).
    i. Anti-human IgA HRP (e.g. Jackson 309035011, 1/1000)
    ii. Anti-human IgG HRP (e.g. Jackson 309035006, 1/1000)
13. The plates were incubated in room temperature for 90 minutes.

Addition of Substrate and plate developing:

14. The plates were washed thrice with PBS-0.05%Tween and once with PBS only.
15. The plates were developed by adding the substrate H_2O_2-OPD (Orthophenylenediamine, *Sigma*), prepared by dissolving 10 mg OPD in 10 ml of 0.1M sodium citrate buffer (pH 4.5), to which 30% H_2O_2 was added immediately before use, 100 µl/well.
16. After 20 minutes, the plates were read at 450 nm in ELISA Reader (Ascent spectrophotometer) and again the optical density (O.D.) was measured at 492 nm by the ELISA reader (Ascent spectrophotometer) in end point mode immediately after stopping the reaction with 25 µl of 1.0 M H_2SO_4.

2.5.4.3 Detection of Tetanus Toxoid (TT) specific IgG antibodies by Enzyme Linked Immunosorbent Assay (ELISA)

Kit: SERION ELISA *classic* Tetanus IgG

Test Procedure:

1. The samples were diluted (1+100) in dilution buffer solution.
2. Diluted samples and ready-to-use control sera/standard sera were pipetted into the microtest wells (100µl).
3. The plate was sealed and wrapped and incubated at 37°C for 60 minutes in moist chamber.
4. The wells were washed four times with 300µl washing solution (1:30 dilution) per well.
5. The ready-to-use conjugate solution (anti-human-IgG) was pipetted into the wells (100µl/well).
6. The plate was sealed and wrapped and incubated at 37°C for 30 minutes in moist chamber.
7. The wells were washed four times with 300µl washing solution (1:30 dilution) per well.
8. The ready-to-use substrate solution (anti-human-IgG) was pipetted into the wells (100µl/well).
9. The plate was sealed and wrapped and incubated at 37°C for 30 minutes in moist chamber.
10. The ready-to-use stopping solution (anti-human-IgG) was pipetted into the wells (100µl/well).
11. The optical density (O.D.) was measured at 405 nm by the ELISA reader.

2.5.4.4 Detection of Measles Virus specific IgG antibody by Enzyme Linked Immunosorbent Assay (ELISA)

Kit: Enzygnost® Anti-Measles Virus/IgG

Test Procedure:

1. The colored sample buffer was prepared (800µl total/strip) by adding 40µl colored solution in 800µl non-colored sample buffer POD (diluent).
2. 5µl of serum sample was mixed well with colored sample buffer solution. 5µl of anti-measles virus reference was also mixed well with colored sample buffer solution.
3. The wells of the strips were filled by non-colored sample buffer POD; 200µl/well.
4. The prediluted serum samples and anti-measles virus reference were added to the wells (20µl/well) and mixed well.
5. The plate was then sealed and wrapped and incubated at 37°C for 60 minutes.
6. The well content was aspirated and washed four times with diluted washing solution (2ml washing solution in 40ml dH_2O).
7. 40µl of conjugate (anti-human IgG/POD) was added to 2ml of Conjugate Buffer Microbial solution and shacked gently to mix well (per strip). 100µl of this conjugate solution is then added to each well.
8. The plate was then sealed and wrapped and incubated at 37°C for 60 minutes.
9. The well content was aspirated and washed four times with diluted washing solution (2ml washing solution in 40ml dH_2O).
10. 200µl of chromogen TMB was added to 2ml of Buffer Substrate TMB (per strip) and mixed. This solution was then kept in dark before use was 100µl was added per well.
11. The plate was then sealed and wrapped and incubated at room temperature for 30 minutes in dark.

12. The reaction was stopped by adding Stopping solution; 100µl/well.
13. The optical density (O.D.) was measured at 450 nm by the ELISA reader.

2.5.4.5 Determination of fecal antibody, immunoglobulin A (IgA)

Plate coating:

1. The antigen Affinipure Goat anti-human IgG ($(Fab')_2$ (Jackson Immune Research Laboratories Inc. Cat# 109-005-097) was diluted with Phosphate buffer saline (PBS) at concentration of 1.0 µg/ml.
2. ELISA plates (*Nunc F, Denmark*) were coated with 100 µl/well of antigen suspension.
3. The plates were kept at 4°C overnight. Following overnight incubation, the ELISA plates could be stored at 4°C up to 2 weeks for further use.

Blocking:

4. The plates were washed thrice with PBS.
5. The plates were blocked with 0.1% Bovine Serum Albumin in PBS (BSA-PBS); 200 µl/well. The plates were then incubated at 37°C for 30 minutes.

Sample loading:

6. The plates were washed thrice with PBS-0.05% Tween and once with PBS only.
7. The fecal extract samples (initial dilution 1:500) diluted in 0.1% BSA-PBS containing 0.05% Tween were placed 150 µl/well and were serially diluted three fold in microtiter plates with the final volume of 100 µl/well.
8. The plates were incubated at Room Temperature (RT) for 90 minutes.

Addition of Conjugate:

9. The plates were washed thrice with PBS-0.05%Tween and once with PBS only.
10. The HRP conjugated goat anti-human IgA (Jackson Immune Research Laboratories Inc. Cat# 109-035-011) were diluted (1:3000) in 0.1% BSA-PBS Tween (0.05%) and added 100 µl / well.
11. The plates were incubated in room temperature for 60 minutes.

Addition of Substrate and plate developing:

12. .The plates were washed thrice with PBS-0.05%Tween and once with PBS.
13. The plates were developed by adding the substrate H_2O_2-OPD (Orthophenylenediamine, *Sigma*), prepared by dissolving 10 mg OPD in 10 ml of 0.1M sodium citrate buffer (pH 4.5), to which 30% H_2O_2 was added immediately before use, 100µl/well.
14. The optical density (O.D) was measured after 20 min at 450 nm in Multiskan Ascent ELISA reader.
15. Endpoint titers were determined as the reciprocal of the interpolated dilution of the test samples giving an absorbance of 0.4 above the background absorbance.

Total IgA Calculation:

After dilution of breast milk pool conc. of IgA is 2.0 µg/ml. So the titer we get in ELISA is equivalent to this conc. For example pool titer is 6750 and sample titer is 3524.

So, 6750 ≡ 2 µg/ml

3524 ≡ (3524*2)/6750≡1.044 µg/ml

So, conc. of 1:50 times diluted stool extract is 1.044µg/ml

Hence, conc. of undiluted stool extract is 50*1.044=52.2 µg/ml

2.5.4.6 Detection of Fecal IgA antibodies against Heat Labile Toxin (LT) of *Escherichia coli* (*E.coli*) by GM1-ELISA

Plate coating:

1. The antigen GM1 (Sigma, cat#G-7641) was diluted with Phosphate buffer saline (PBS) at concentration of 120nmol/ml.
2. ELISA plates (*Nunc F, Denmark*) were coated with 100 µl/well of antigen suspension.
3. The plates were kept at 4°C overnight. Following overnight incubation, the ELISA plates could be stored at 4°C up to 2 weeks for further use

Blocking:

4. The plates were washed thrice with PBS
5. The plates were blocked with 0.1% Bovine Serum Albumin in PBS (BSA-PBS); 200 µl/well. The plates were then incubated at 37°C for 30 minutes.

Sample loading:

6. The plates were washed thrice with PBS-0.05% Tween and once with PBS only.
7. The purified LTB (0.5µg/ml in PBS) diluted in 0.1% BSA-PBS were added, 100µL per well and incubated at Room Temperature (RT) for 60 min at 37°C.
8. The plates were washed 3 times with PBS - Tween (0.05 %) and once with PBS.
9. The undiluted (neat) fecal extract samples were placed 150 µl/well and were serially diluted three fold in microtiter plates with the final volume of 100 µl/well.
10. The plates were incubated at Room Temperature (RT) for 90 minutes.

Addition of Conjugate:

11. The plates were washed thrice with PBS-0.05%Tween and once with PBS only.
12. The conjugate (rabbit anti-human IgA conjugated to horse radish peroxidase (HRP), Jackson Immune Research Laboratories Inc.) were diluted (1:1000) in 0.1% BSA-PBS containing 0.05% Tween, and added to the wells (100 µl/well).
    I. Anti-human IgA HRP (e.g. Jackson 309035011, 1/1000)
13. The plates were incubated in room temperature for 90 minutes.

Addition of Substrate and plate developing:

14. The plates were washed thrice with PBS-0.05%Tween and once with PBS only.
15. The plates were developed by adding the substrate H_2O_2-OPD (Orthophenylenediamine, *Sigma*), prepared by dissolving 10 mg OPD in 10 ml of 0.1M sodium citrate buffer (pH 4.5), to which 30% H_2O_2 was added immediately before use, 100 µl/well.
16. The reaction was stopped by adding 25µl H_2SO_4 after 20 minutes.
17. The optical density (O.D.) was measured at 492 nm by the ELISA reader in end point mode.

2.6.5 Vibriocidal Antibody Assay

Vibrio cholerae is exceedingly susceptible to the bactericidal effect of O antibody in the presence of complements. Vibriocidal tests, in principle, depend on exposure of dilutions of plasma to a carefully standardized constant inoculum of *Vibrio cholerae* in the presence of excess guinea pig complement, an incubation period to permit killing of

bacteria, and subculture of the dilutions of plasma and appropriate controls on agar or in broth.

Vibriocidal antibody assays were performed using guinea pig complement and with *V. cholerae* O1 Ogawa (X-25049) and *V. cholerae* O1 Inaba (strain 19479 El Tor) as the target organisms. Vibriocidal titer was defined as the reciprocal of the highest dilution resulting in >50% reduction of the optical density when compared to that of control wells without serum. Individuals showing a ≥4-fold increase in vibriocidal responses one or three weeks post infection (if any) or vaccination were considered responders.

Procedure

a) *Vibrio cholerae* O1 Ogawa (X-25049), *Vibrio cholerae* O1 Inaba (strain 19479 El Tor) were cultured overnight on blood agar plates at 37°C.
b) A loopful of bacteria from the plates was inoculated in 15 ml Bovine-Heart Infusion (BHI) medium in a conical flask with cotton plug. This was incubated on a shaker at 37°C for 3-4 hours.
c) The culture was centrifuged at 3000rpm for 10 minutes and the supernatant was thrown away. The sediment was resuspended in sterile saline.
d) This was again centrifuged for another 8-10 minutes. The pellet was resuspended in sterile saline.
e) Bacterial concentration was adjusted optical density by spectrophotometer at 600 nm.

   For *V. cholerae* O1 (X25049 and 19479) ➔ adjusted at 0.3

f) Heat-inactivated (56° C, 30 minutes) sera was diluted 2-fold in sterile saline in flat-bottom microtiter plates (Nunc, F) as follows:

- 25 µl of cold saline was dispensed in all wells except column #2.
- 45 µl of cold saline and 5 µl of test sera were dispensed in column #2. The sera was serially diluted (initial dilution 1:10) 2-fold by using a multi-channel dispenser. The dilution was accomplished by mixing the solution in column #2, aspirating 25 µl and dispensing and

mixing the sample in column #3 and so on, till column #12 (this equals to 1:10240). The last 25 µl was discarded from the last well on each row. The plates were kept at 4°C (on ice) until used.

g) The indicator (bacteria-complement [Guinea pig Sera, Sigma]-saline mixture) was prepared. The composition for each plates is as follows:

Bacterial Strains	Sterile Saline	Bacteria	Complement
***V. cholerae* 01 (X25049 and 19479)**	2.55 ml	150 µl	300 µl

h) The indicator was used immediately after preparation.

i) 25 µl of the indicator was added to all wells except wells in row A, B, C and D in column #1. The plate was incubated on a shaker at 37°C for 1 hour (50 revolutions /min).

j) After incubation, 150 µl BHI broth/well were added. The plates were again incubated for another 3-4 hours at 37°C without shaking.

k) The plates visually and spectrophotometrically. The absorbance for control wells should reach 0.20 to 0.28 at 595 nm.

l) Vibriocidal antibody titer is defined as the reciprocal of the highest serum dilutions resulting in greater than 50% OD reduction when compared to control wells without serum.

2.7 Statistical Analysis of the Data

Data analyses were carried out using the Microsoft Excel and GraphPad Prism 5.02. Following acquisition by the flow cytometer, the acquired cells were analyzed by using the multipurpose analysis software Flowjo (Tree Star, Inc., version 8.5.3). It was used for making different gating and plots. GraphPad Prism 5.02 was used for graph preparation and measurement of significance level as comparison between the values of different experiments were then analyzed by, unpaired t-test, Mann-Whitney Rank Sum test to evaluate statistical differences between and/or within study groups, where appropriate. A P-value of ≤ 0.05 was the criterion for a statistically significant difference. Data were expressed as median, arithmetic, geometric mean and standard error of mean (±SEM).

CHAPTER 3

RESULTS

3.1 Baseline data of study children

A total of fifty children were enrolled in this study. Forty children were taken from the Mirpur area, and ten cord blood specimens were collected from the same area and considered as the neonate specimen. All the subjects are children of between 6 months to 24 months of age and newborn, were divided into five groups depending upon age. The children of different groups were comparable with respect to their age and gender (Table 3.1).

Table 3.1: Baseline data of study participants

Study Participants						
Characteristics		**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**
Total enrollment		10	10	10	10	10
Age (month)		**0** (Cord blood specimen)	**6**	**12**	**18**	**24**
Sex	Female	5	7	4	3	7
	Male	4	3	6	7	3

3.2 Comparison of T cell proliferation of study participants

The T cell response of infants of all groups to tetanus toxoid (TT) and phytohemaglutinin (PHA) was measured by T cell proliferation Assay. Proliferation was assessed by determination of radiolabelled tritiated thymidine (^{3H}T) incorporation into the T cells after 5 days incubation of in-vitro cultures and expressed by comparison with cells incubated in absence of antigen (unstimulated) as stimulation index (SI).

In case of T cell response against Tetanus toxoid (TT) antigen, gradual increase of response was seen among the groups. Although newborns showed significantly lower response when compared with 6 months (P= 0.013), 12 months (P =0.003), 18 months (P =0.01), and 24 months old subjects (P <0.0001) (Fig-3.1).

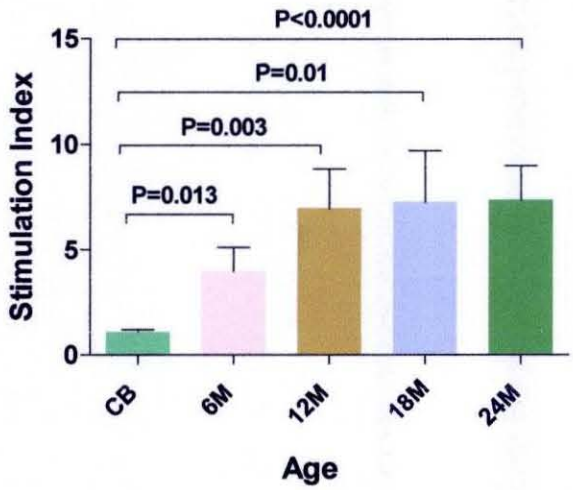

Figure 3.1: Comparison of T cell responses to tetanus toxoid (TT) antigen for infants of different ages. The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the mean values whereas the error bars represented the SEM.

However, T cell response of the study children to phytohemaglutinin (PHA), 6 months infants showed lowest response where 18 months old children showed the highest response among the groups. Again, 24 months children response was significantly higher than 6 months (P= 0.02) (Fig-3.2).

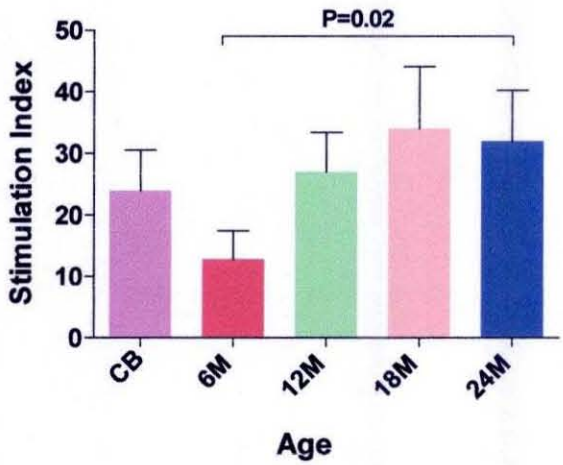

Figure 3.2: Comparison of T cell responses to phytohemaglutinin (PHA) antigen for infants of different ages. The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the mean values whereas the error bars represented the SEM.

Intra-group analysis for change in T cell response to unstimulated (US), tetanus toxoid (TT) and phytohemaglutinin (PHA) showed gradual increase in response to these antigens respectively in all groups of age.

In newborn, PHA response was significantly higher than TT (P= 0.003) where, US response to PHA was 30 fold lower when median was consider (Fig-3.3 A). T cell response of 6 months old infants to phytohemaglutinin (PHA) was 13 and 7 fold higher than unstimulated (US) and tetanus toxoid (TT) respectively (Fig-3.3 B).

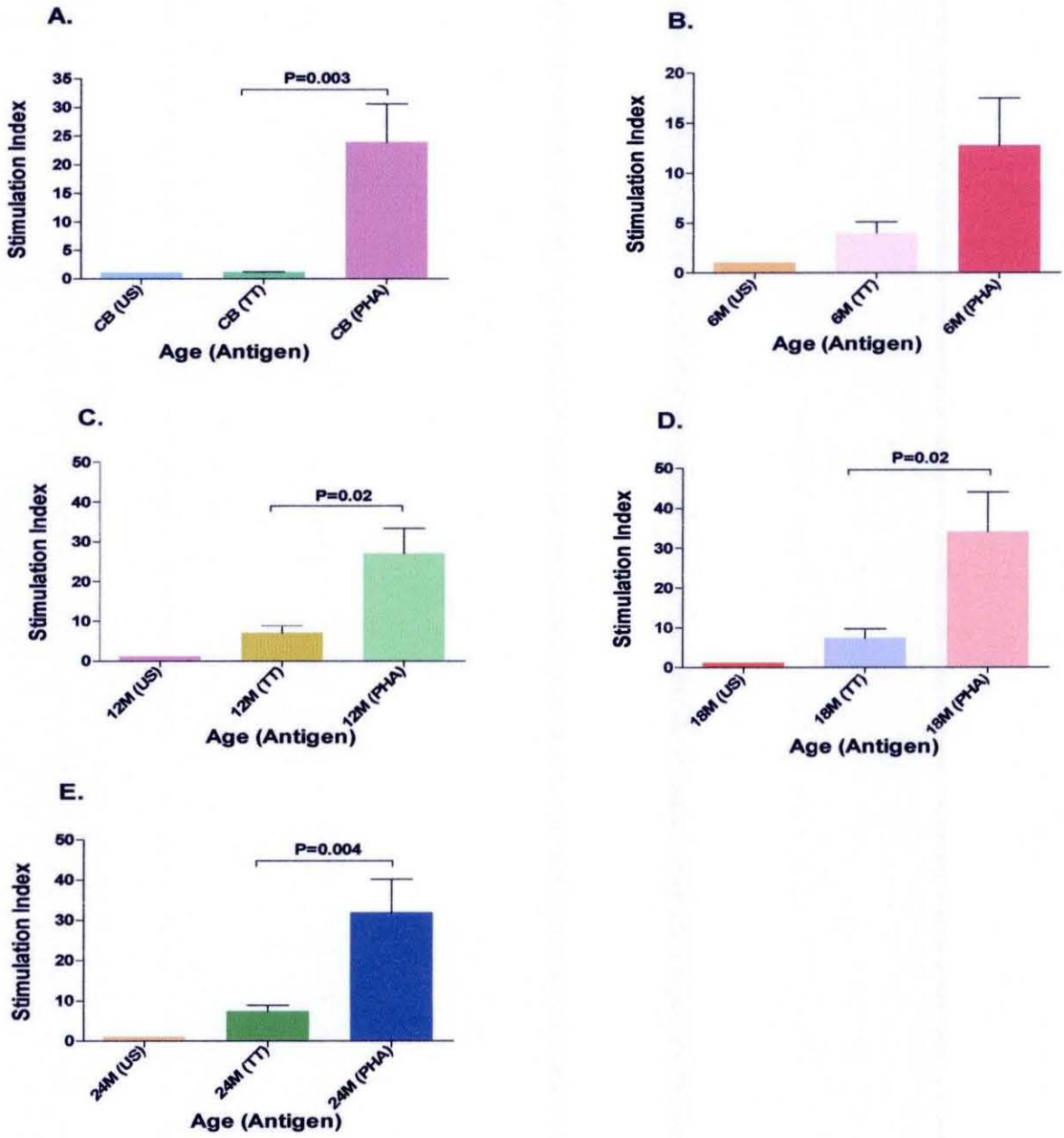

Figure 3.3: Intra-group Comparison of T cell responses to unstimulated (US), tetanus toxoid (TT) and phytohemaglutinin (PHA). The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the mean values whereas the error bars represented the SEM.

Children of 12 months of age showed significantly lower T cell response to US than TT and PHA which were 12 and 35 folds respectively, where PHA response were higher than TT (P= 0.02) (Fig-3.3 C).

However, phytohemaglutinin (PHA) response of T cell was found to be significantly elevated, around 30 folds than in unstimulated (US) and TT (P= 0.02) in 18 months children(Fig-3.3 D). Similar response was found in case of 24 months old children, where PHA response were significantly higher than TT (P= 0.004) (Fig 3.3 E).

3.3 Distribution of T-cell and B-cell specific Surface proteins, Clusters of differentiation (CD) markers

The distribution of T-cell and B-cell specific surface proteins clusters of differentiation (CD) markers on lymphocytes of cord blood and peripheral blood was measured by Flow cytometry (FACS).

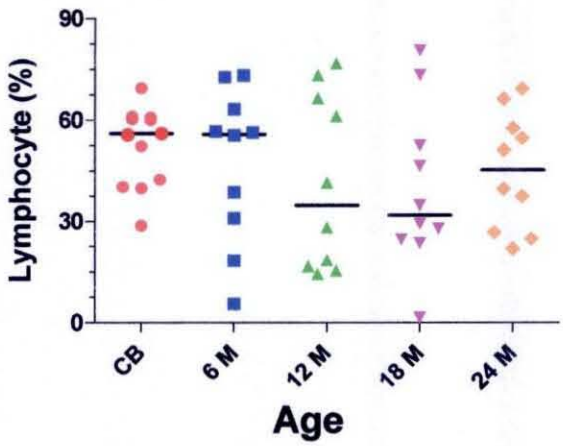

Figure 3.4: The Distribution of lymphocytes in infants of different ages. The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the median values whereas the error bars represented the SEM.

Total lymphocyte population was found to be higher in newborns, 6 months and 24 months children than 12 and 18 months old children.

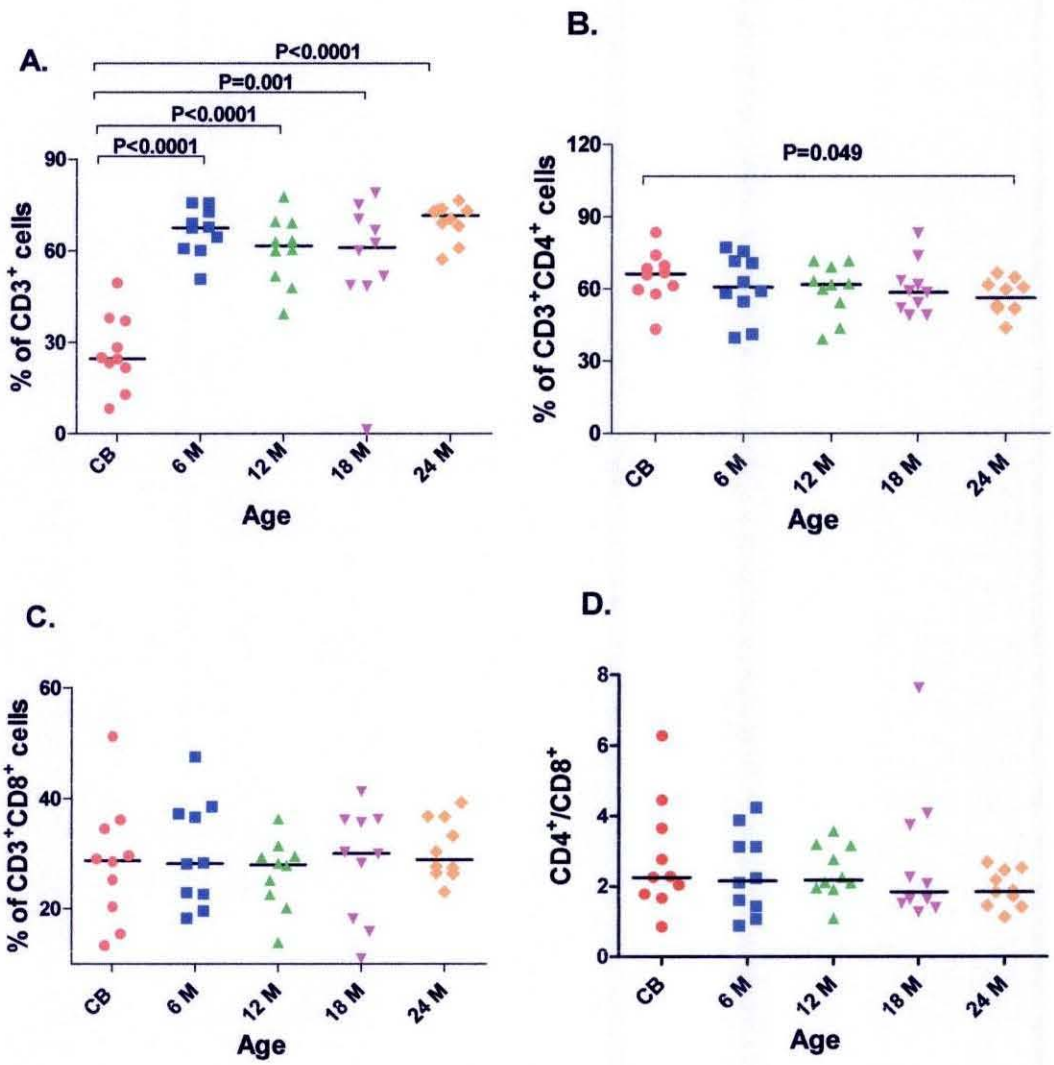

Figure 3.5: The Distribution T-cell specific surface proteins, clusters of differentiation (CD) markers on lymphocytes and ratio of Helper and cytotoxic T cells. The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when P was ≤ 0.05. The bars represent the median values whereas the error bars represented the SEM.

Total T cell ($CD3^+$) population was also found to be significantly lower in newborns than 6 months ($P< 0.0001$),12 months ($P< 0.0001$),18 months ($P= 0.001$) and 24 months ($P< 0.0001$) old children (Fig- 3.5 A.).

Among the T cells, percentage of T helper cells ($CD3^+$ $CD4^+$) were similar in 6 months, 12 months 18 months and 24 months old children although it was significantly higher in newborns than 24 month children ($P= 0.049$) (Fig- 3.5 B.).

Distribution of cytotoxic T cells ($CD3^+$ $CD8^+$) was found not to be significantly different in all the age groups. (Fig- 3.5 C).

However, age-dependent decrease of helper and cytotoxic T cells ratio ($CD4^+/CD8^+$) was observed among the study chlidren. (Fig-3.5 D).

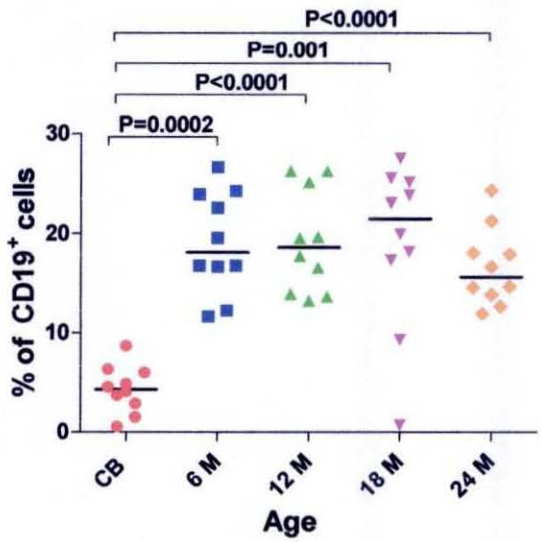

Figure 3.6: The Distribution B-cell specific surface proteins, clusters of differentiation (CD) marker on lymphocytes. The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when P was ≤ 0.05. The bars represent the median values whereas the error bars represented the SEM.

However, percentage of B cell (CD 19^{+} cells) population was notably lower in newborns than 6 months (P= 0.0002), 12 months (P< 0.0001), 18 months (P= 0.001) and 24 months (P< 0.0001) old children (Fig- 3.6).

3.4 Cholera toxin (CT) specific Plasma Immunoglobulins

The level of plasma response to cholera toxin (CT) of *Vibrio cholerae* specific immunoglobulins (IgG and IgA) was quantitatively determined by the GM1-Enzyme linked immunosorbent assay (ELISA).

3.4.1 Plasma response for cholera toxin (CT) specific immunoglobulin G (IgG)

The CT-specific plasma IgG response was similar in infants of 6 months and 12 months. Plasma IgG response to CT was significantly lower in newborns than 18 months (P= 0.001) and 24 months (P< 0.0001) where 24 months children had significantly elevated level of CT-specific IgG than 6 months old infants (P= 0.03) (Fig-3.7 A.).

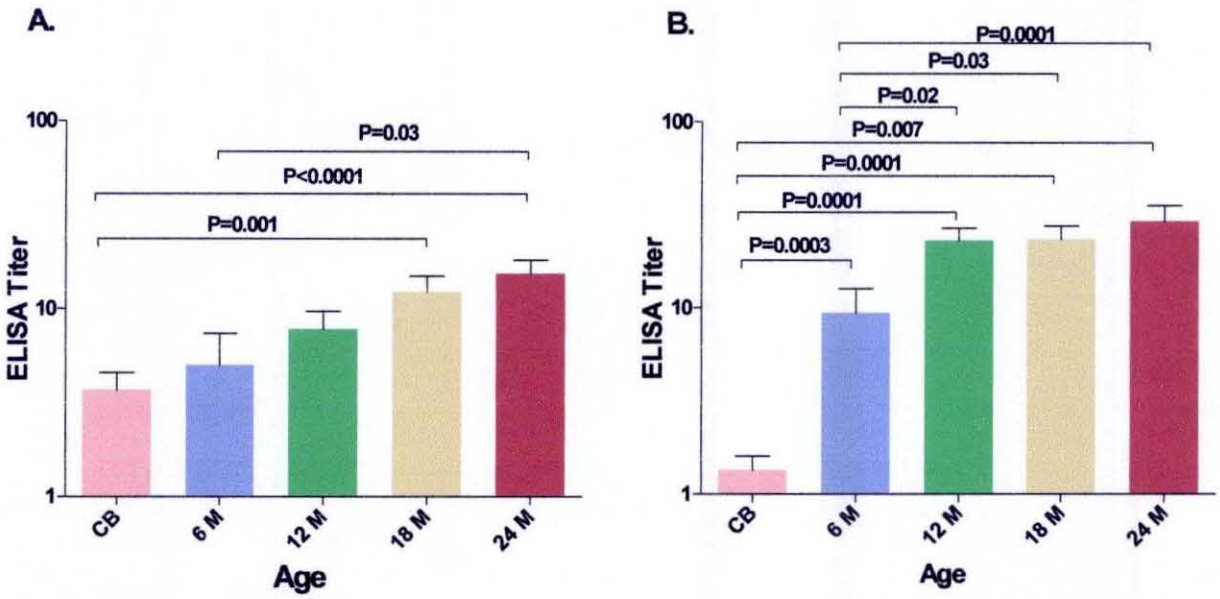

Figure 3.7: Plasma response to CT-specific IgG and IgA. The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the mean values whereas the error bars represented the SEM.

3.4.2 Level of Plasma response for cholera toxin (CT) specific immunoglobulin A (IgA)

Again, CT-specific plasma IgA response was found to be significantly lower in newborns than in 6 months (P= 0.0003), 12 months (P= 0.0001), 18 months (P= 0.0001), and 24 months (P= 0.0001). However, infants of 6 months showed significantly lower IgA response than 12 months (P= 0.02), 18 months (P= 0.03), and 24 months (P= 0.007). (Fig-3.7 B).

3.5 Level of Heat Labile toxin (LT) of *Escherichia coli* specific plasma immunoglobulins

The plasma response to Heat Labile toxin (LT) specific immunoglobulins (IgG and IgA) was quantitatively determined by the GM1-Enzyme linked immunosorbent assay (ELISA).

3.5.1 Level of Plasma response for Heat Labile toxin (LT) specific immunoglobulin G (IgG)

The LT-specific plasma IgG response was found to be gradually increased with age of the subject infants. Plasma IgG response to LT was significantly lower in newborns than in 6 months (P= 0.02), 12 months (P= 0.0005), 18 months (P= 0.0003) and 24 months (P< 0.0001) children (P= 0.03) (Fig-3.8 A.).

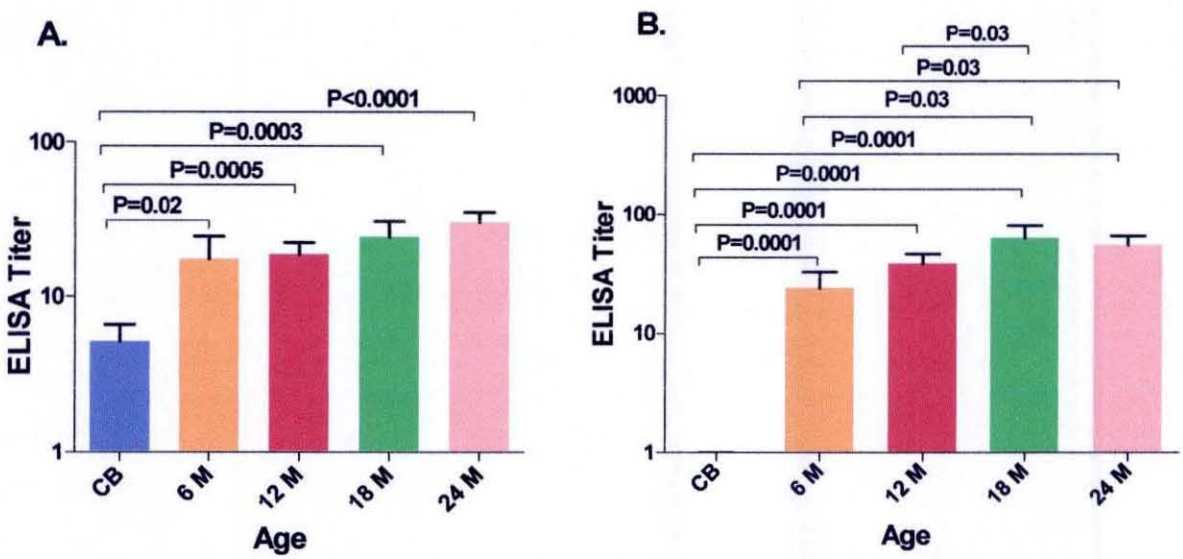

Figure 3.8: Plasma response to LT-specific IgG and IgA. The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the mean values whereas the error bars represented the SEM.

3.5.2 Level of Plasma response for Heat Labile toxin (LT) specific immunoglobulin A (IgA)

However, LT-specific plasma IgA response was found to be significantly lower in newborns than in 6 months (P= 0.0001), 12 months (P= 0.0001), 18 months (P= 0.0001), and 24 months (P= 0.0001). Although, 6 months infants showed notably lower IgA response than 24 months (P= 0.03). Moreover, 18 months old children had elevated IgA level when compared with 6 months (P= 0.03) and 12 months (P= 0.03) (Fig-3.8 B.).

3.6 Vaccine specific Plasma Immunoglobulin G (IgG) response to Tetanus toxoid (TT)

Tetanus toxoid (TT) specific plasma immunoglobulin G (IgG) response was measured by commercially available Enzyme linked immunosorbent assay (ELISA) system, SERION ELISA *classic* Tetanus IgG kit.

TT-specific IgG level was found to be elevated in newborns among the infants which gradually decreased till 18 month of age. Newborn IgG level was appreciably higher than 6 months (P= 0.04), 12 months (P= 0.02), 18 months (P= 0.007) and 24 months (P= 0.02) old children. (Fig-3.9).

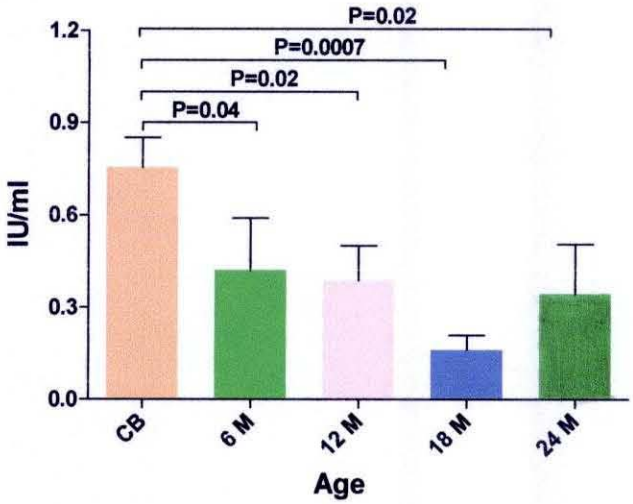

Figure 3.9: Vaccine specific plasma response to TT-specific immunoglobulin G (IgG). The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the mean values whereas the error bars represented the SEM.

3.7 Vaccine specific Plasma Immunoglobulin G (IgG) response to Measles virus

Measles virus specific IgG was determined by commercially available Enzyme linked immunosorbent assay (ELISA) system, Enzygnost® Anti-Measles Virus/IgG.

Plasma anti-measles IgG level was significantly elevated in newborns than 6 months (P= 0.001), 12 months (P= 0.03), 18 months (P= 0.009) and 24 months (P= 0.015) old children. Moreover, 6 months old infants had notably lower level of IgG than 18 months (P= 0.04) (Fig-3.10).

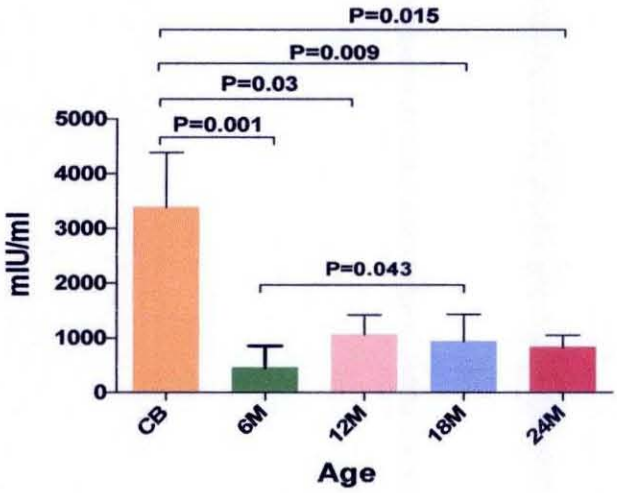

Figure 3.10: Vaccine specific plasma response for Measles virus specific immunoglobulin G (IgG). The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the mean values whereas the error bars represented the SEM.

3.8 Vibriocidal Antibody Responses

Vibriocidal antibody response is an indication of *Vibrio cholerae* killing activity of plasma. It was measured by Vibriocidal antibody assay. The assay was carried out for Ogawa (Strain X-25049) and Inaba (Strain-19479) serotypes of *Vibrio cholerae* O1.

The vibriocidal plasma response was expressed as antibody titer. For Ogawa serotype, there was no significant change of response was found in all age groups, (Fig-3.11 A.). Similarly, for Inaba the antibody titer near the base line in all age groups was observed (Fig-3.11 B.).

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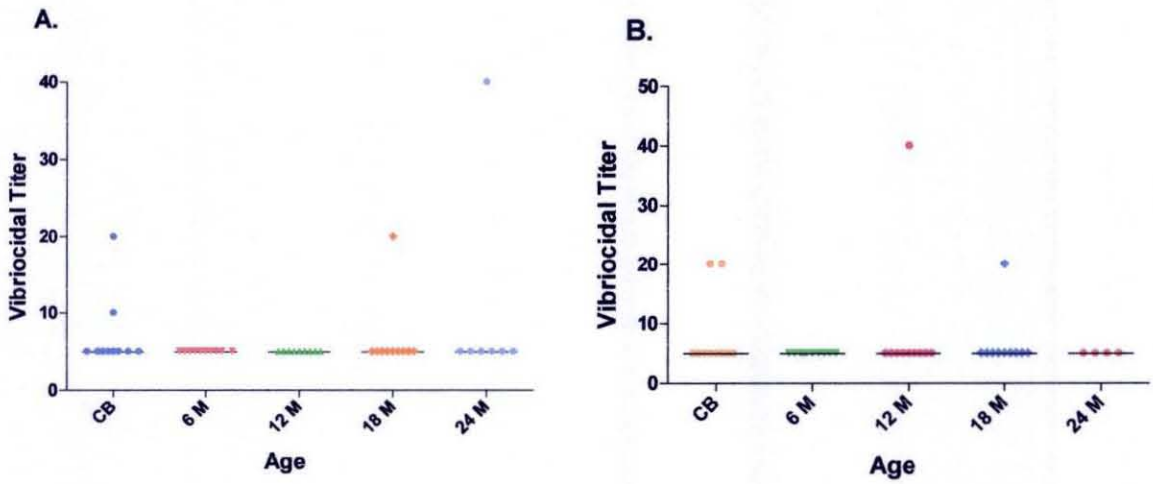

Figure 3.11: Vibriocidal antibody response in plasma A. Ogawa, B. Inaba The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the median values whereas the error bars represented the SEM.

3.9 Fecal antibody, Immunoglobulin A (IgA) Profile

Fecal antibody, immunoglobulin A (IgA) of the study participants (except newborns) was measured by Enzyme linked immunosorbent assay (ELISA).

3.9.1 Fecal total IgA Content

Fecal total IgA content was found to be differing insignificantly. However, among the infants, 6 months showed lowest IgA content but highest in 12 months old a children, which was decreased gradually with the increasing age up to 24 months (Fig-3.12 A.).

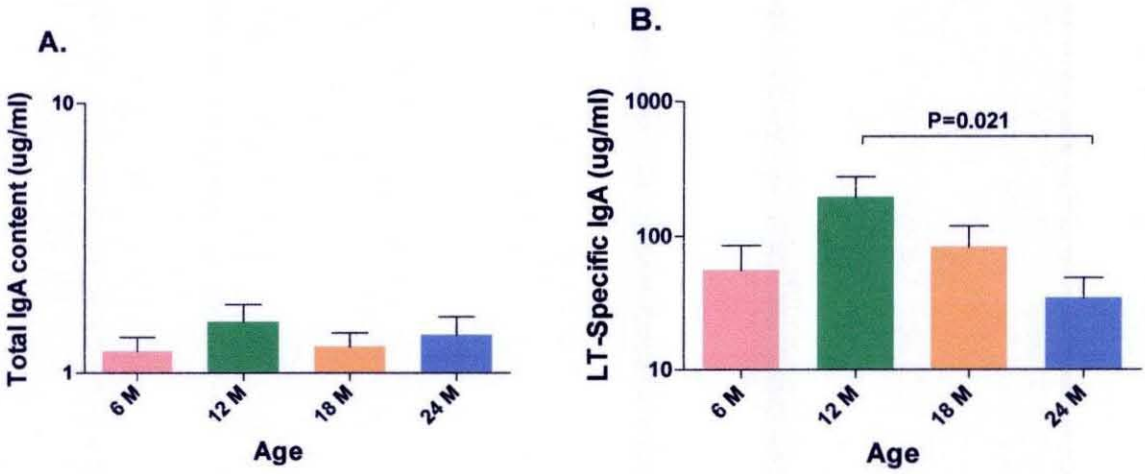

Figure 3.12: Fecal total IgA and LT-specific IgA. The statistical analysis was done by Mann-Whitney Rank Sum Test for comparison of two groups. The results were considered significant when $P \leq 0.05$. The bars represent the mean values whereas the error bars represented the SEM.

3.9.2 Heat labile toxin (LT) of *Escherichia coli* specific fecal immunoglobulin A (IgA)

The LT-specific fecal IgA response was found to be low at 6, 18 and 24 months old children , although it was significantly elevated at 12 months than 24 months children (P= 0.021) (Fig-3.12 B.).

CHAPTER 4

DISCUSSION

Human neonates are highly susceptible to infections by a wide range of bacteria, viruses, protozoa, and fungi {Marodi, 2006 #11} and remain as the most common infectious agents responsible for death in early life. The role of the immune system has recently been reported to be specifically adapted rather than immature {Wood, 2011 #291} at birth is to protect neonates from those pathogens during postnatal life {Trivedi, 1997 #119}.

Recognition of the high burden of disease in early life and advances in the understanding of neonatal immunology has resulted in renewed interest in the study of the functional maturation of neonates. In view of these observations, the present study was undertaken to evaluate the development of immune response of healthy infants of different ages between birth to two years.

The knowledge of the kinetics of the changes occurring in postnatal period, and associated qualitative/ quantitative changes in individual cellular functions, is exceedingly sparse {Holt, 2000 #83}. In our present study, the postnatal immune functions development in human until 2 years was compared with cord blood that represents the neonatal blood.

We analyzed the immune function of cord blood mononuclear cells (CBMC) in comparison to peripheral blood mononuclear cells (PBMC) of infants in different ages.

The inability of neonates to respond to antigen stimulation in vitro that has been proposed by Lewis D.B. and Wilson C.B may be due to less antigen-experience and exposure {Lewis DB, 1995 #120}. However, T cell hypo responsiveness in neonates reported by Pirenne H.Y. et al reflects an inherent functional immaturity of T cells {Pirenne, 1992 #128}.

In our study, poor T cell proliferative responses to mitogen, phytohemagglutinin (PHA) was found in newborns and young infants of 6 months and 12 month than the elderly children of 18 month and 24 months (P= 0.02) which presumably could be due to evolution of antigen primed T cells from naïve neonatal T cells and their increase in number with age.

Further, we evaluated T cell proliferative responses induced by a vaccine antigen, tetanus toxoid (TT), which is currently in use at different stages of immunological maturation (from birth to 14 weeks of age). Age dependent increase in T cell response to tetanus toxoid (TT) was observed in our study, which suggests the age-dependent progressive maturation of T-cell responses in infants.

However, delayed or less responsiveness to antigen due to immaturity of neonatal T cell was also reported by Harris, D. T *et al* in another study conducted in North America {Harris, 1992 #173}.

In this study, the T and B cell specific cell surface protein, cluster of differentiation (CD) on lymphocytes were analyzed immediately upon isolation and antibody staining of CBMC and PBMC by flow cytometry (FACS). We found that cord blood contained lower numbers of $CD3^+$, $CD4^+$ and $CD8^+$ cells than peripheral blood of study participant infants. More importantly, we also observed that newborn T cells expressed the $CD3^+$ antigen at lower level than 6 months ($P< 0.0001$), 12 months ($P< 0.0001$),18 months ($P= 0.001$) and 24 months ($P< 0.0001$) old children T cells and may represent maturational intermediates that may or may not be fully functional. In addition, gradual fall in $CD4^+/CD8^+$ ratio following increase of age was found in our study, which demonstrates maturation of cytotoxic capacity of infant T-cell over age.

However, in another neonatal study, Chirico G. *et al* showed similar age dependent maturation of T cell through increase in cytotoxic function of T cell and decrease in ratio of T helper and cytotoxic T cells ($CD4^+/CD8^+$) upon age {Chirico, 2005 #163}.

Percentage of B lymphocytes ($CD19^+$) was also found significantly low in cord blood than in peripheral blood of children at 6 months ($P=0.0002$), 12 months ($P< 0.0001$), 18 months ($P= 0.001$) and 24 months of age ($P< 0.0001$). The presence of B cell specific CD antigens other than $CD19^+$ like $CD5^+$, $CD20^+$, $CD21^+$ and $CD22^+$ in newborns is perhaps a reason for its low percentage of $CD19^+$ than infants blood as presence of high percentage of $CD5^+CD19^+$ B cells was reported by Randolph D A in 2005 {Randolph, 2005 #211}.

In this study, we have analyzed the systemic immune responses of the IgG and IgA antibodies against cholera toxin (CT) of *V. cholerae*. The IgG ELISA method was found to be particularly suitable for seroepidemiological studies of cholera {Levine, 1981 #277}. Radically elevated level of CT-specific IgG response was seen in infants of other age groups when compared with newborns. In contrast, the 24 month aged children had significantly elevated levels of IgG than the 6 months old infants (P= 0.03). Besides, CT-specific plasma IgA response was found to be significantly lower in newborns than in 6 months (P= 0.0003), 12 months (P= 0.0001), 18 months (P= 0.0001), and 24 months (P= 0.0001) old children. However, 6 months old infants showed much lower IgA response than 12 months (P= 0.02), 18 months (P= 0.029), and 24 months (Fig 3.7) old ones, possibly due to lack of placental transfer of maternal IgA to fetus during pregnancy.

Bangladesh is an endemic country for enterotoxigenic *Escherichia coli* (ETEC) infection, Significant difference in LT-specific IgA responses (Fig 3.8) was observed among the group of infants than IgG responses in our study, which may be due to previously explained placental transfer of IgG rather IgA during gestational life.

In our study, similar antibody responses were observed with the B subunits of LT and CT using GM1 ELISA. Since LT has antigenic determinants (about 80% sequence homology {Moseley, 1980 #274} {Dallas, 1979 #275}) in common with the B subunits of CT, these could share several properties at the molecular level like common colonization mechanism {Clements, 1979 #261}{Clements, 1980 #262}{Field, 1979 #263}{Geary, 1982 #264}{Kantor, 1975 #267}{Clements, 1978 #265}{Donta, 1974 #272}{Honda, 1981 #271}{Holmgren, 1973 #270}{Gyles, 1974 #269}{Klipstein, 1977 #268}. Most likely due to the presence of cross-reactive antibodies, our study has found similar antibody responses with the B subunits of LT and CT.

Vibriocidal antibody assay is a sensitive tool for measuring vibriocidal antibodies that are the best studied and reliable surrogate markers of protective immunity to cholera {Finkelstein, 1962 #248} {Qadri, 1995 #257}.

In current study, vibriocidal plasma antibody response was measured against two common serotypes of *V. cholerae*, Ogawa (X-25049) and Inaba (Strain-19479). We found plasma vibriocidal antibody response expressed as vibriocidal titer, did not differ significantly in infants of all age groups for both the target serotypes of *V. cholerae* O1 organisms.

A recently conducted study on both children and adult cholera patients in our country also confirmed the fact that, infants have the lowest baseline vibriocidal antibody levels compared to children and adults, presumably reflecting the previous exposure to *V. cholerae* infections with growing age, as living in an area of cholera endemicity {Leung, 2011 #283}.

A standard measure of successful immunization is the development of antigen specific humoral immunity {Ochsenbein AF, 2000 #245}. In our study, vaccine specific immune response to tetanus toxoid (TT) was determined by quantification of TT specific IgG with a commercially available sensitive and specific enzyme-linked immunosorbent assay (ELISA) system. Gradual decline of IgG from newborns to 18 months of age was seen probably due to the 2 dose vaccination {WHO, 2010 #64} {WHO, 2011 #235} {WHO, 1996 #281} to mother during pregnancy and 3 dose vaccination to neonate during early infancy {WHO, 2011 #235}.

To evaluate vaccine specific immune response to measles, our study found high levels of cord blood-measles virus IgG responses than in infants of 6 months of age. This can be explained by the presence of high titer of maternal antibodies in newborns which have influence on the response. In contrast, responses were seen consistent in the higher age groups (Fig 3.10) upon vaccination at around 9 months {WHO, 1996 #281} when presence of maternal antibodies is very less of no longer can be detected.

Earlier, Albrecht, P *et al* described the presence of maternal antibody as a possible reason of measles vaccine failure in early infancy and presence of high anti-measles virus antibody titer {Albrecht, 1977 #42} {Siegrist, 1998 #39} {Siegrist, 1998 #20}.

The main protective immunoglobulin at intestinal mucosal surfaces is IgA. The level of protective fecal IgA was measured in this study to assess the development of infant intestine. Among the study infants, 12 months old showed maximum IgA content possibly be due to influence of breast milk derived IgA as cord blood donor neonates were excluded from this part of the study because of unavailability of collection of fecal sample at the moment of blood collection.

It is becoming increasingly clear from recent studies that the seeds for expression of a variety of immunologically mediated diseases in adulthood are sown during infancy i.e. early postnatal life. During this period, the immune system is fine-tuning a variety of key functions, in the face of direct stimulation from environmental signals not previously encountered during fetal life, and the response patterns ''learned'' during this period persist into adult life.

The present study was conducted to observe development of infant immune response in different time periods from various aspects which may in future be helpful for analyses of mechanisms that underlie differential immune responses in newborns and infants so that prevention and treatment of neonatal infections can more safely be targeted to address UN Millennium Development Goal 4 on reducing childhood mortality. These baseline results will also help in understanding the reason children in Bangladesh and in other developing countries show differential responses to vaccines and infections compared to those living in industrialized settings. Such studies need to be undertaken.

CHAPTER 5

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APPENDICES

Laboratory Apparatus

1. Eppendorf tubes and micropipette tips were taken from Eppendorf® and Sigma, and were sterilized by autoclaving at 121°C for 20 minutes.
2. Petridishes used in the experiments were provided by either Sterilin or Gibco. Screw capped tubes and other glass wares were taken from Pyrex® Labware, USA.
3. Plastic tubes and pipettes were of Falcon®; both were the brands of Becton, Dickinson and Company, USA.
4. 96F Microwell low affinity ELISA plates and 96U T-cell culture plates were obtained from Nunc™, Sweden.
5. Micropipettes were from Thermo Labsystems.
6. Multi-channel dispensers (Lab Systems, USA).
7. Mini scale centrifugations were carried out in eppendorf® Centrifuge 5417R, large-scale centrifugation were carried out in a Sorvall® RT 6000B Refrigerated Centrifuge. ELISA and vibriocidal reading were taken using ASCENT Multiskan® reader. T-cell proliferation was measured by LS6500 Multipurpose Scintillation Counter, Becton, USA. Flow cytometry is done by FACS Calibur instrument, BD, USA.
8. Heparin-coated sterile vacutainer tubes (Becton Dickinson, Ruther-ford, NJ).

1. BSA (Bovine Serum Albumin), Gibco BRL- 16140-071.
2. Ficoll-Isopaque, GE Healthcare, Uppsala, Sweden.
3. Rabbit anti-human immunoglobulins, Affinipure (goat-antihuman IgG), horse radish peroxidase (HRP), Jackson Immuno Research, West Grove, P. A., USA
4. H_2O_2 (Hydrogen Peroxide), Fisher Scientific, H-325.
5. Orthophenyline diamine (OPD) Sigma- 7786-30-3.
6. Brain-heart infusion Media, Becto™, BD, USA.
7. Guinea pig complement sera, Sigma.

BUFFERS AND SUBSTRATE SOLUTIONS

Preparation of phosphate buffer saline (PBS) (Vacutainer System; Becton Dickinson, Rutherford, NJ) (pH 7.2)	
NaCl *(Fischer Scientific, Pittsburgh, PA, USA)*	80.00 g
Na_2HPO_4	11.50 g
KH_2PO_4	2.00 g
KCL (*Fischer Scientific, Pittsburgh, PA, USA*)	2.00 g
Deionized water	1000.0 ml

The concentrated solution (10xPBS) was diluted ten times and was used as working solution.

Preparation of 0.1% BSA in PBS (500 ml)	
Phosphate Buffer Saline (PBS)	500 ml
Bovine Serum Albumin (BSA)	0.5 g

Preparation of 0.1% BSA in PBS-Tween (500 ml)	
Phosphate Buffer Saline (PBS)	500 ml
Bovine Serum Albumin (BSA)	0.5 g
Tween	250 µl

Tri-natrium citrate ($Na_3C_6H_5O_4.2H_2O$)	29.40 gm
Deionized water	1000.0 ml

Preparation of Orthophenylene diamine - H_2O_2 substrate (10 ml)	
Orthophenylene diamine (OPD)	10.0 mg
0.1 M sodium citrate (pH 4.5)	10.0 ml
30% H_2O_2	4.0µl

MEDIA

Modified DMEM media (50 ml)	
DMEM	47 ml
AB Serum	2.5 ml
Gentamycin	0.5 ml

Brain-Heart infusion Broth (BHIB) (500ml)	
Brain-Heart infusion media	18.5 gm
Deionized water	500 ml

Normal Saline (1000ml)	
NaCl	8.50 gm
Deionized water	1000 ml

APPENDIX D

PREPARATION OF REAGENTS AND CHEMICALS

For Tetanus Toxoid (TT)-IgG ELISA

Preparation of Washing Solution (1:30 dilution) 30 ml	
Buffer concentrate	1.0 ml
Distilled water	29.0 ml

Ready-to-use:

- Control sera and standard sera
- Anti-human-IgG-conjugate
- Dilution buffer
- Substrate
- Stopping solution

For Measles Virus-IgG ELISA

For one test plate:

Preparation of Anti-Human IgG/POD Conjugate (1+50 dilution)	
Conjugate	250µl
Conjugate Buffer Microbiol	12.5 ml

Preparation of Coloured Sample Buffer POD (1+20 dilution)	
Colour Solution (blue)	2.5 ml
Sample Buffer POD	50.0 ml

Preparation of Washing Solution POD (1+20 dilution)	
Washing Solution POD	20.0 ml
Distilled/deionized water	400.0 ml

Preparation of working Chromogen Solution (1+10 dilution)	
Chromogen TMB	1.0 ml
Buffer/Substrate TMB	10.0 ml

Antibodies and their dilutions used in flow cytometry (FACS)

Antibody	Dilution	Company
anti-CD3-allophycocyanine (anti-CD3-APC), Cat#347543	1:100	BD
anti-CD4-peridinin chlorophyll protein (anti-CD3-PerCP), Cat#347324	1:10	BD
anti-CD8-fluorescein isothyocyanate (anti-CD8-FITC), Cat#340692	1:5	BD
anti-CD19-phycoerythrin (anti-CD19-PE), Cat#120457	1:5	BD

APPENDIX E

SOFTWARES

1. MS word
2. Microsoft Excell
3. Graphpad Prism (version 5.02, , USA).
4. Flowjo (version 5.8.3)
5. Endnote X
6. Ascent software