NATURALLY ACQUIRED IMMUNITY TO *PLASMODIUM FALCIPARUM* ANTIGENS IN CHILDREN LESS THAN FOUR YEARS OF AGE IN A MALARIA HOLOENDEMIC REGION OF WESTERN KENYA.

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ABSTRACT.

The mechanism of age-dependent acquisition of immunity to *P. falciparum* in malaria endemic areas is less clear. Understanding how immunity is acquired with age in humans requires a rational design and development of methodology for evaluating the key features of naturally acquired immunity especially in infants and young children. However, immunological studies in infants and young children have been hampered by the ethical requirement that only small blood volumes can be drawn for the studies. To circumvent this problem, this study was designed to use finger prick blood samples to investigate the level and age dependent development of immunological responses to *Plasmodium falciparum* antigens in children less than 4 years of age from western Kenya. A total of 110 individuals were enrolled and stratified into four age groups: 0-12 months, 13-24 months, 25-36 months, and 37-48 months. 0.5-1ml of finger prick blood was collected from children and separated by Ficol-Hypaque density gradient centrifugation to obtain the peripheral blood mononuclear cells (PBMC) and the plasma. Varying concentrations of PBMC ($1x10^5$, $2x10^5$, and $5x10^5$) were plated per well and cultured for 72 and 120 hours with each mitogen (phytohaemoglutinin [PHA], and phorbal 12 meristate 13 acetate-ionomycin [PMA-I]) and *Plasmodium falciparum* antigenic peptides (Liver stage antigen-1 [LSA-1] and Merozoite surface protein-1 [MSP-1]) in humidified incubator at 37.2^0C and 5% carbon dioxide in cRPMI 1640 media. After incubation, the supernatant from each well was tested for the presence of interleukin 10 (IL-10) and interferon gamma (IFN-γ) cytokines and plasma was used to test for the presence of total immunoglobulin M (IgM), IgG and IgG subclass antibodies to *Plasmodium falciparum* malaria antigens by enzyme linked immunosorbent assays (ELISAs).

The results showed that the frequencies of IFN-γ responses to both antigens showed an increasing trend while the frequencies of IL-10 were similar across the age groups. The frequencies and levels of total IgG, IgM and IgG subclass responses to pre-erythrocytic and erythrocytic antigens were variable. For IgG subclasses, only IgG1 and IgG3 but not IgG2 and IgG4 to all antigens were found at detectable levels. Total IgG, IgG1 and IgG3 frequencies to circumsporozoite protein (CSP) were similar across the age groups. IgG1 and IgG3 frequencies to thrombospondin-related adhesive protein (TRAP) were similar in all age groups as compared with total IgG that was higher in age group 0-4 months and 37-50 months ($\chi^2 = 8.93$, $P=0.0302$). Total IgG and subclass responses to LSA-1 showed a significant strong trend of increase across the age groups ($\chi^2 = 21.69$, $p<0.0001$). On the other hand, frequencies of total IgG to apical membrane antigen-1 (AMA-1), glycosylated and non-glycosylated erythrocyte binding antigens-175 (EBA-175 G and EBA-175 NG) were significantly higher in 0-4 months, decreased in 5-20 months and increased progressively up to 50 months ($\chi^2 = 21.90$, $p=0.0001$; $\chi^2 = 25.12$, $p<0.0001$ and $\chi^2 = 12.45$, $p=0.006$ respectively). Only frequencies of IgG1 to EBA-175 G was significantly different across the age groups ($\chi^2 = 6.24$, $p=0.0256$), while IgG3 frequencies to these erythrocytic antigens were similar across the age groups with over 50% responding.

MSP-1_{19} results showed that total IgG frequencies to all MSP-1 alleles were similar across the age group. Frequencies of IgG1 to Q-KNG ($\chi^2 = 12.19$, $p=0.0256$) and IgG3 to E-KNG, Q-TSR and E-TSR allelic peptides ($\chi^2 = 9.54$, $p=0.023$; $\chi^2 = 9.59$, $p=0.0224$, and $\chi^2 = 17.14$, $p=0.0007$ respectively) significantly varied across age groups. The frequencies and levels of IgM responses to both pre-erythrocytic and erythrocytic

antigens significantly increased across age groups ($p<0.05$) except for two MSP-1 allelic peptide (Q-KNG and Q-TSR) that were similar across the age groups.

This study suggests that age and /or exposure to malaria infection affects development of anti-malarial cytokines and antibodies and that there is differential acquisition of antibodies to pre-erythrocytic and erythrocytic antigens by children less than four years of age. These findings may be useful for future studies in malaria vaccine design and assessment and evolution of immunity in young children from malaria endemic areas.

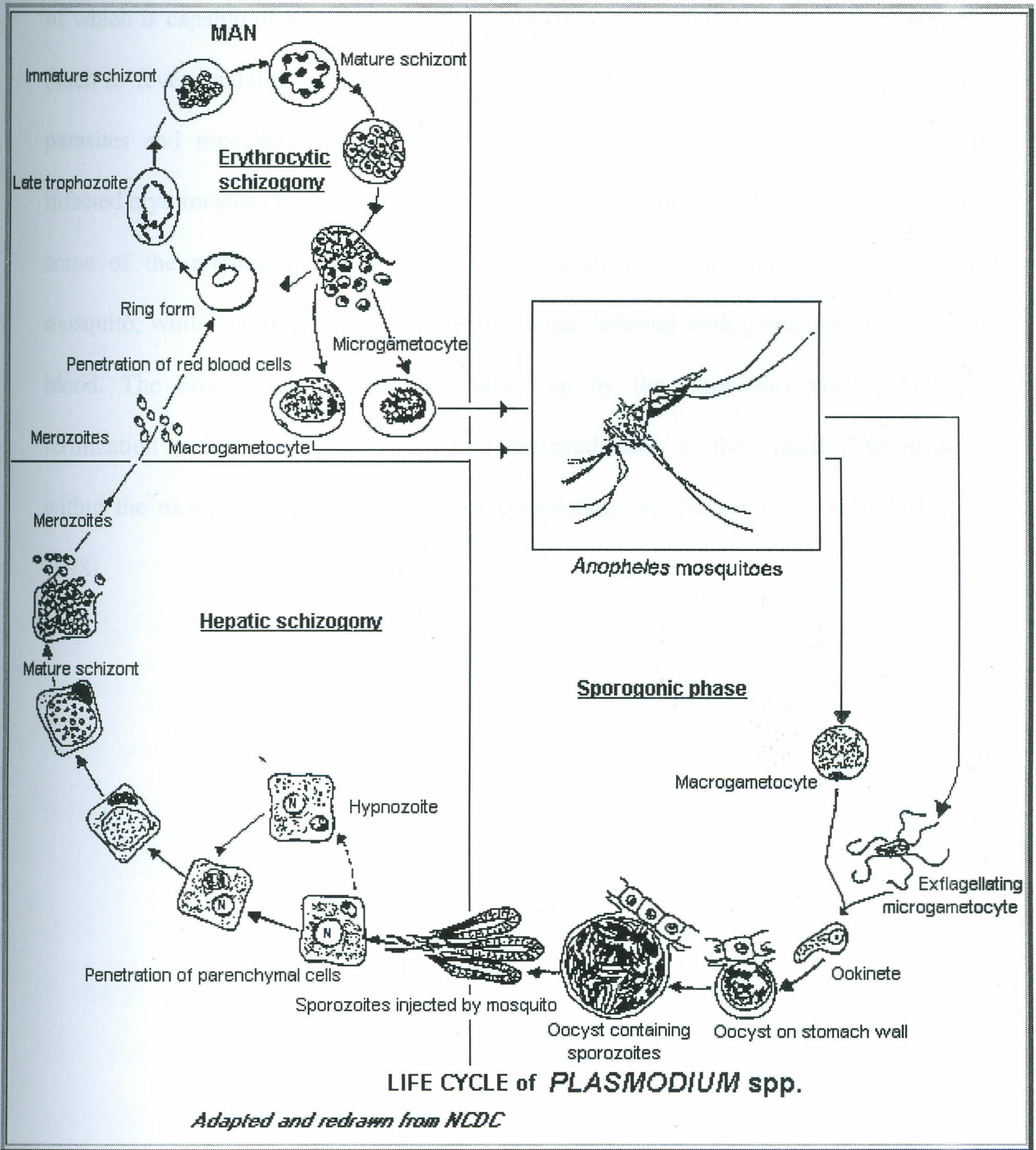
CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1. Human *Plasmodium* species and life cycle

Malaria is a significant health problem in many parts of the world with more than 500 million clinical cases and over 3 million deaths reported annually (Taylor-Robinson, 2000; Patten-Hitt, 2001). In sub-Saharan Africa alone, it is estimated that more than 150 million clinical cases of malaria occur annually, and that about 2 million people die from the disease every year (Hviid, 1998). At-risk groups include those in whom immunity has not yet developed (travelers, young children in endemic areas, etc) and those in whom immunity has diminished (pregnant women and people from endemic areas who have ceased to be routinely exposed to infection). Hence malaria is often cited as a substantial impediment to economic and social development in endemic regions (James and Miller, 2001; Hviid, 1998).

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium.* Four species of *Plasmodium* cause malaria in humans: *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malarie*. Of all these, *Plasmodium falciparum* is the most important, both in terms of the number of clinical cases and the severity of the disease it causes (Hviid, 1998). Malaria infection is acquired when infected female *Anopheles* mosquitoe injects sporozoite-stage malaria parasites from its salivary glands into the human blood stream while feeding as shown in the life cycle, Figure 1. Once injected, the sporozoites infect specifically the liver cells, where an initial round of parasite multiplication takes place. Initially, the sporozoites develop into exo-erythrocytic forms or liver schizonts. After approximately 5-8 days, the infected liver cells

Figure 1. Life cycle of *Plasmodium falciparum*.



(hepatocytes) bursts, releasing thousands of merozoite stage parasites, each of which is capable of invading erythrocytes. Within the erythrocytes, the parasites continues to multiply and approximately every 48 hours releases up to 32 "daughter" merozoites, each of which is capable of invading a new erythrocyte. All the clinical symptoms of malaria (such as fever, malaise, anaemia) are associated with this multiplication of blood-stage parasites and especially with the, often synchronous, bursting of large numbers of infected erythrocytes (Hviid, 1998; Patten-Hitt, 2001; Nardin *et al*, 1993). After ten days, some of the merozoites mature into sexual forms (gametocytes). At this time the mosquito, while taking a blood meal, will become infected with gametocytes in human blood. The blood-stage gametocytes, taken up by the *Anopheles* vector, undergo fertilization and sporogony resulting into the production of the infective sporozoites within the mosquito salivary glands thus completing the life cycle of malaria (Hviid, 1998).

1.2. Malaria vectors

Human malaria is a disease transmitted by female *Anopheles* mosquitoes. Mosquitoes that transmit human malaria belong to the Order Diptera, Class Insecta, family Culicidae, and genus *Anopheles*. Depending on the species, mosquitoes seem to show preferential habitat colonization though different species are known to co-exist in one habitat (Minakawa *et al.*, 2002). Previous studies have shown that the main malaria vectors in western Kenya are *Anopheles gambiae, Anopheles arabiensis* and *Anopheles funestus* (Gimning *et al.*, 2002). Some studies have also demonstrated that malaria can be transmitted through infected blood as well as transplacentally, though both modes contribute to very low cases (Ericsson *et al.*, 2002).

1.3. *Plasmodium* diagnosis

Proper diagnosis of the *Plasmodium* parasites in the blood samples of an infected individual is fundamental to treatment, control and prevention of malaria. Currently, there are several methods of identifying the *Plasmodium* parasites. The most common conventional method of microscopic examination of thick and thin blood smears stained with Giemsa or field stains requires skilled manpower, is laborious and time-consuming (Lee *et al.*, 2002). However, newer techniques of malaria diagnosis include: the quantitative buffy coat method, which employs fluorescent staining of the parasites after an enrichment step for the infected erythrocytes, the *para*sight F and the malquick tests, which are based on the immunological capture of the *P. falciparum* histidine-rich protein 2 in whole blood (Lek-Uthai *et al.*, 2000), and the OptiMal assay, which is an antibody-based detection of parasite lactate dehydrogenase. Polymerase chain reaction (PCR)-

based methods for detection of *Plasmodium* DNA and RNA have recently been developed and are more sensitive and specific than other tests for malaria parasites detection (Phillips, 2001; Lee *et al.*, 2002). Some of these new diagnostic techniques may have specific applications in particular settings, depending on the purpose and location of testing, cost, speed, ease of use, and desired sensitivity and specificity (Lee *et al.*, 2002).

1.4. Malaria control and prevention strategies

Before implementation of malaria control measures, various factors should be put into consideration. These include: the biological, anthropological, cultural, and social characteristics of the population; the intensity and the periodicity of malaria transmission; the species of malaria parasites and their sensitivity to antimalarial drugs; the species of the mosquitoe vector, their behavior, and their susceptibility to insecticides; the presence of social and ecological change and the type of health services available (Phillips, 2001). All strategies of malaria control can be grouped into three areas: vector control, diagnosis and treatment of infected people, and early detection and warning on epidemics followed by rapid application of appropriate control measures (Lindsay *et al.*, 1996; Lek-Uthai *et al.*, 2000; Phillips, 2001; Shiff, 2002; Fischer and Bialek, 2002).

1.4.1. Environmental management

Mining, logging and land clearance for agricultural activities are some of the major operations that can have rapid negative impact on the environment. Roads that are constructed to clear way for these activities as well as extensive pits dug alongside the roads causes accumulation of water. Open cast mining creates water catchment areas

while felling trees exposes fragile topsoil, which quickly erodes hence creating water catchment areas. Siltation in local rivers due to soil erosion leads to shallow river margins. All these activities lead to creation of new mosquitoe breeding sites. Furthermore, workers that lack an effective level of acquired immunity may be imported from areas where malaria is absent or infrequent and may lead to insurgence in malaria incidence (Phillips, 2001). However, other environmental manipulation activities such as filling in ditches, covering or destroying water containers, flushing irrigation channels, and slashing long grass are beneficial in malaria control efforts since they reduce the breeding sites.

1.4.2. Chemical control

Various chemicals can be used to reduce mosquitoe vector population and thus help in reducing malaria incidence. These include petroleum oils, larvicides and insecticides such as organochlorines, organophosphates and carbamates (Rose, 2001). Petroleum oils and derivatives applied onto water form a film that prevents mosquitoe larvae and pupae from breathing through the surface of the water. Larvicide such as paris green (copper acetoarsenite) contains very small particles that are filtered in by mosquitoe larvae while feeding leading to poisoning and death of the larvae. Insectides including the natural components of flowers (pyrethrum and pyrethrin) and their synthetic derivatives (pyrethroids), and also organochlorines (e.g. dichlorodiphenyltrichloroethane and dieldrin), organophosphates (e.g. malathion and temephos), and carbamates (e.g. propoxur) are used as dust or sprays against adult mosquitoes. Since the rationale for using these insecticides is to kill or repel the mosquitoes, they should be formulated to be

active on the sprayed surface for weeks or months. However, several species of mosquitoes have developed resistance to these insecticides, and coupled with the environmental contamination by these chemicals, their use has greatly been limited. These insecticides have also been demonstrated to cause modifications in mosquitoe behaviour. For example, one study in Amazon region has shown that *Anopheles darlingi* that is normally endophilic rests inside houses for only a few minutes before and after feeding if DDT is applied (Phillips, 2001; Shiff, 2002).

1.4.3. Biological control

This involves use of natural enemies and predators to control the population of mosquitoes which transmit malaria. Use of naturally occurring bacteria to control mosquitoe larvae have been applied safely in the field and shown to be effective. *Bacillus sphaericus* and *Bacillus thuringensis* synthesize potent larvicidal toxins that are toxic when ingested by mosquitoe larvae while feeding (Boyd, 2001). The protoxins produced by these bacteria are solubilized in the alkaline pH of the larval midgut, proteolytically activated and bind to specific receptors on the epithelial cells leading to cell lysis, which makes the larvae to stop feeding leading to death. However, the use of these bacteria as biological control agents has not been widespread due to the following reasons: (a) spores of *Bacilli* sediment rapidly from the larval feeding zones, especially for *Anopheles* larvae that feed on the surface; (b) the *Bacilli* Spores are sensitive to ultraviolet light from the sun; (c) the rate of larvicidal activity is lower as compared to use of insecticides; and (d) the *Bacilli* lack broad-spectrum effect on mosquitoes and are relatively species-specific and also very expensive to formulate. To add on these, the public resistance on

the use of genetically modified organisms further aggravates this situation (Phillips, 2001).

1.4.4. Human protection from mosquitoe contact

Several methods have been used to reduce the incidence of human contact, with mosquitoes. These include the use of insect repellents, protective clothing, insect screens, siting of dwellings away from mosquitoe breeding sites, improvement of design and construction of dwellings, and use of insecticide impregnated bed nets. Although the use of impregnated bed nets has been shown to significantly reduce morbidity and mortality due to malaria (AMREF, 1999), its effects on the acquisition of natural immunity is doubted (Perera, 1997). Acquired immunity to clinical malaria is thought to develop with time and to be a function of the frequency of infections. Therefore, use of bed nets might reduce exposure and consequently the multiplicity of infections leading to a corresponding delay of acquired immunity (Phillips, 2001; Carnevale, 1995).

1.4.5. Climatic changes and disease prediction

Mosquitoe population has been shown to be dependent on climatical changes (Minakawa *et al*., 2002). Therefore monitoring of climatic variables (rainfall, temperature and, humidity) may be relevant for predicting malaria epidemics. Also, use of remote sensing techniques (such as local aerial photography and satellite-based sensors) to identify mosquitoe breeding sites can help in prediction of malaria epidemics (Hay *et al*., 1998). This is because early prediction of epidemics is important in the context of early planning and implementation of antimalarial control measures.

Global warming is also thought to have an impact on malaria transmission in subtropical areas. This is because slight increases in temperature in these areas where malaria is unstable or currently absent might increase transmission. In contrast, areas with stable transmission may have little or no change in transmission with temperature rise (Phillips, 2001; Lindsay *et al.*, 1996).

1.4.6. Transgenic mosquitoes

Manipulation of mosquitoe genome has been identified as another strategy of reducing malaria prevalence (Ghosh *et al.*, 2002). Focus has been placed on main malaria vectors: *Anopheles gambiae*, *Anopheles arabienses* and *Anopheles funestus* in an attempt to replace their natural population with populations which are unable to complete the development of *Plasmodium*. The aim is to identify genes for refractory as well as those that inhibit development of *Plasmodium* in the mosquitoe vectors. However, the public responses on the environmental effects on the use of genetically modified mosquitoes might be a drawback to these efforts (Phillips, 2001).

1.4.7. Antimalarials and drug resistance

Malaria is a curable disease, and early treatment will significantly reduce the morbidity and mortality cases due to *Plasmodium* infection. If the parasites are successfully transmitted to a person and becomes established in the system, drug intervention would eventually be required to reduce or prevent clinical malaria. For the last 50 years, there have been two main groups of antimalarial agents in use. These are antifolates and *Cinchona* alkaloids or quinoline-containing drugs (Ramachandran, 2002).

The antifolates include diaminopyrimines (such as pyrimethamine), biguanides (such as proquanil) and sulphur drugs (such as sulfones). The qiunoline-containing drugs include quinine and halofantrine. Sometimes, mefloquine and halofantrine are used in combination with antibiotics such as tetracycline and doxycycline to treat malaria infections (Phillips, 2001; Shiff, 2002).

Initially, these drugs used to clear blood infection and hence reduce clinical malaria. However, recent studies have demonstrated the existence of widespread resistance to the commonly used antimalarials by the *Plasmodium* parasites (Nardin *et al.*, 1995; Phillips, 2001; Ramachandran, 2002). These trends of resistance seem to be drug specific and vary from one region to another. But the fact that resistance to cheap and affordable drugs such as chloroquine has been reported in almost all parts of the world and especially by *Plasmosdium falciparum* (Nardin *et al.*, 1995; James and Miller, 2001) shows that malaria poses a great threat to humans and urgent effective intervention measures should be developed to reduce morbidity and mortality associated with severe malaria.

It has also been noted that lack of medical facilities and the unavailability of adequate medication for local populations in malarious regions compound the difficulties of malaria control (Nardin *et al.*, 1993).

As a result of the spread of drug-resistant malaria parasites and insecticide-resistant mosquitoe vectors, there are now fewer tools to control malaria than existed 20 years ago. Because of growing malaria global burden, both socially and economically, its control is however essential. Historically, vaccines have been one of the most cost-effective and easily administered means of controlling infectious diseases, yet no licensed

vaccines exist for malaria. Accumulating basic and clinical research suggest that effective vaccines for malaria can be developed and could significantly reduce morbidity and mortality, and potentially reduce the spread of infection (James and Miller, 2001).

1.4.8. Malaria vaccines

It is ironical that the only recent option in controlling clinical malaria is based on upregulating the level of immunity against malaria parasites since the use of insecticides and drugs have proved to be largely ineffective. From some studies, it has been shown that under natural exposure to *Plasmodium* parasites, partial immunity to malaria is developed which never provides total protection against new infections (Amador and Patarroyo, 1996). Since immunity acquired through constant exposure to malaria infections is never complete (Hogh, 1996), a prospective antimalarial vaccine to be developed should therefore be able to provide a level of protection that is never achieved by natural exposure. However if natural immunity could be mimicked, then vaccination could prevent severe malaria morbidity and mortality but not give complete protection against new infections.

Malaria parasites have complex life cycles and distinct developmental stages each of which has multiple antigens that could serve to elicit host immune responses. For example, a pre-erythrocytic vaccine would protect against the infectious forms (sporozoites) injected by a mosquito and/or inhibit parasite development in the liver. And in a previously unexposed individual, if a few parasites were to escape the immune defenses induced by a pre-erythrocytic vaccine, they could eventually multiply and result in a full-blown disease. An erythrocytic vaccine would inhibit parasite multiplication in

the red cells, thus preventing (or diminishing) severe disease during the blood infection. Lastly a sexual stage vaccine will not protect the person being vaccinated, but instead will interrupt the cycle of transmission by inhibiting the further development of parasites once they are ingested by the mosquitoe along with the antibodies produced in response to the vaccine. Hence an optimal vaccine should have the ability to elicit protective immunity that blocks infection as well as prevent pathology and interrupt transmission of parasites, and would most likely be a combination or cocktail vaccine comprised of sub-units from different developmental stages of the parasite (James and Miller, 2001).

1.4.8.1. Preerythrocytic vaccines

Complete species specific protection can be induced when infected mosquitoes are irradiated and allowed to feed on volunteers, (Clyde, 1975; Herrington *et al.*, 1991). Though this method is ideal, it is impractical because vaccinating each individual will require thousands of irradiated mosquitoes. As a result, studies on the proteins expressed on the surface of the sporozoites have been thought to be a better alternative. The first protein expressed on the surface of the *Plasmodium falciparum* sporozoite to be discovered was circumsporozoite protein (CSP). This protein has been used to vaccinate humans and animals and has been shown in some studies to elicit high antibody levels that completely protected some individuals hence indicating it as a viable vaccine candidate (Chatterjee *et al.*, 1999; Rieckmann, 1990; Herrington *et al.*, 1991; Hoffman *et al.*, 1990; Hoffman *et al.*, 2002).

Other *Plasmodium falciparum* pre-erythrocytic stage vaccine antigens include sporozoite surface protein 2 (SSP-2/TRAP), and liver stage antigen-1 (LSA-1) that have

also been tested in animal models and human beings and shown to evoke both humoral and cell mediated immune responses (Nardin *et al.*, 1993; John *et al.*, 2000; Joshi *et al.*, 2000). While some studies have correlated these responses to protection (John *et al.*, 2000; Kurtis *et al.*, 1999; Dolo *et al.*, 1999; Luty *et al.*, 1998) some have shown no protective role of these responses (Hoffman *et al.*, 1987).

1.4.8.2. Blood-stage vaccines

Clinical cases of malaria are mainly due to invasion of the erythrocytes by merozoites released after bursting of infected hepatocytes and erythrocytes. The parasites invade the erythrocytes leading to breakdown of haemoglobin and eventual release of toxins and other by products, which stimulate immune system to produce pyrogenic cytokines such as tumor necrosis factor that eventually lead to clinical manifestation of malaria (Kwiatkowski *et al.*, 1997). During invasion of erythrocytes, various *Plasmodium* proteins are expressed on the apical region and on the surface of infected erythrocytes (O'Donnell *et al.*, 2001; Saul *et al.*, 2000) and these are targeted by the immune system. Several of these proteins have been characterized (including MSP-1, AMA-1, and erythrocyte binding proteins) and have being evaluated as potential vaccine candidates (Egan *et al.*, 1996; Kabilan *et al.*, 1994). These proteins have been shown in human and mice studies to elicit high levels of specific immunoglobulins (IgG and IgM) that inhibit invasion of erythrocytes by *Plasmodium* parasites (Ndungu *et al.*, 2002; Sim *et al.*, 2001; Garraud *et al.*, 1999; Bouharoun-Tayoun *et al.*, 1990; Branch et al., 1998).

1.4.8.3. Transmission blocking vaccines

To date, there are three target antigens of transmission blocking immunity: pre-fertilization antigens expressed predominantly on gametocytes (such as pf230 and pf48/45), post-fertilization antigens expressed predominantly on zygotes or ookinetes (such as P25 and P28), and late-midgut-stage antigens (such as chitinase inhibitors) required by the ookinete to penetrate through the peritrophic membrane. Antibody as well as complement-dependent activity have been reported as the possible mechanism for protection (Kwiatkowski *et al.*, 1997).

1.4.8.4. Multistage and multivalent vaccines

Stage specific expression of antigens suggests that only stage specific immunity can be developed. However, failure of a vaccine directed to a specific stage would lead to the parasite progression to the next stage and eventually to clinical malaria. Hence, a vaccine should be effective if it contains antigens against more than one stage. Due to genetic restriction within a population, some individuals will be unable to generate $CD8^+$ or $CD4^+$ T cell or B cell responses to specific epitopes in different *Plasmodium* parasite populations. Therefore a vaccine with multiple epitopes should reduce or eliminate this difficulty. These information have led to a suggestion that a multistage and multivalent vaccine would possibly be a solution to malaria control and prevention. One such vaccine (NYVAC-Pf7, a genetically engineered vaccine candidate) has gone through I-IIa safety, immunogenicity and efficacy trials and although it elicited poor antibody responses, cellular immune responses was detected in >90% of volunteers (Ockenhouse *et al.*, 1998;

Shi *et al.*, 1999). It also completely protected one volunteer and delayed time to parasite patency in other volunteers as compared to controls.

1.5. Malaria immunity in animal models

Over thirty years ago, mice immunized with irradiated sporozoites followed by subsequent exposure to infected mosquitoes were protected from clinical malaria (Nussenzweig *et al.*, 1967). This pioneered studies into circumsporozoite protein (CSP), which is the predominant protein expressed by the sporozoites. This study and subsequent ones (Anders *et al.*, 2000; Hoffman *et al.*, 2002; Herrington *et al.*, 1990) have highlighted the protective role of CSP. Irradiated sporozoites seem to confer protection by priming T cells that eventually provide help to B-lymphocytes to trigger the production of high antibody titers that inhibit liver stage development *in vitro* (Chatterjee *et al.*, 1999). This was demonstrated by immunization of C57BL6 mice with 12 krad dose-irradiated sporozoites and post immunization challenge with 100 live sporozoites. However, protection may be dose-dependent as was shown by the ability of 12 krad dose to confer protection as compared to 20 krad dose which did not protect (Chatterjee *et al.*, 1999).

Previous studies on blood stage antigens in mice and rabbits suggests that parasite invasion of erythrocytes can be blocked by antibodies against *Plasmodium* proteins that are expressed during asexual blood stage infection (e.g. MSP-1, AMA-1, EBA-175, MSP-2, RESA). DNA based vaccine plasmids encoding EBA-175 region 11 inhibited invasion of erythrocytes and elicited high anti-EBA-175 antibody titers (Sim *et al.*, 2001)

while apical membrane antigen-1 (AMA-1) has been tested in mice and shown to protect against *Plasmodium chabaudi* (Anders *et al.*, 2000)

Several studies have been done on non-human primates models to evaluate the protective immunity to pre-erythrocytic and erythrocytic antigens. Immunization of chimpanzees with pre-erythrocytic LSA-1, LSA-3 and TRAP has been shown to induce protective cellular and humoral responses against successive heterologous challenges with large numbers of *P. falciparum* sporozoites (Daubersies *et al.*, 2000; Schneider *et al.*, 2001).

Antibodies against *Plasmodium* proteins that are expressed during asexual blood stage infection (MSP-1, AMA-1, EBA-175, MSP-2, RESA) protects against infection by simian parasites. The first demonstration of the protective role of malaria erythrocytic antigens in a non-human primate was shown by immunization of owl monkeys with *P. falciparum* merozoites in 1977 by Siddiqui. These monkeys were protected from subsequent challenge with homologous strains. Apical membrane antigen-1 has been tested in monkeys and shown to protect against *Plasmodium fragile* (Anders *et al.*, 2000). Although some studies suggest some level of heterologous protection (Collins *et al.*, 1994; Daubersies *et al.*, 2000), each vaccine should be tested against parasites of diverse genotypes as demonstrated by ability of antibodies against recombinant AMA-1 to be effective against invasion by homologous (3D7) merozoites but less effective to heterologous, HB3 (Anders *et al.*, 2000). Antigenic diversity can reflect allelic polymorphism (e.g. the presence of MSP-1, MSP-2 parasite population) as well as antigenic variation (i.e. in situation where a clone changes phenotypes due to gene-switching leading to expression of variant forms of antigens on surface of erythrocytes

containing mature asexual forms). Evaluation of EBA-175 region 11 DNA in *Aotus* monkeys have been shown to elicit high antibody titers that significantly reduce parasite density after challenge with live sporozoites hence suggestive of antiparasitic protection (Jones *et al.*, 2000; 2001; 2002). MSP-1 DNA based vaccine has also been shown to protect murine and simian models against sporozoite challenge by inducing high antibody levels which seem to vary depending on the region of terminal sequences. Becker *et al.* (1998) reported that C-and N terminus sequence in PyMSP-1 elucidated higher antibody responses in mice when compared to use of C terminus alone.

In an attempt to demonstrate multiantigen effect of vaccines, Jones *et al.* (2002) immunized *Aotus* monkeys with either single or a combined DNA plasmids expressing AMA-1, EBA-175 or MSP-1. Results by antigen-specific enzyme-linked immunosorbent assays (ELISAs) showed no significant differences in the antibody titer induced to the three antigens when each was used as a separate vaccine compared with the titer induced to the same antigens by the trivalent preparation. However, use of immunofluorescent antibody assays indicated that each of the three monovalent vaccines induced significant antibody titers while trivalent vaccine induced antibody titers 3-12 fold higher than those induced by any of the single vaccines, suggesting that multiantigen vaccine may be more protective than a single antigen vaccine. It also suggests that the method of evaluating the antibody titers affect the results.

Although initial studies on protective role of irradiated sporozoites were focused on antibodies, studies using mice lacking B cells but not T cells were immunized with irradiated sporozoites demonstrating that cellular immune mechanisms are also induced during immune protection. Further studies showed that both $CD8^+$ T and $CD4^+$ T cells

mediated this protection by cytotoxic activity directed at the infected hepatocyte, and by secreting gamma interferon, which in turn induces nitric oxide-dependent killing of the parasite within the hepatocyte (Weiss *et al.*, 1993; Hoffman *et al.*, 1990; Guebre-Xabier *et al.*, 1999).

In murine, Th1 CD4 T cells produce IL-2 and IFN-γ, which are essential for a cell-mediated immune response, while IL-10 which is also known to augment humoral immune responses is produced by Th2 cells (Ho *et al.*, 1995). Further studies have revealed that the Th1 response is dependent on nitric oxide production, while IgG1 antibodies mediate Th2 response (Ho *et al.*, 1995). Therefore, both cellular and humoral immune responses are involved in protection of animal models against malaria infection.

1.6. Acquired immunity in humans

It is well documented that cellular and humoral protective responses to malaria infections can be induced in humans, though partially, by use of stage specific *Plasmodium* antigens (Rieckmann, 1990; Herrington *et al.*, 1991; Egan *et al.*, 1993; Egan *et al.*, 1999; Hoffman *et al.*, 2002). Previous studies on immunization with radiation attenuated *P. falciparum* sporozoites were shown to elicit strain-transcendent protective immunity that persisted for some months (Rieckmann, 1990; Herrington *et al.*, 1991; Egan *et al.*, 1993; Hoffman *et al.*, 2002). However, as with murine and non-human primates, over irradiated sporozoites did not confer any protection suggesting that exposing sporozoites to excess radiation destroys the antigenic epitopes (Silvie *et al.*, 2002). More studies on synthetic peptides circumsporozoite protein conjugated vaccine (NANP) 3-TT and *Plasmodium* malaria circumsporozoite (CS) protein showed that some

volunteers had significant levels of cellular and humoral responses that correlated with protection as depicted by delayed parasitaemia when challenged with bites of infective mosquitoes (Vreden *et al.*, 1991; Herrington *et al.*, 1990; Hoffman *et al.*, 1989; Hoffman *et al.*, 1987). But some studies in Kenya did not show any correlation between high parasite density and vaccines induced anti-sporozoite antibodies (Sherwood *et al.*, 1996). TRAP was shown to elicit high levels of protective antibodies and also IFN-g and IL-4 cytokine production (Flanagan *et al.*, 1999; Dolo *et al.*, 1999). Some *in vitro* studies on LSA-1 have also suggested the protective role of IFN-g and IL-10 cytokines and antibodies to this peptide (Connelly *et al.*, 1997; Kurtis *et al.*, 1999; Domarle *et al.*, 1999; John *et al.*, 2000; Luty *et al.*, 1998).

The roles of antibodies and cytokines in protection against asexual blood stage *Plasmodium* infections have also been evaluated using *in vitro* inhibition assays (Egan *et al.*, 1999; Bouharoun-Tayoun *et al.* 1990; Chappel *et al.*, 1994; Hodder *et al.*, 2001) and by passive transfer of immunoglobulin G from immune adults (Kumaratilake *et al.* 1997; Bouharoun-Tayoun *et al.* 1990). Higher levels of IgG and IgM antibodies to MSP-1 and CSP have been correlated with lower levels of parasitaemia in infants and adults under natural infection in malaria endemic areas (Branch *et al.*, 1998; Egan *et al.*, 1996) though some studies suggested protection without an increase in the serum levels of IgG and IgM (Clyde, 1975; Hoffman *et al.*, 1987; Anderson *et al.*, 1997). Antibodies against AMA-1, EBA-175, and MSP-1 inhibit invasion of erythrocytes by homologous and heterologous strains of *P. falciparum* (Hui *et al.*, 1993; Hodder *et al.*, 2001; Kocken *et al.*, 2002; Pandey *et al.*, 2002). Most antibody studies in endemic areas have implicated cytophilic

antibodies (IgG1 and IgG3) in acquisition of antimalarial immunity against asexual blood stages (Aribot *et al.*, 1996; Oketch *et al.*, 2001).

Although for a long time it has been held that antibodies are mainly involved in protection from malaria, the mechanism of protection is not clearly understood. However, recent studies have suggested that antibodies co-operate with T lymphocytes against both erythrocytic and pre-erythrocytic stage *Plasmodium* infection. It has been shown that IFN-γ, TNF-α and serum or plasma containing anti-*P. falciparum* antibodies increase the rate of merozoite phagocytosis by neutrophils (Bouharoun-Tayoun *et al.*, 1990; Kumaratilake *et al.*, 1992; Bergman *et al.*, 1997). Neutrophils kill merozoites via phagocytosis, and also by production and release of oxygen-derived reactive species.

Interferon gamma (IFN-γ) and interleukin 4 (IL-4) have been shown to up regulate and down regulate antimalarial effects of monocytes, respectively, through expression of Fcγ receptors on monocytes. Monocytes on the other hand may be acting against parasites via the production of tumor necrosis factor (TNF-α). Moreover, IFN-g production has been shown to be significantly higher in severe malaria than uncomplicated infections (Ho *et al.*, 1995). But IL-10 seems to be the predominant cytokine that down regulates the production of inflammatory cytokines such as TNF-a during acute *falciparum* infection. It also induces activated B cells to secrete large amounts of IgG, IgA, and IgM, and the combination of IL-10 and IL-4 results in the secretion of the four immunoglobulin isotypes (Rousset *et al.*, 1993). In contrast with murines, both Th1 and Th2 human T cell clones produce IL-10 in humans and it down regulates the functions of both Th1 and Th2 cells (Ho *et al.*, 1995).

Since the role of single stage specific antigens to elicit protective immunity against malaria has been elusive, efforts are being made to design a multistage-multivalent vaccine. Recent trials of a cocktail vaccine, NYVAC-Pf7, gave promising results as it was capable of evoking both cellular and humoral immune responses that delayed reinfection and protected some individuals (Ockenhouse *et al.*, 1998; Shi *et al.*, 1999). Hence more studies should focus on multistage vaccines.

1.7. Age-related T cell responses to mitogens and *P. falciparum* antigens

The study of lymphocyte function is of great significance in clinical research for evaluation of the immunological responses both for diagnostic significance and therapeutic implications for all geriatric diseases in which immunity may play a role (Lavanchy and Rosenthal, 1978). Adaptive immune responses to various disease conditions have been evaluated through mitogenic and antigenic stimulation of lymphocytes. Mitogens are polyclonal hence are non-specific stimulators of lymphocytes and induce mitosis in lymphocytes of different specificities or clonal origin. On the other hand, antigens are specific and cause restricted stimulation to a clone of lymphocytes (Janeway *et al.*, 1999). Because of their polyclonal nature, mitogens are normally used as a measure of immune competence to determine the best conditions for optimum immune responses before any specific antigen is used to investigate a clinical condition (Froebel *et al.*, 1999). Studies on different diseases have shown that optimal conditions for evaluating T cell function depends on the concentration of the specific mitogen used as well as culture duration (Barten *et al.*, 2001), clinical state of an individual (Yoshino *et al.*, 2001; Sasdelli *et al.*, 1981) and age (Fetisova, 1977).

It is evident that *P. falciparum* infections lead to immunosuppression as depicted by lower or non-responsive T cells to malaria antigens during acute infection (Ho *et al.*, 1986; Chemtai *et al.*, 1989; Braga *et al.*, 2002). However, after treatment and during convalescence, T cell proliferative responses are recovered. Comparison of T cell responses to mitogens and *P. falciparum* antigens in The Gambian children and adults showed that intense exposure to malaria suppresses T cell responses to PHA but not to concanavalin A (Riley *et al.*, 1988). It was also clear that crude *P. falciparum* antigens elicited higher IFN-γ responses as compared to purified antigens. These studies did not specify the specific peptide tested. In contrast, one study has shown that IFN-γ responses to LSA-1 is rather enhanced during acute infection compared to convalescence (Luty *et al.*, 2001).

A one-year prospective longitudinal study of children from two different malaria endemic areas (Gabon and Cameroon) reported correlation between clinical protection and high proliferative and antibody responses to LSA-1, MSA-1, MSA-2 and RAP-1 independent of age, though age accounted for higher antibody prevalence to RESA in Gabon (Migot-Nabias *et al.*, 1999; Migot-Nabias *et al.*, 2000). The immunological differences observed between children in the 2 study sites suggests that different endemic areas may have different response dynamics to the same peptides.

Recent studies involving children and adults resident in areas with seasonal epidemic malaria have shown age-related increase in IFN-γ and IgG responses to LSA-1 but not in lymphocyte proliferation, and IL-10 responses (John *et al.*, 2000; 2002), though IL-10 was significantly associated with protection against malaria. In a study in west Africa, it was shown that, lymphocyte proliferation, antibody production, and IFN-γ

responses to LSA-1 increased with age in children though significant associations were only observed between antibody production and lymphoproliferative responses to proteins from the C terminus of the LSA-1 molecule (Riley *et al.*, 1992). In the same study, resistance to episodes of fever was found to be associated with high parasitaemia in partially immune children. Also in a study in endemic areas of Gabon, IFN-γ but not IL-10 responses to LSA-1 was reported to be higher in adults than in children (Bongartz *et al.*, 2002), though it was not clear from the report whether this was associated with protection against malaria.

Immunization of non-exposed human volunteers with either of the two allelic forms of recombinant MSP-1_{19} induced high levels of antigen-specific Th1 (IFN-γ) and Th2 (IL-10) type lymphokines showing that these two cytokines are involved in protection against malaria infection (Lee *et al.*, 2002). Furthermore, IFN-γ production in response to recombinant proteins representing polymorphic regions of the MSP-1 molecule was shown to increase with age although there was no significant correlation with protection (Riley *et al.*, 1992).

1.8. Age related antibody responses to *Plasmodium falciparum* antigens

It is hypothesized that natural immunity to malaria in endemic areas develops gradually with age/or intensity of exposure and is first characterized by decreases in the severity of the disease episodes but not levels of parasitaemia (Luty *et al.*, 1994). This hypothesis of anti-disease immunity, although not firmly accepted, has been attributed to the presence of antibodies to *P. falciparum* toxins released at schizogony and to

differential regulation of innate immune responses in different age groups (Kremsner *et al.*, 1996).

Previous studies of antibody levels to CSP and MSP-1 in transmigrants from areas of low or no malaria (Java) to malaria hyperendemic (Irian Jaya) areas in Indonesia showed age-dependent acquired protection against malaria. Adults developed immunity to clinical malaria within a two-year period of intense exposure to infection while children took longer time to develop antimalarial immunity (Hoffman *et al.*, 1986; Baird *et al.*, 1991; Andersen *et al.*, 1997; Baird, 1998). This age-dependent level of protection among the Javanese adults was of the same magnitude as that of Irian Jaya (residents). Comparative study in Kenya also suggested that adults have superior immune responses than children as shown by the difference in time of reinfection between the adults and children after a radical cure of *Plasmodium* infection (Hoffman *et al.,* 1987).

A number of prospective longitudinal studies have been done in areas of different malaria transmission intensities in an attempt to dissect the mechanisms involved in acquisition of immunity to malaria infections. Although some of these studies support antibody dependent acquisition of age-related immunity, some of them contradict these reports. While IgG antibodies to CSP in endemic areas were higher in adults than children (Tapchaisri *et al.*, 1983; Hollingdale *et al.*, 1989), antibody prevalence and levels to RESA and CSP in hypoendemic areas were shown to increase gradually with age (Iqbal *et al.*, 1994). RESA responses did not correlate with parasite levels but CSP responses negatively correlated with parasitaemia. A longitudinal analysis of antibody responses to TRAP in a malaria endemic area with seasonal transmission in West Africa showed seasonal fluctuations as well as age-dependent quantitative differences in the

levels of specific antibodies to TRAP which correlated with protection against malaria (Dolo *et al*., 1999; Scarselli *et al*., 1993). In contrast, a longitudinal study by use of monthly serum samples collected from birth to age 1 year, initiated to characterize antibody responses to repetitive epitopes of the CSP, and LSA-1, of *Plasmodium falciparum* in infants residing in a *P. falciparum*-hyperendemic area of western Kenya (Zhou *et al*., 2002) showed no association between maternally transferred anti-LSA-1, and anti-CSP antibodies and an infants first detected malaria infection. There was also no significant correlation between an infant's antibody responses to the two repetitive epitopes of these antigens and protection against malarial parasitemia during the first year of life. It also showed that antibodies passively transferred from mothers were detectable for CSP, and LSA-1 repeat epitopes and infants were able to mount and maintain a strong antibody response against LSA-1 but much lower antibody titer to CSP in their first year of life.

In malaria endemic areas, cases of clinical malaria and presence of parasitaemia are very low in the first few months of infants life and peaks of clinical cases occurs at the age of 1-2 years and thereafter reduces progressively as the age increases (Rogier and Trape, 1993). The mechanism of early protection is not clearly understood but presence of maternal antibodies to asexual blood stage parasites has been suggested as one possible reason for this protection. In line with this hypothesis several studies have been done in malaria endemic areas that either support or contradict this view. In prospective longitudinal studies on antibody responses to MSP-1 in malaria endemic population in Papua New Guinea and West Africa it was shown that the prevalence and concentration of antibodies to MSP-1 increased with age and was associated with reduced cases of

clinical malaria (Sehgal *et al.*, 1989; Riley *et al.*, 1992; Al Yaman *et al.*, 1996). Other support for antibody-related protection came from longitudinal investigations of IgG and IgM antibodies responses to MSP-1 domain of *P. falciparum* in infants from Western Kenya, which revealed that these antibodies were protective in infants and pregnant mothers and were also short lived as depicted by several peaks that correlated with parasitaemia level (Branch *et al.*, 1998). This study also showed that IgG and IgM levels highly coincide during the first year of an infant life. However, other studies (Riley *et al.*, 2000; Achidi *et al.*, 1996; Kitua *et al.*, 1999) have shown that high antibody levels against MSP-1, MSP-2, AMA-1 and Pf155 during birth may not be protective but may act as markers of intensity of exposure to malarial infections.

Antibody evaluation to recombinant erythrocyte binding antigen 175 (EBA-175) in a large population study in The Gambia showed that prevalence of IgG antibodies increased with age and there was no significant association between total IgG and its subclasses to clinical protection, though higher levels of IgG showed a trend indicating protection from clinical malaria (Okenu *et al.*, 2000).

A longitudinal study of natural immune response to *P. falciparum* AMA-1 in a malaria holoendemic region in Western Kenya (Udhayakumar *et al.*, 2001) showed stable antibody responses to various B cell epitopes tested. Younger children (<10 years), and older children (10-18 years) responded more frequently to some epitopes than the adults (>18 years) while adults responded more significantly to different epitopes than the two groups.

These studies suggest that the degree of acquired protective immunity against *Plasmodium falciparum* may be governed by recent exposure and age, independent of

history of chronic heavy exposure (Baird *et al.*, 1991; Baird *et al* 1993; Rogier and Trape, 1993). Therefore acquired immunity may be linked to features of immune system that change with time during the normal course of development and ageing. Such changes may establish differences between children and adults that profoundly affect the course of infection by *P. falciparum*. The ratio of naive to memory T cells gradually diminishes during ageing, as a result of the cumulative effect of exposure to the myriad antigens encountered throughout the normal course of life. Moreover, the gradual involution of the thymus progressively limits the production of naive T cells. The likelihood of stimulating memory T cells with cross-reactive antigens may increase with age and this may bias the immune response to the relative benefits of the host under chronic exposure, or to the detriment of the host under acute exposure. Intrinsic features of the immune system that change with age may determine key characteristics of the immune response to infection by *P. falciparum*, and whether that response is relatively harmful or beneficial may depend upon the conditions of exposure i.e. acute or chronic (Baird, 1998).

Normally, higher levels of immune responses to stimulators (mitogens and antigens) have been associated with immune competence or rather protective immunity to various disease conditions (Froebel *et al.*, 1999; Branch *et al.*, 1998; Migot-Nabias *et al.*, 2000). However, immune responses to stimulators normally vary within and between different age groups. Infants have lower immunity to infectious diseases as compared to older children and adults possibly due to immature immune system (Siltanen *et al.*, 2002; Oddy, 2001). This means that infants need more protection against many pathogens as compared to the other age groups. It is therefore essential to investigate how immune

responses develop over time in infants before any specific antigens or vaccines are used to stimulate their immune system against specific diseases.

This research work focused on malaria, which is a global problem especially in non-immune individuals. Since one of the modern approaches to combat malaria is the design and development of vaccines, it is essential that immune responses of each age-group is determined before introducing any vaccine. For the few years that research work on malaria vaccine has been carried out, much of the work has been on older children and adults while little has been done on infants, yet they are the most susceptible group. The available studies show that age-dependent acquisition of immunity to malaria in endemic areas are complicated by the fact that the age of an individual and the total time of exposure to parasites can be correlated. This has made it difficult to disentangle the effect of one from the other. However, based on available epidemiological and clinical data, it has been hypothesized that children might develop resistance to malaria at a lower rate than adults (Baird, 1998). This hypothesis suggests that the constitutive differences in the immune responses between children and adults, rather than differential exposure to a sufficient number of protective antigenic variants may explain the slow acquisition of protective immunity in children. Determining which hypothesis is correct, or whether both contribute to the multi-faceted complex of immune factors, constitutes a fascinating challenge for researchers interested in the natural acquisition of protective immunity to malaria in humans.

Most studies on evaluation of host resistance to malaria in endemic and epidemic areas have been done on infants, older children or adults as separate entities or by comparing immune responses between two or all of these age categories (Branch *et al.*,

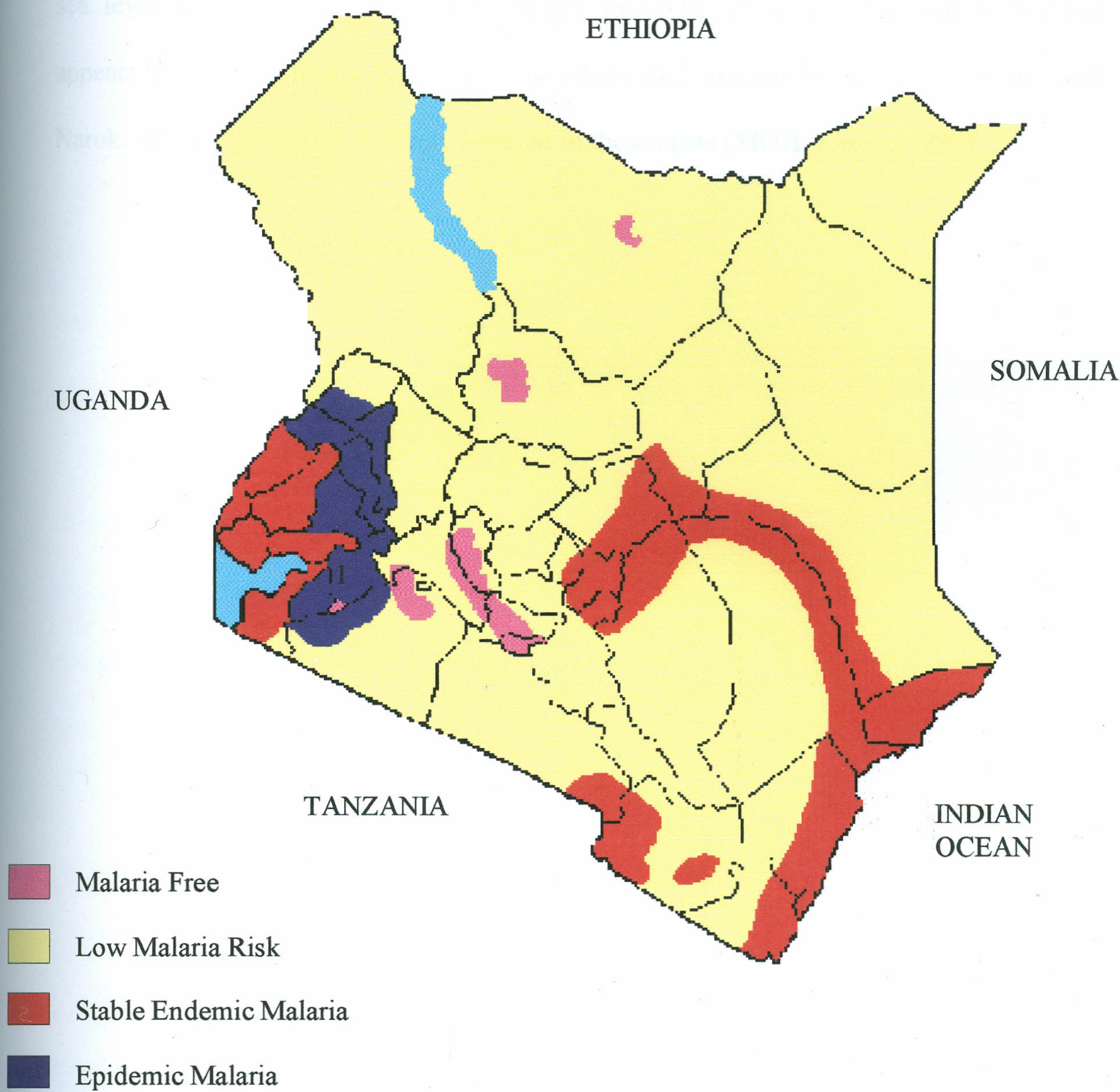
1998; John *et al.*, 2000; Udhayakumar *et al.*, 2001). Some of these studies have followed the groups prospectively for one year and correlated the results with clinical or parasitological status of individuals (Branch *et al.*, 1998). Comparing infants with adults or infants with older children as two separate groups may not give an insight on how anti-malarial immunity is developed with age or exposure. Because malaria immunity has been shown to develop gradually with age and/or intensity of exposure in endemic areas, the present cross-sectional study was therefore designed to compare immune responses in different age groups of children less than four years of age.

1.9. Occurrence and distribution of malaria in Kenya

The level of endemicity of malaria in Kenya varies from region to region as shown in Figure 2. It ranges from hyper-endemic areas (e.g. the coastal and the lake regions) to the malaria free areas (e.g. the very high grounds on Aberdares ranges and around Mt. Kenya). In the lake and coastal regions at altitude not exceeding 300m above sea level, malaria is transmitted all year round and is described as stable. Severe infection and high mortality are most common in the first five years of life. Partial immunity develops due to recurrent exposure to the parasite affording protection against severe disease in the majority of the older children and adults (MOH, Kenya, 1998).

Unstable malaria occurs in several areas in Kenya. In these areas, clinical manifestation of malaria appear seasonally during periods of transmission and the people of all age groups may suffer from severe clinical attacks. It is characterized by intermittent transmission, which may be annual, biannual or variably epidemic. Areas of

Figure 2. Malaria endemicity in Kenya



Adapted from Ministry of Health, Kenya (1998)

seasonal malaria include parts of Eastern and Rift Valley provinces (MOH, Kenya, 1998).

In the highlands regions situated at altitudes ranging from 1,700m-2,500m above sea level and, semi arid regions especially those bordering endemic zones, malaria appears in epidemic forms. These areas include Kisii, Uasin-Gishu, Kericho, Turkana and Narok. All age groups are severely affected in these areas (MOH, Kenya, 1998).

CHAPTER TWO.

2.1. RATIONALE OF THIS STUDY

Plasmodium falciparum causes the highest cases of malaria morbidity and mortality in sub-Saharan Africa. Of late, malaria has become a threat to human health globally due to vector resistance to insecticides, drug resistant *Plasmodium* strains coupled with poor infrastructure and lack of well-trained health personnel in Africa (Shiff, 2002; Carnevale, 1995). As a result, urgent intervention measures are required, one of which is developing a vaccine.

In areas where malaria is seasonal such as the highlands of western Kenya, clinical cases due to *Plasmodium falciparum* are reported in all age groups without much disparity between different age groups (MOH, Kenya, 1998; Malakooti *et al.*, 1998). However, in malaria holoendemic areas, where malaria is stable and intense throughout the year, clinical cases due to *Plasmodium falciparum* are highly concentrated in children less than five years. Above the age of five years, children in endemic areas seem to show a progressive reduction in clinical cases (MOH, Kenya, 1998). This phenomenon of age-related acquisition of naturally protective immunity to *Plasmodium falciparum* malaria infection is not clearly understood.

Immunological studies in infants and young children have been hampered by the ethical requirement that only small blood volumes can be drawn for the studies. Since development of immunity is thought to involve both cellular and humoral immune responses, studies on cytokine and antibody profiles in children resident in malaria holoendemic zones should shed some light into this age-related naturally acquired immunity to *Plasmodium falciparum* infection. Assessing how these responses develop

over time would be important in determining how the malaria-exposed infant's immune system responds to malaria infection. Therefore a practical method that can appropriately assess these responses would be very helpful in field studies. Blood samples collected by finger prick technique have been used successfully to obtain PBMC and serum from young children. However, the optimal PBMC concentration required for detectable IFN-γ and IL-10 cytokine responses to mitogens and *Plasmodium falciparum* antigens is not known. Therefore, this study was designed to determine the optimal test mitogen for IFN-γ and IL-10 production in infants and young children, the optimal concentration of the mitogen, and the optimal cell concentration for determining IFN-γ and IL-10 responses to two malaria peptides, T3 (from LSA-1) and M1 (from MSP-1).

It is well documented that protective immunity to malaria involves multiple multistage antigens, which should be identified in each malaria endemic setting before a cocktail vaccine is produced. Hence assessment of the prevalence of antibody responses to different pre-erythrocytic and erythrocytic *P. falciparum* antigens by age in this age-stratified cross sectional study allowed identification of antigens recognized by children less than four years in this malaria endemic region of Kisumu district, western Kenya where the study was conducted. Identification of *P. falciparum* antigens recognized by infants could be used as a precursor for future studies looking into the evolution of immune responses against malaria and development of a multi-antigen malaria vaccine for young children in malaria holoendemic areas.

2.2. HYPOTHESIS AND OBJECTIVES OF THE STUDY

2.2.1. Alternate Hypothesis

This study was designed to test the alternate hypothesis that the prevalence and levels of cellular and humoral immune responses to mitogens and *Plasmodium falciparum* antigens increase with age in children less than four years in malaria holoendemic areas.

2.2.2. Aim and objectives of the study

2.2.2.1. General objective

The aim of this study was to determine the optimal conditions for assessing immune responses to mitogens and to *Plasmodium falciparum* antigenic peptides in peripheral blood mononuclear cells (PBMC) from children less than four years and asses how immunity develops over time.

2.2.2.2. Specific objectives of the study

1. To determine optimal concentration of two mitogens (PHA, PMA-I) for T cell IFN-γ and IL-10 cytokine production by PBMC collected from children less than four years.
2. To determine optimal cell count for T cell responses to PHA and PMA-I in children less than four years.

3. To determine optimal cell count for T cell responses to *Plasmodium falciparum* antigenic peptides, LSA-1 (T3) and MSP-1 (M1) in children less than four years.
4. To determine the cross-sectional prevalence and levels of antibodies, total IgM, IgG, and IgG subclass to malaria antigens: CSP, TRAP, LSA-1, AMA-1, EBA-175 (glycosylated and non-glycosylated) and MSP-1 in children aged 0 to 48 months.

CHAPTER THREE: EXPERIMENTAL DESIGN AND METHODS

3.1. Study area

Blood samples for this study were collected from Kanyawegi in Kisumu district, which is situated at the shores of lake Victoria western Kenya. This region has intense and perennial malaria and has been documented as a malaria holoendemic zone. Majority of the residents are from the Luo ethnic community.

3.2. Human subjects

A total of 110 young children from Kanyawegi were randomly recruited and age-stratified as follows: 0-12 months, 13-24 months, 25-36 months, and 37-48 months. Of these samples, 50 were used for optimizing cytokine assays and the remaining 60 were used for cross-sectional cytokine prevalence study. All 110 samples were tested for the presence of antibodies to *Plasmodium falciparum* antigens. The clinical officer took the medical information mainly on malaria from each individual or guardian and clearly explained the purpose of the study in the language that they best understood before obtaining informed consent from guardians of those who participated in the study.

Ethical approval was obtained from Ethical Review Committee at the Kenya Medical Research Institute and Human Investigations Institutional Review Board at the University Hospitals of Case Western Reserve University, Cleveland, OH.

3.3. Blood collection and transportation

Approximately 0.5-1ml volume of blood was collected from each individual by finger prick method. In this method, the finger to be pricked was cleaned with 70% alcohol and pricked using a sterile lancet. The first drop of blood was wiped using dry gauze the finger was then squeezed gently to get 0.5-1ml of blood in a microtainer containing anticoagulant (ethylenediaminetetra acetic acid) EDTA while mixing. Collected blood samples was placed in boxes and transported within the next six hours from the field to the Case Western Reserve University laboratory at the Center for Vector Biology and Control Research, KEMRI, at Kisumu.

Also, *beta*-haemoglobin level was measured in the field using Hemocue (Hemocue AB, Angelholm Sweden). Treatment was prescribed if a child had malaria symptoms and/or haemaglobin level of less than 5g/dl.

3.4. Microscopic investigations of *Plasmodium* parasites

Thick and thin blood smears for identification and enumeration of the malaria parasites were prepared in the field and also transported to the laboratory at Kisian. The prepared smears were stained using 5% Giemsa solution and read by two microscopists to determine if the study volunteer was infected with malaria parasites. Parasite density in the positive slides were enumerated against 200 white blood cells and the final density per microliter of blood was calculated by counting the number of parasites per 200 white blood cells and multiplying by 40.

3.5. Collection of plasma and isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were separated from the whole blood by Ficoll-Hypaque density gradient centrifugation. Separation by this technique was done as follows: Blood anticoagulated with EDTA in purple top tubes was spinned at 7000 rpm (eppendorf centrifuge 5417 R, Germany) for 10 minutes and plasma transferred to prelabelled sarstedt tubes leaving the buffy coat intact. Blood plasma was frozen at -20^0C for later evaluation of IgG and IgM antibodies specific to *Plasmodium falciparum* antigenic peptides. The remaining pellet was resuspended in 2 ml of sterile 1x PBS and carefully overlayed on 2 ml of Ficoll in 15 ml conical tube. This was then centrifuged for 30 minutes in a swing bucket rotor (Jouan SA, Germany) at 1500 rpm at 20^0C, with no break. Using 5 ml pipette, the mononuclear cell layer was transferred to another 15 ml conical tube. Mononuclear cells were washed by adding sterile 1x PBS to bring the total volume in the conical tube to 10ml and spinned for 15 minutes at 1300 rpm. The supernatant was removed, mononuclear pellet broken, cells resuspended in 10 ml sterile 1x PBS, and spinned for 10 minutes at 1300 rpm to remove most of platelets. The final pellet was diluted in 0.5 ml of media (cRPMI 1640 containing gentamicin, Hepes, and glutamine) and cell count adjusted accordingly (cell count on haematocytometer x 0.5 x 10^5). This was the total cell count in the 0.5 ml of media.

3.6. Optimization of mitogen concentration for *In vitro* T cell cytokine production and ELISA

After PBMC separation and cell counting as explained above, the number of cells in 0.5 ml of culture media was then diluted to final concentration of $0.5x10^6$ cells/ml, plated in duplicates at 200 microlitres/well in a 96 well U-bottom microtiter plates (Microtest^{TM}, Becton Dickson, USA). Different concentrations of PHA i.e. 1, 2, and 5μg/ml, and of PMA i.e. 10, 20, and 25ng/ml plus ionomycin (1μg/ml) were then added to the wells of different rows in duplicates. The seeded plates were then incubated for 120 hours at 37^0C in 5% $C0_2$ under humidified conditions.

After 120 hours, 200 microliters of the supernatants from each well was aliquoted and tested freshly or transferred into the wells of appropriately labeled separate tissue culture plate, and frozen at -20^0C until tested for presence of IL-10 and IFN-g cytokines by two-site enzyme-linked immunosorbent assay (ELISA). ELISA technique employed was as follows: 96 well ELISA microtiter plate (Immulon^R 4 HBX, Thermo Labsysytems, USA) was coated with 50 microliters/well of the primary antibody and incubated at 37^0C for 2 hours or 4^0C overnight. The plate was washed twice using 0.05% tween 20 in 1x PBS, blocked with 50 microliters/well of 3% BSA and incubated at 37^0C for 1 hour. It was then washed twice, and samples, standards and blanks added at 50 microliters/well. The plate was incubated at 37^0C for 2 hours then washed twice. 50 microliters/well of secondary antibody (biotinylated) was added, incubated at room temperature for 45 minutes and washed twice. 50 microliters/ well of streptavidin alkaline phosphatase (Jackson ImmunoResearch) at 1:2000 dilution was added and incubated for 30 minutes at room temperature and washed six times. 50 microliters/well

of alkaline phosphatase substrate (5mg tablet/5ml of alkaline phosphatase buffer) (Sigma Diagnostics, inc. st. lous, MO) was added and developed for approximately 20 minutes. When the colour started forming, the plate was taken to the ELISA plate reader (OpsysMR Dynax Technologies, USA) and read at 405 nm using Revelation Quicklink software. When the highest standard had reached an OD of 1.0 to 1.4, the plate was stopped with 50 microlitres of 3M NaoH. The read results were saved in the computer hard disk and in zip disk to be analysed to determine the best mitogen and their optimal concentrations which can elicit IL-10 and IFN-γ cytokine production.

The preliminary results showed that the optimal PHA concentration was 5μg/ml and PMA plus ionomycin concentrations were 20ng/ml and 1μg/ml, respectively. Since both mitogens gave similar frequencies and levels of cytokine production, both were used in the subsequent studies.

3.7. Optimization of cell numbers for *In vitro* T cell cytokine production

After PBMC separation and cell counting as previously explained, cells in 0.5 ml of media were diluted to final concentration of 0.5×10^6, 1×10^6, and 2.5×10^6 cells/ml, plated in duplicates at 50 microlitres/well in a 96 well U-bottom microtiter plates (Microtest™, Becton Dickson, USA). Optimal concentration of PHA (5μg/ml) and PMA-I (20ng/ml-1ug/ml) and two *P. falciparum* antigenic peptides (MSP-1, designated M1, and LSA-1, designated T3 [both at 10μg/ml]) were then added to the wells of different rows having different concentrations of cells. The seeded plates were then incubated for 120 hours at 37^0C in 5% CO_2 under humidified conditions. After incubation, 200 microliters of the supernatants was harvested from each well and tested by ELISA, as

previously explained, to determine the optimum cell count that can elicit IL-10 and IFN-γ cytokine production. These preliminary experiments showed that $2x10^5$, and $5x10^5$ cells per well elicited higher and similar cytokine responses. For subsequent studies, $2x10^5$ cells per well was used because it was suitable for the lower volume of blood obtained by finger prick from children less than four years of age.

3.8. Optimization of incubation period for *In vitro* T cell cytokine production

Using optimal mitogen concentrations and cell numbers as described above, M1 and T3 peptides were added to the wells at the final concentration of 10μg/ml and the plates incubated for either 120 and 72 hours at 37^0C in 5% $C0_2$ under humidified conditions. At the end of incubation periods, the supernatants were tested for IL-10 and IFN-γ cytokine production by ELISA. These preliminary experiments showed that 72 hours of cell culture was optimal for IL-10 and IFN-γ cytokine production as compared to 120 hours of cell culture though the difference was not statistically significant ($p > 0.05$). Because the difference in cytokine production was not significant, all subsequent experiments were done using both culture durations.

3.9. *In vitro* assays for T cell IL-10 and IFN-γ cytokine production across age groups

The optimized conditions were now used for carrying out the *in vitro* assays for T cell IL-10 and IFN-γ cytokine production using field samples collected from children less than four years of age.

After field collection, PBMC were separated from the whole blood samples, cells counted and diluted to final concentration of $2x10^5$ cells per well, plated, and mitogens and peptides added. Plates were then incubated for 72 hours and 120 hours and ELISA done as previously described.

3.10. *Plasmodium falciparum* antigens for antibody tests

The following *P. falciparum* antigens were used for the antibody ELISA tests: CSP peptide, amino acid sequence, $(NANP)^5$ at a concentration of 10µg/ml (Genosys Biotechnologies). Recombinants AMA-1 and EBA-175 NG both expressed in *Pichia pastoris* (EntreMed), LSA-1 (1.0µg/ml), EBA-175 G (EntreMed), TRAP (0.5µg/ml), and MSP-1_{19} corresponding to four variants designated as Q-KNG, E-KNG, Q-TSR, and E-TSR alleles expressed in *Saccharomyces cerevisiae* (MRA/ATCC), were also used to evaluate antibody responses at the concentration of 0.1 µg/ml. MSP-1_{19} nomenclature refers to constructs containing the amino acid Q or E at codon 1644, and KNG or TSR at codons 1691, 1700, and 1701.

3.11. ELISA for quantifying antibodies to *Plasmodium falciparum* antigens

Plasmodium falciparum malaria antigens (CSP peptide, recombinants TRAP, LSA-1, EBA-175 G, EBA-175 NG and MSP-1_{19} (alleles) were tested to detect IgG and IgM antibodies in plasma collected in children aged 0 to 4 years (stratified as 0-4 months, 5-20 months, 21-36 months, and 37-48 months) using ELISA technique. Control plasma was obtained from nine healthy North American adults who had never traveled outside

the United States and had no prior exposure to malaria. Both test and control plasma had previously been stored at -20^0C.

ELISA method for quantifying these antibodies was carried out as described below: For total IgG Immulon-4 ELISA plates was coated with 50 microliters/well of antigens diluted to appropriate concentration in 1x PBS and incubated at 4^0C overnight, washed twice with 0.05% Tween 20 in1x PBS (designated 0.05% PBS-T) followed by blocking with 50 microliters of Blotto (5% w/v dry milk in 1x PBS). This was incubated at room temperature for 1 hour and washed x 2 with 0.05% PBS-T. The plasma was diluted 1:100 in 5% nonfat dry milk and 50 microliters/well added to the microtiter plates in duplicates (for control plasma) and in singlicates (for the field collected plasma samples) then incubated at room temperature for 2 hours. It was washed x 3 with 0.05% PBS-T and alkaline phosphatase-conjugated goat anti-serum antibody (Jackson ImmunoResearch) added, diluted 1:1000 in 1x PBS-5% nonfat milk (50 microliters/well) then incubated for 1 hour at room temperature. Plates were washed x 6 with 0.05% PBS-T, 50 microliters alkaline phosphatase substrate solution (5mg tablet of p-Nitrophenyl phosphate/5ml alkaline phosphatase solution) (Sigma™ 104-105) added, and the reaction stopped after approximately 15 minutes by addition of 3 N NaOH. The plates were then read at 405 nm and data saved in the computer hard disk and in the Zip disk.

For IgM, evaluation was similar to IgG with the following modifications: Immulon-1 plates were used instead of Immulon-4 plates, seablock buffer was used instead of Blotto, and goat anti-human IgM-AP diluted 1:4000 in 1x Seablock was used. Antibody levels were measured as arbitrary units (AU), which was calculated by dividing the OD of the sample plasma by the OD plus three standard deviations (3 SD) of control

plasma from nine North American adults who had never been exposed to malaria. AU values ≥1.0 were considered positive.

For IgG subclasses, Immulon-4 ELISA plates was coated with 50 microliters/well of antigens diluted to appropriate concentration in 1x PBS and incubated at 4^0C overnight, washed x3 with 0.05% PBS-T followed by blocking with 50 microliters of 3% BSA. This was incubated at room temperature for 1 hour and washed x3 with 0.05% PBS-T. The sera was diluted 1:100 5% in 1% BSA and 50 microliters/well added to the microtiter plates in duplicates (controls) and in singlicates (samples) then sealed and incubated over night at 4°C. It was washed x3 with 0.05% PBS-T and secondary mouse anti-human IgG 1-4 biotinylated antibodies (Zymed Laboratories, Inc., San Francisco, USA), diluted 1: 1000 in 1% BSA added and incubated at room temperature for 45 minutes. After washing x3 with 0.05% PBS-T, 50 microliters/well of streptavidin conjugated alkaline phosphatase (Jackson ImmunoResearch) diluted 1:2000 in 1% BSA was added, then incubated for 30 minutes at room temperature. Plates were washed x 3 with 0.05% PBS-T, 50 microliters alkaline phosphatase substrate solution (5mg tablet p-Nitrophenyl phosphate/5ml alkaline phosphatase solution) (Sigma™ 104-105) added, and the reaction stopped after approximately 15 minutes by addition of 3 N NaOH. The plates were then read at 405 nm and data saved in the computer hard disk and in the Zip disk. Antibody levels for controls and samples were measured as arbitrary units (AU) as described above for the total IgG assays.

3.12. Statistical Analysis

ELISA results were processed using Revelation Quick link software for linear regression analysis with data extrapolation (log-lin) then transferred to Microsoft excel. Using excel, optical density values were obtained by subtracting the mean values of baseline (unstimulated) culture from the mean optical density values of stimulated cultures. For cytokines, a positive response to a mitogen was used as a precursor of characterizing individuals as positive or negative responders. Any mitogen and peptide which showed a mean optical density value $\geq$ 20 ng/ml above the baseline optical density was taken as a positive T cell response. Individuals who showed optical density values of $\geq$ 20 ng/ml to at least one mitogen and had peptide response $\geq$ 20 ng/ml were scored as positive responders while those who had optical density $\leq$ 20 ng/ml were regarded as non-responders.

Antibody levels to recombinant and antigenic peptides were measured as arbitrary units (AU) and any AU optical density value of the sample plasma that was $\geq$1.0 were considered a positive response.

Cytokine production between the different test conditions: i.e., cell numbers ($1x10^5$, $2x10^5$, or $5x10^5$ cells/well), mitogens and antigens (PHA, or PMA-I and *P. falciparum* peptide antigens), and age groups (0-12, 13-24, 25-36 and 37-48 months old) were compared. The prevalence and median optical density levels of antibodies responses were also compared between different age groups. Cytokine and Antibody levels across four different age categories were analysed by non-parametric Kruskal-Wallis analysis of variance. Chi-square analysis-of-r x c contingency table was used to determine the prevalence of cytokine and antibody production across the age groups. Correlation

coefficient between cytokine and antibody responses to *P. falciparum* antigens was calculated using Spearman's rank correlation test. The difference was considered statistically significant if the probability (*p*) value was less than 0.05. Stata 7.0 programme (Stata corporation, Texas, USA) was used for all statistical analysis.

CHAPTER FOUR: RESULTS

4.1. Frequency and density of parasitaemia across age groups

Frequency of parasitaemia was statistically low in age category 0-4 months as compared with older age categories, which had higher and similar frequencies ($p<0.0001$) (Table 1). Median parasitaemia density increased progressively from 0 to 3 years and thereafter decreased. This increase was statistically significant ($p=0.0001$).

Also, there was a weak correlation between parasite densities with an increase in age (Spearman $\varphi = 0.322$, $p= 0.0006$).

Table 1: Frequency and median levels of *P. falciparum* parasitaemia in children aged 0-50 months

Age group (months)	Num. of parasitaemia positive / total (%)[a]	Median parasitaemia level, (range)[b]
0-4	5/21 (23.81)	0 (0, 6000)
5-20	28/34 (82.35)	640 (0, 257000)
21-36	23/30 (76.67)	2780 (0, 186600)
36-50	20/25 (80.00)	1880 (0, 47120)

[a] $p<0.0001$, frequency of parasitaemia, χ^2 test.

[b] $p=0.0001$, median parasite density, Kruskal Wallis test.

4.2. Optimization of mitogen concentration, cell numbers, and incubation period

The concentration of PHA and PMA-I at 5μg/ml and 20ng/ml respectively had higher frequencies and levels of IFN-γ and IL-10 responses (Tables 2 and 3). There was no significant difference in the frequencies and levels of cytokine responses between $1x10^5$ and $2x10^5$ cells per well (Tables 4 and 5). Frequencies of cytokine responses to PHA and PMA-I were similar at 72 hours and 120 hours. However, frequencies of cytokine responses to M1 and T3 were slightly higher at 72 hours than at 120 hours, though the difference was not statistically significant (Tables 6 and 7). Hence, when comparing frequencies and levels of cytokines across ages, the 72 hour cytokine values were used.

Table 2: Frequency and levels of IFN-γ to different concentrations of mitogens after 5 days of cell culture

	IFN-γ					
	PHA (1 μg/ml)	PHA (2 μg/ml)	PHA (5 μg/ml)	PMA-I (10 ng/ml)	PMA-I (20 ng/ml)	PMA-I (25 ng/ml)
Frequency (%)	4/7 (57.1)	5/7 (71.4)	7/7 (100.0)	5/5 (100.0)	5/5 (100.0)	5/5 (100.0)
Median (pg/ml)	46.22	169.69	268.54	2440.69	2892.87	2686.79
Mean (pg/ml)	318.77	805.15	1008.28	2303.41	2934.67	2857.59

Table 3: Frequency and levels of IL-10 to different concentrations of mitogens after 5 days of cell culture

	IL-10					
	PHA (1 μg/ml)	PHA (2 μg/ml)	PHA (5 μg/ml)	PMA-I (10 ng/ml)	PMA-I (20 ng/ml)	PMA-I (25 ng/ml)
Frequency (%)	1/7 (14.3)	1/7 (14.3)	1/7 (14.3)	0/5 (0.0)	1/4 (25.0)	2/5 (40.0)
Median (pg/ml)	0.00	0.00	0.00	0.00	0.00	0.00
Mean (pg/ml)	13.65	9.64	15.84	0.38	66.45	46.31

Table 4: Frequency and levels of IFN-γ responses to mitogens and *P. falciparum* antigens using different number of cells and 5 days of cell culture

	IFN-γ					
Cell number	100,000			200,000		
Condition	PHA	PMA-I	M1	PHA	PMA-I	M1
Frequency (%)	5/5 (100.0)	5/5 (100.0)	0/5 (0.0)	8/8 (100.0)	8/8 (100.0)	1/8 (12.5)
Median (pg/ml)	2985.79	3422.76	0.49	3625.61	4153.22	20.78
Mean (pg/ml)	2599.60	4120.52	0.00	3881.05	4058.02	0.00

Table 5: Frequency and levels of IL-10 responses to mitogens and *P. falciparum* antigens using different number of cells and 5 days of cell culture

	IL-10					
Cell number	100,000			200,000		
Condition	PHA	PMA-I	M1	PHA	PMA-I	M1
Frequency (%)	3/5 (60.0)	2/5 (40.0)	0/5 (0.0)	5/8 (75.0)	4/8 (50.0)	0/8 (0.0)
Median (pg/ml)	54.64	120.71	0.00	116.66	114.92	1.48
Mean (pg/ml)	32.99	12.99	0.00	77.70	34.46	0.00

Table 6: Temporal prevalence of IFN-γ responses to mitogens and *P. falciparum* antigens

Condition	PHA		PMA-I		M1		T3	
Incubation time (hours)	72	120	72	120	72	120	72	120
Age (months)								
0-12	11/11 (100%)	12/12 (100%)	6/8 (75.00%)	7/9 (77.78%)	1/10 (10.00%)	1/12 (8.33%)	0/9 (0.00%)	1/11 (9.09%)
13-24	12/12 (100%)	12/14 (85.71%)	8/8 (100.00%)	14/14 (100.00%)	1/10 (10.00%)	2/14 (14.29%)	1/9 (11.11%)	3/14 (21.43%)
25-36	13/13 (100%)	13/13 (100%)	8/10 (80.00%)	10/13 (76.92%)	3/11 (27.27%)	4/13 (30.77%)	4/11 (36.36%)	2/13 (15.38%)
37-48	12/12 (100%)	13/14 (92.86%)	7/9 (77.78%)	10/13 (76.92%)	4/10 (40.00%)	2/14 (14.29%)	4/10 (40.00%)	4/13 (30.77%)
Overall	100.00%	94.00%	85.00%	84.00%	22.00%	17.00%	23.00%	20.00%

Table 7: Temporal prevalence of IL-10 responses to mitogens and *P. falciparum* antigens

Condition	PHA		PMA-I		M1		T3	
Incubation time (hours) Age (months)	72	120	72	120	72	120	72	120
0-12	7/11 (63.64%)	11/15 (73.33%)	1/8 (12.5%)	4/12 (33.33%)	2/10 (20%))	1/14 (7.14%)	0/9 (0.00%)	1/14 (7.14%)
13-24	9/12 (75.00%)	6/15 (40.00%)	3/8 (37.5%)	3/14 (21.43%)	3/10 (30.00%)	1/15 (6.67%)	1/9 (11.11%)	1/15 (6.67%)
25-36	10/13 (76.92%)	10/16 (62.50%)	4/10 (40.00%)	2/16 (12.50%)	0/11 (0.00%)	2/16 (12.25%)	0/11 (0.00%)	0/16 (0.00%)
37-48	10/12 (83.33%)	8/15 (53.33%)	3/9 (33.33%)	6/13 (46.15%)	2/10 (20.00%)	1/15 (6.67%)	2/10 (20.00%)	1/14 (7.14%)
Overall	75.00%	57.00%	32.00%	27.00%	17.00%	8.00%	8.00%	5.00%

4.3. Frequency and levels of IFN-γ and IL-10 cytokines across age groups.

Frequencies and levels of IFN-γ and IL-10 responses to PHA were higher than that of PMA-I (Figures 3, 5). The variation in frequencies was highly marked for IL-10 responses with at least 64% responding to PHA and at most 40% responding to PMA-I across the age groups. Frequencies and levels of IFN-γ responses to MSP-1 (i.e. M1) and LSA-1 (i.e. T3) were higher than those of IL-10 (Figures 4, 6). There was also a clear trend towards increased IFN-γ responses with increased age to M1 (p=0.069) and T3 (P=0.019, χ^2 test for trend) as compared to IL-10 which was similar across the age groups.

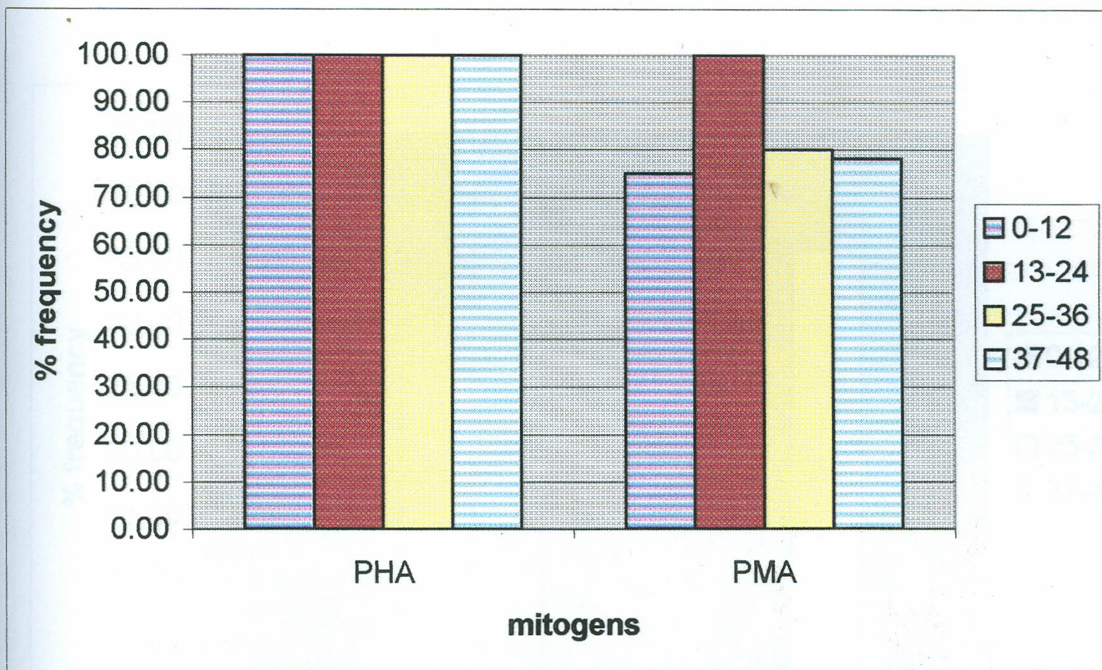


Figure 3. Prevalence of interferon gamma responses to mitogens in children aged 0-48 months after 72 hours of incubation

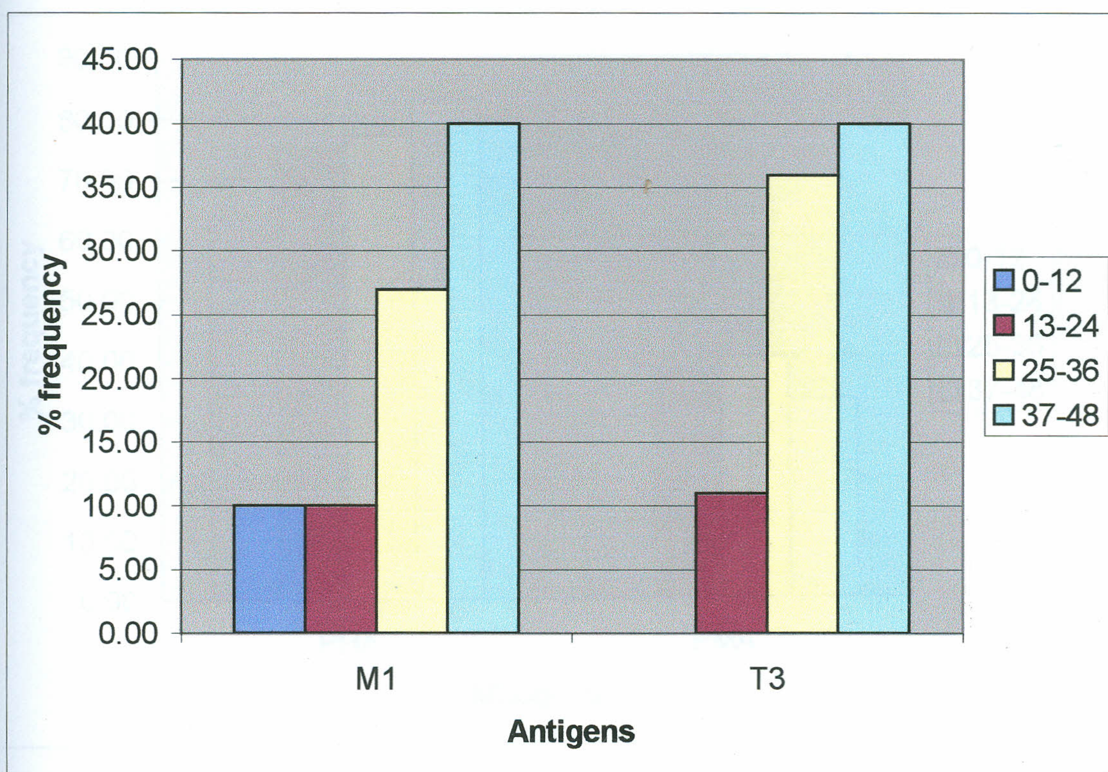


Figure 4. Prevalence of interferon gamma responses to *P. falciparum* antigens in children aged 0-48 months after 72 hours of incubation

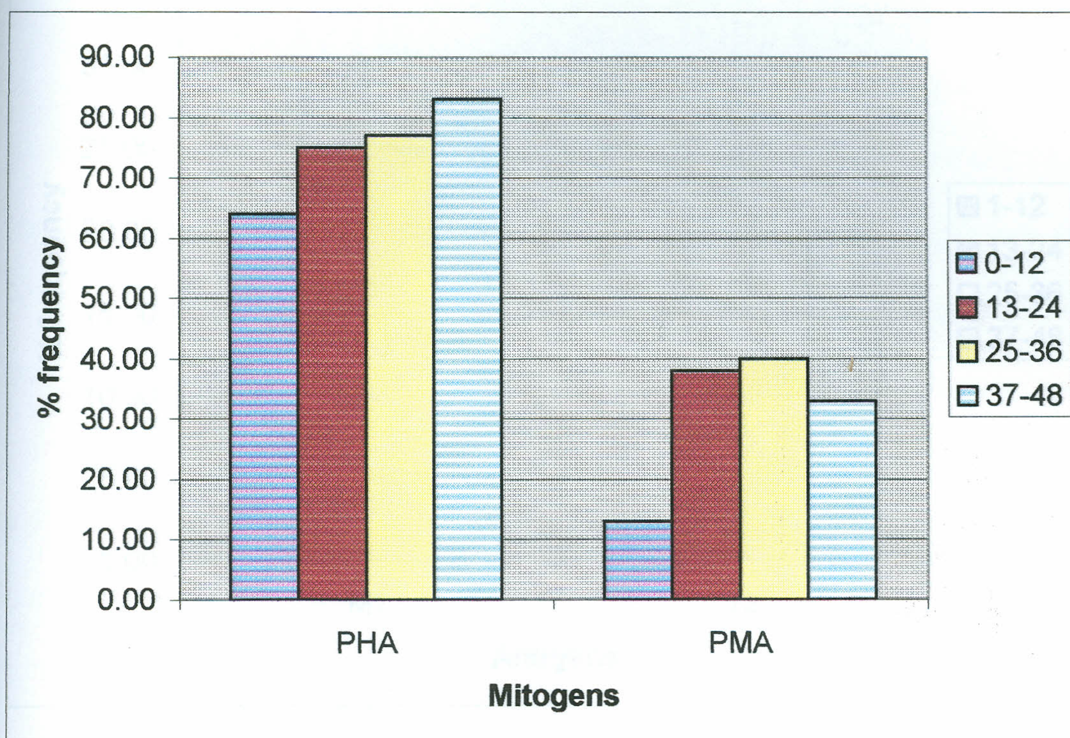


Figure 5. Prevalence of interleukin-10 responses to mitogens in children aged 0-48 months after 72 hours of incubation

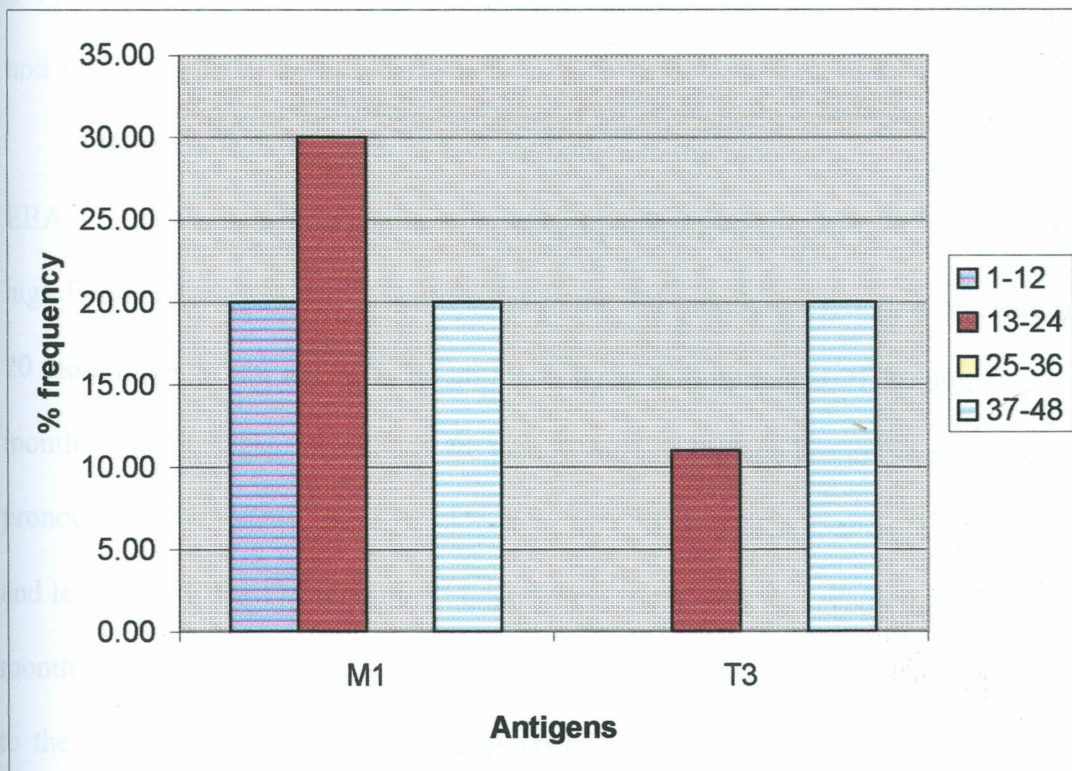


Figure 6. Prevalence of interleukin-10 responses to mitogens in children aged 0-48 months after 72 hours of incubation

4.4. Frequency and levels of total IgG across age groups

The frequencies and levels of IgG antibodies to the pre-erythrocytic antigens, CSP and TRAP demonstrated a similar pattern i.e., high frequencies and levels of antibodies in the 0-4 month age group, with a decrease in the 5-20 month old children and subsequent increases in children aged 21-36 months and 37-50 months. In contrast, frequency of IgG antibodies and antibody levels to the pre-erythrocytic stage LSA-1 were very low in the 0-4 month age group, and subsequently increased with age from 5 to 50 months (Figure 7 and Table 8).

Frequencies and levels of IgG antibodies to the blood stage antigens, AMA-1 and EBA-175, demonstrated a similar age-related pattern to that seen for CSP and TRAP i.e., high frequencies and levels of antibodies in the 0-4 month age group, a decrease in the 5-20 month age group, and subsequent increases in children aged 21-36 months and 37-50 months. The decrease in antibody frequency and level at age group 0-4 months was more pronounced for AMA-1 and EBA-175 than for CSP and TRAP, and antibody frequencies and levels for EBA-175 remained higher in the 0-4 month age group than even the 37-50 month age group (Figures 8 and Table 8). In contrast, frequencies and levels of antibodies to the 4 variants of MSP-1 antigen PfMSP-1_{19} did not differ significantly in any age group (Figures 8 and Table 8).

A statistically significant difference across age groups was seen for frequencies of IgG antibodies to LSA-1 (p=0.0001), TRAP (p=0.0302), AMA-1 (p=0.0001), EBA-175 NG (p=0.006) and EBA-175 G (p<0.0001), and the differences in the frequencies of CSP approached significance (p=0.0561). More highly significant differences were seen between age groups and IgG antibody levels to LSA-1, TRAP, AMA-1, and EBA-175

(all, $p<0.004$), and trend towards a difference was again seen with CSP ($p=0.055$) but none with any PfMSP-1_{19} allele (Table 8).

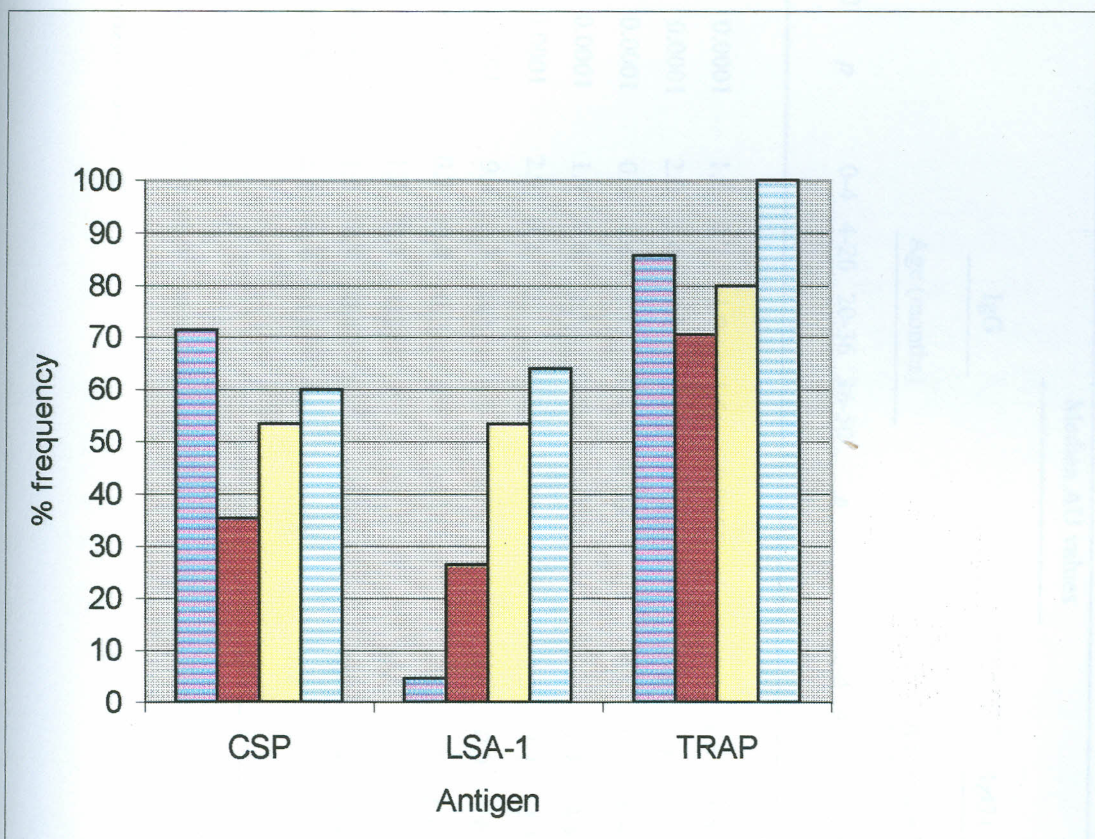


Figure 7. Prevalence of total IgG to *P. falciparum* pre-erythrocytic stage antigens in children aged 0-50months

Table 8. Median antibody levels to *P. falciparum* antigens in children aged 0 to 50 months

	Median AU values																			
	IgM					IgG					IgG1					IgG3				
	Age (months)					Age (months)					Age (months)					Age (months)				
Antigen	0-4	4-20	20-36	36-50	*p*	0-4	4-20	20-36	36-50	*p*	0-4	4-20	20-36	36-50	*p*	0-4	4-20	20-36	36-50	*p*
CSP	0.1	0.4	0.6	0.7	0.0001	1.6	0.7	1.0	1.6	0.0555	1.4	1.6	1.5	2..3	0.0286	1.4	1.2	2.4	5.4	0.0011
TRAP	0.1	0.9	0.8	1.1	0.0001	2.0	1.4	2.2	2.3	0.0041	1.8	1.6	1.7	2.2	0.4511	1.5	3.0	3.8	4.0	0.1235
LSA-1	0.3	0.7	0.7	0.8	0.0001	0.3	0.6	1.1	1.4	0.0001	0.5	1.3	1.5	1.9	0.0001	0.6	1.7	2.6	3.9	0.0001
AMA-1	0.2	0.5	0.6	0.8	0.0001	1.9	0.6	2.2	3.0	0.0001	5.0	1.9	4.9	5.8	0.0005	1.2	2.4	2.6	3.3	0.0825
EBA-175 G	0.1	0.7	0.8	0.9	0.0001	2.9	0.2	0.3	0.7	0.0005	2.3	1.1	1.5	2.0	0.0028	1.2	1.8	1.6	1.8	0.4401
EBA-175 NG	0.1	0.5	0.6	0.6	0.0001	9.4	0.7	0.9	1.6	0.0001	2.7	0.9	1.0	1.7	0.041	1.3	2.0	1.6	2.7	0.3835
E-KNG	0.2	0.5	0.6	0.6	0.0001	0.7	0.8	0.7	1.0	0.7162	1.1	1.5	1.1	1.7	0.2935	0.7	1.7	1.4	1.8	0.0356
Q-TSR	0.2	0.6	0.6	0.8	0.0001	1.2	1.1	1.3	1.8	0.3732	1.4	1.5	1.7	1.6	0.2131	0.4	1.1	1.3	1.7	0.0049
Q-KNG	0.1	0.5	0.6	0.7	0.0001	1.0	0.9	0.9	1.1	0.7694	0.7	1.4	1.4	1.1	0.0404	0.7	1.1	1.0	1.3	0.1891
E-TSR	0.1	0.5	0.7	0.9	0.0001	0.8	0.9	0.8	1.3	0.5518	1.1	2.1	1.9	1.9	0.0924	0.4	1.9	1.7	1.9	0.0027

Individual antibody levels were calculated in AU. (See materials and methods).

$p<0.05$, was regarded as significant, Kruskal Wallis test.

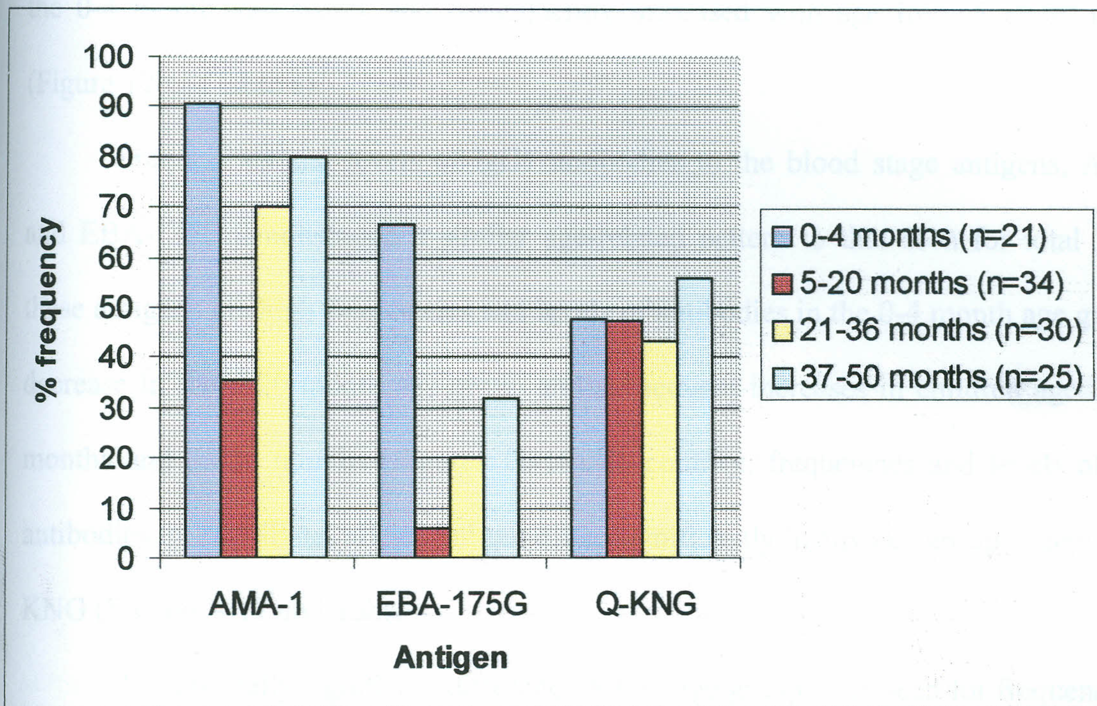


Figure 8. Prevalence of total IgG to *P. falciparum* erythrocytic stage antigens in children aged 0-50 months

4.5. Frequency and levels of IgG subclasses (IgG1, IgG2, IgG3, IgG4) across age groups

The frequencies and levels of IgG1 antibodies to CSP and TRAP demonstrated a similar pattern of high frequencies which was maintained across the age groups with over 70% responding to both antigens (Figures 13 and 15, and Table 8). In contrast, frequency of IgG1 antibodies and levels to LSA-1 were similar to that of total IgG i.e. very low in the 0-4 month age group, and subsequently increased with age from 5 to 50 months (Figure 12 and table 8).

Frequencies and levels of IgG1 antibodies to the blood stage antigens, AMA-1 and EBA-175, demonstrated a similar age-related pattern to that seen for total IgG to these antigens i.e. high frequencies and levels of antibodies in the 0-4 month age group, a decrease in the 5-20 month age group and subsequent increases in children aged 21-36 months and 37-50 months (Figures 16-18). In contrast, frequencies and levels of IgG1 antibodies to PfMSP-1_{19} alleles did not differ significantly in any age group except for Q-KNG (Figures 9-12 and Table 8).

A statistically significant difference across age groups was seen for frequencies of IgG1 antibodies to LSA-1 ($p<0.0001$), EBA-175 G ($p=0.0256$), and Q-KNG ($p=0.0068$). Significant differences were also seen between age groups and IgG1 antibody levels to CSP, LSA-1, AMA-1, EBA-175, and Q-KNG, $p<0.05$ (Table 8).

The pattern of IgG3 responses to pre-erythrocytic and erythrocytic antigens was unique from that of total IgG and IgG1. The frequencies and levels of IgG3 to LSA-1 significantly increased with age, as was the case with total IgG and IgG1. However, the frequencies of IgG3 to CSP, TRAP, AMA-1, EBA-175G, and EBA-175 NG were similar

across the age groups with over 50 % responding (Figures 13-18). The frequencies and levels of IgG3 to all PfMSP-1_{19} variants had a similar pattern i.e., low in 0-4 month age group, an increase in 5-20 months to over 50% responders and subsequent maintenance at over 50% responders in children aged 21-50 months.

Significant statistical difference across the age groups were seen in frequencies of IgG3 responses to LSA-1, p=0.0001; E-KNG, p=0.023; Q-TSR, p=0.0224, and E-TSR p=0.0007 (Figures 8-10). The levels of IgG3 responses to CSP, LSA-1, E-KNG, Q-TSR, and E-TSR varied significantly across the age groups, all $p<0.05$ (Table 8).

There was almost complete absence of IgG2 and IgG4 responses to all *P. falciparum* antigens in this age-stratified study.

4.6. Frequency and levels of total IgM across age groups

There were significantly higher frequencies ($p<0.05$) and levels ($p<0.0001$) of IgM antibodies to both pre-erythrocytic and erythrocytic antigens in older age groups. However, IgM frequencies to two MSP-1_{19} alleles (Q-TSR and Q-KNG) were similar across age groups (Figures 9-18 and Table 8).

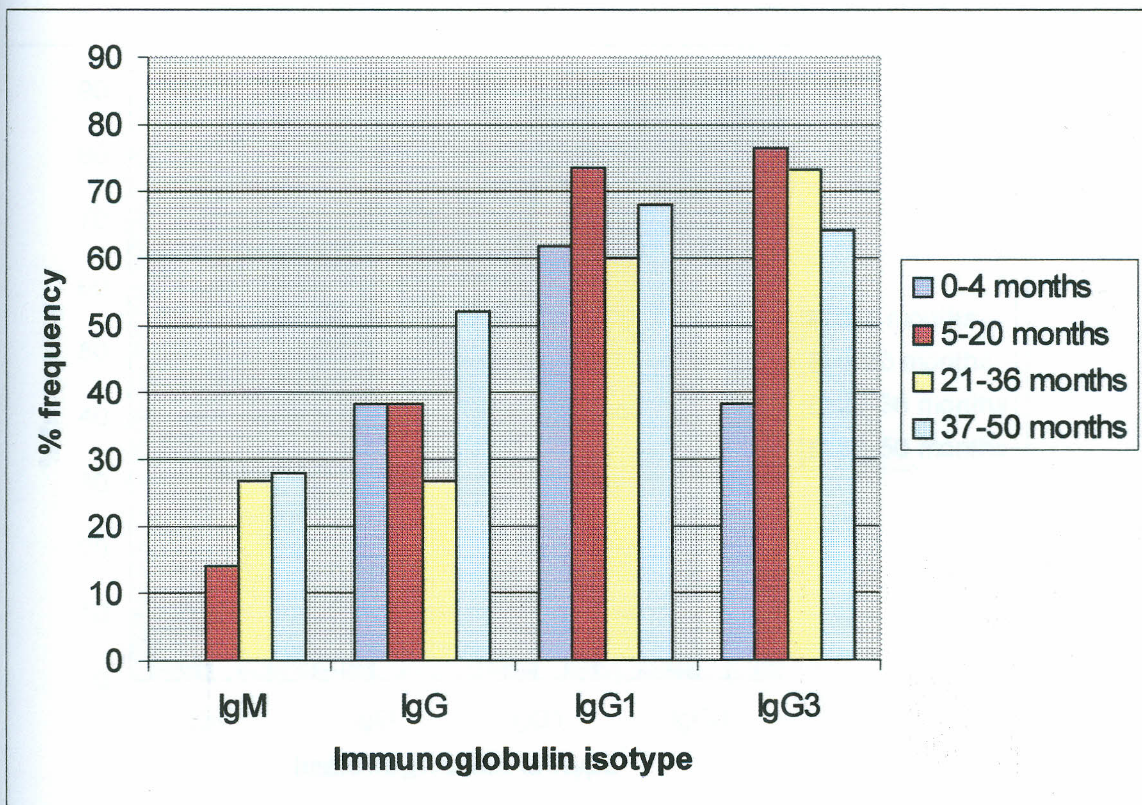


Figure 9. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* merozoite surface protein-1 (E-KNG) in children aged 0-50 months

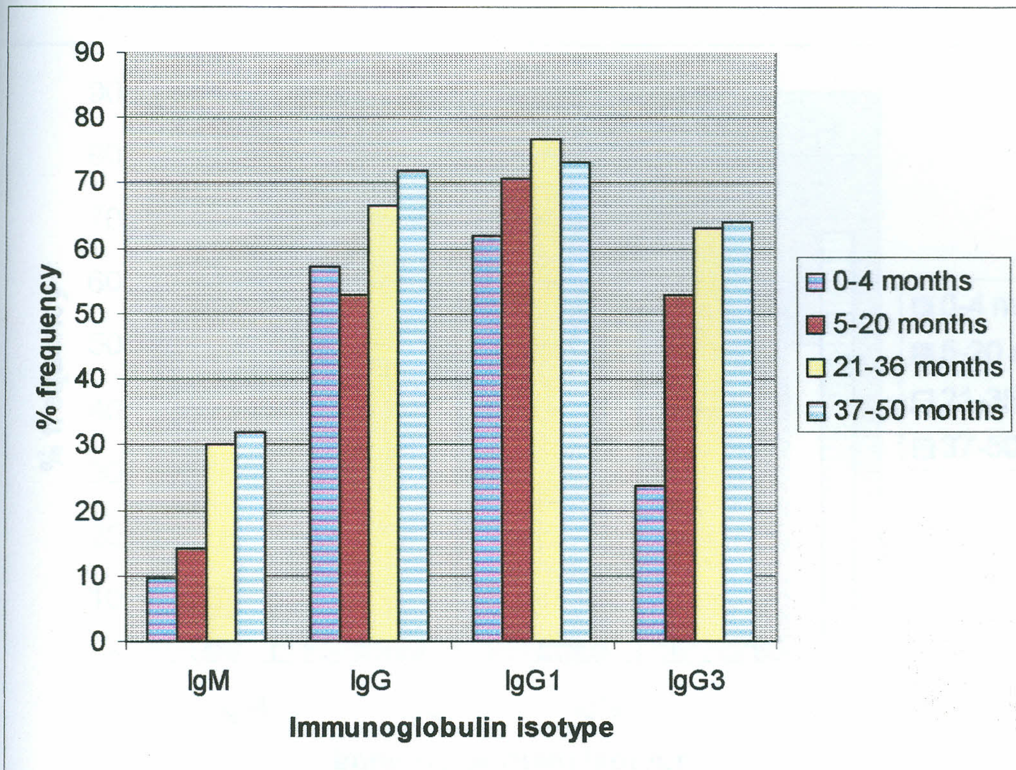


Figure 10. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* merozoite surface protein-1 (Q-TSR) in children aged 0-50 months

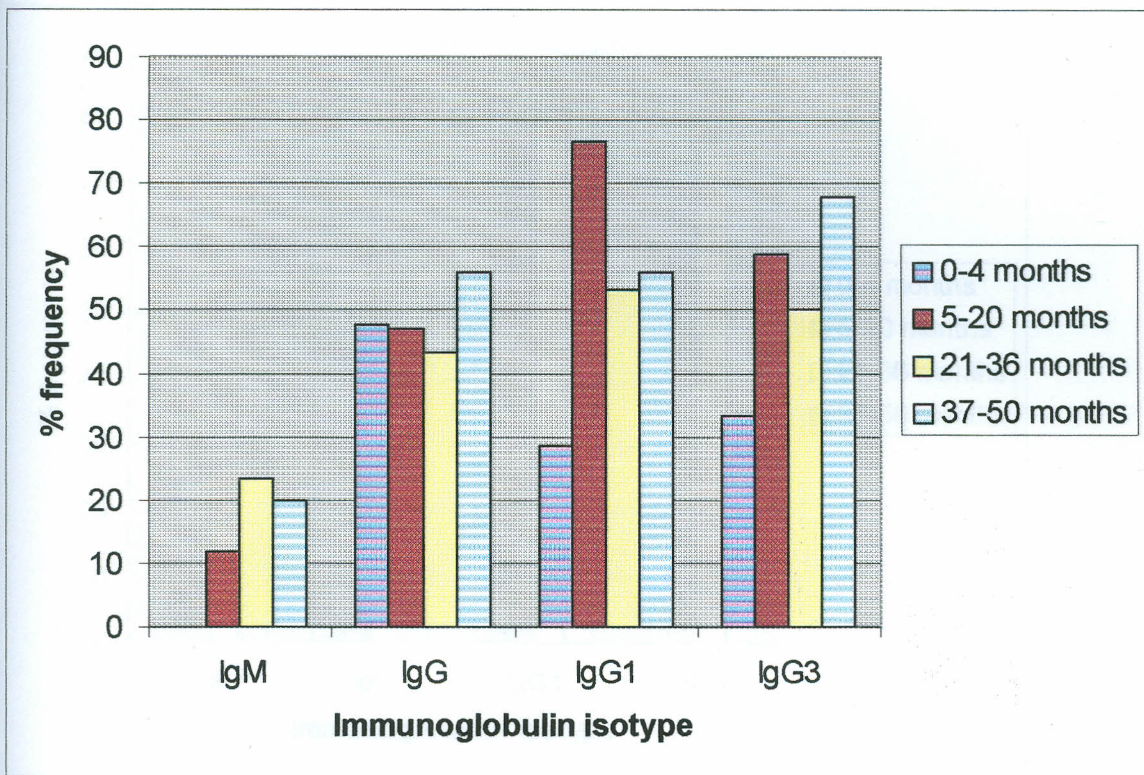


Figure 11. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* merozoite surface protein-1 (Q-KNG) in children aged 0-50 months

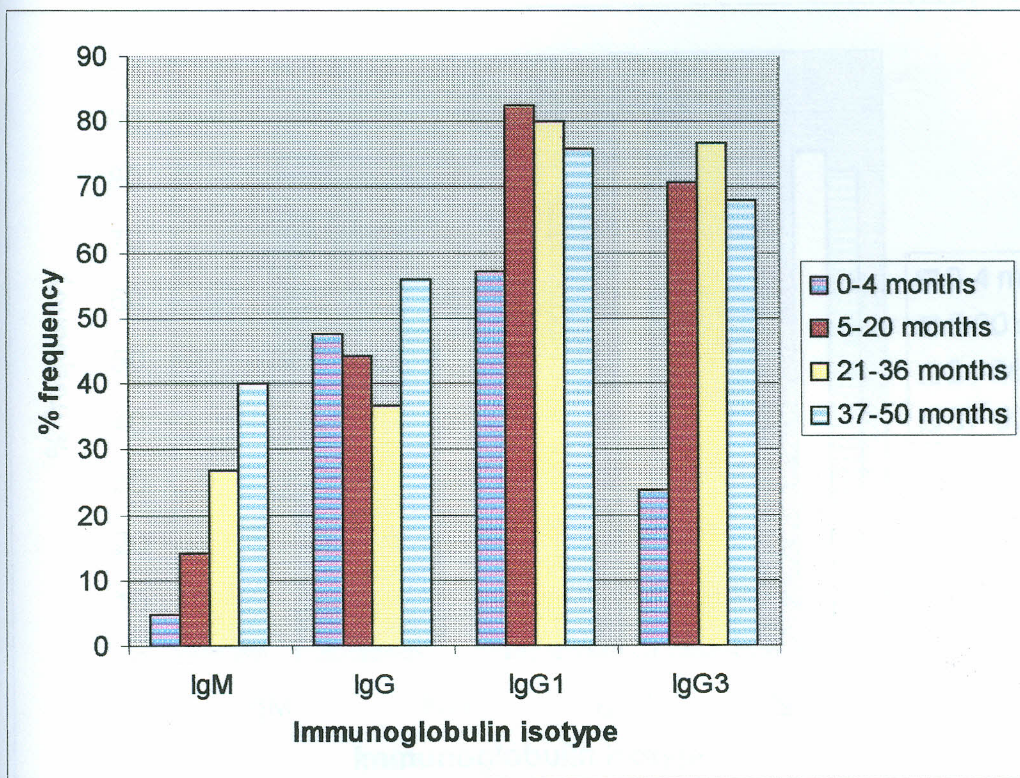


Figure 12. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* merozoite surface protein-1 (E-TSR) in children aged 0-50 months

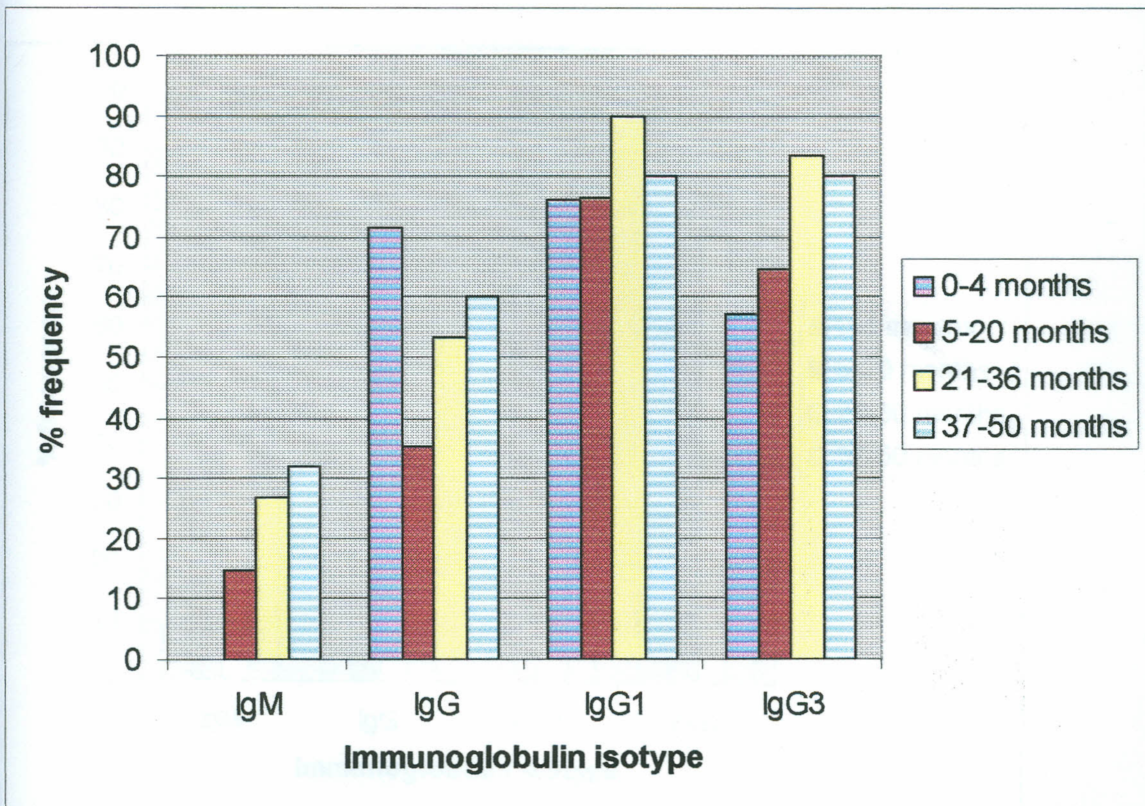


Figure 13. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* circumsporozoite antigen in children aged 0-50 months

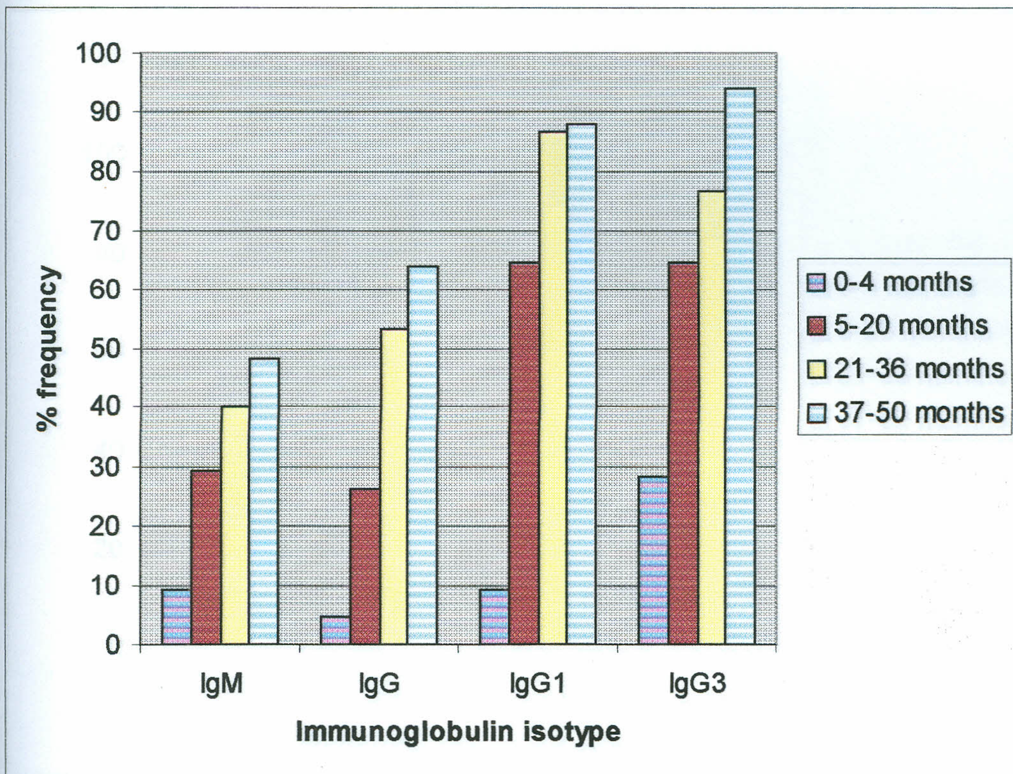


Figure 14. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* liver stage antigen-1 in children aged 0-50 months

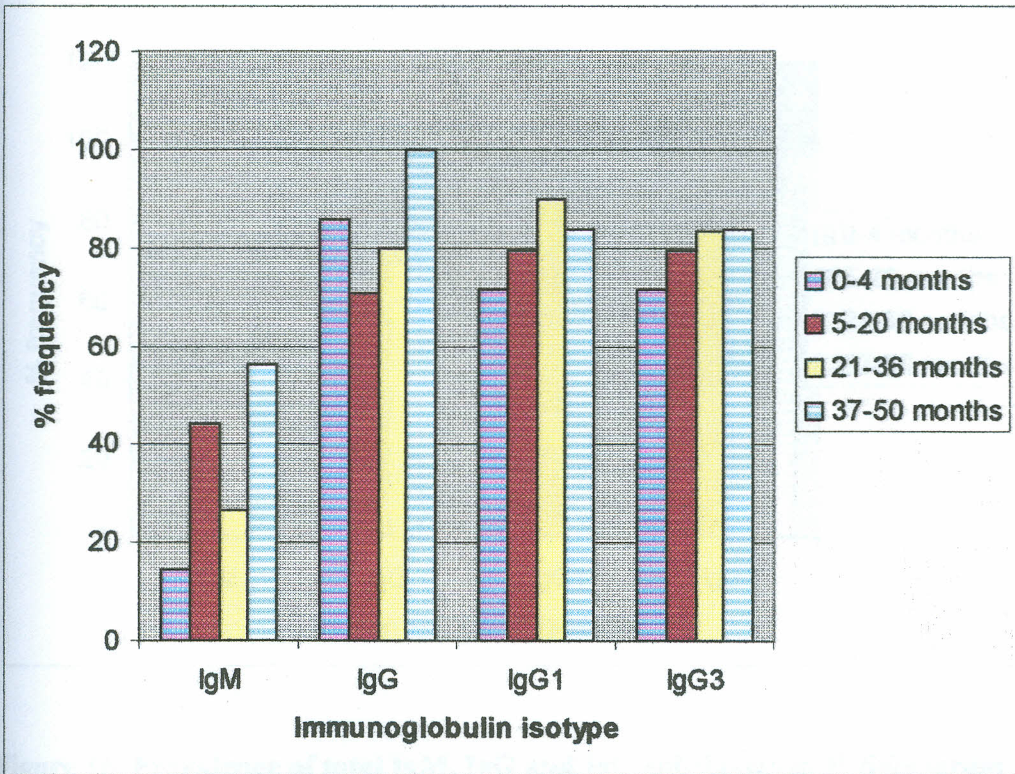


Figure 15. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* thromboposdin related adhesive protein in children aged 0-50 months

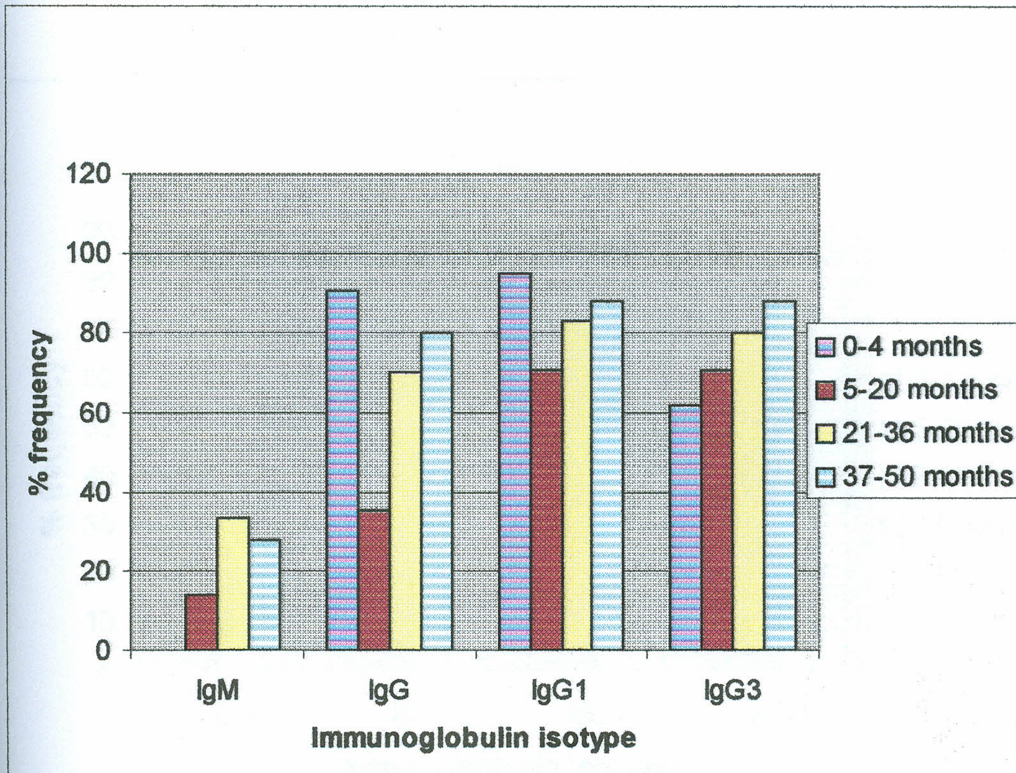


Figure 16. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* apical membrane antigen in children aged 0-50 months

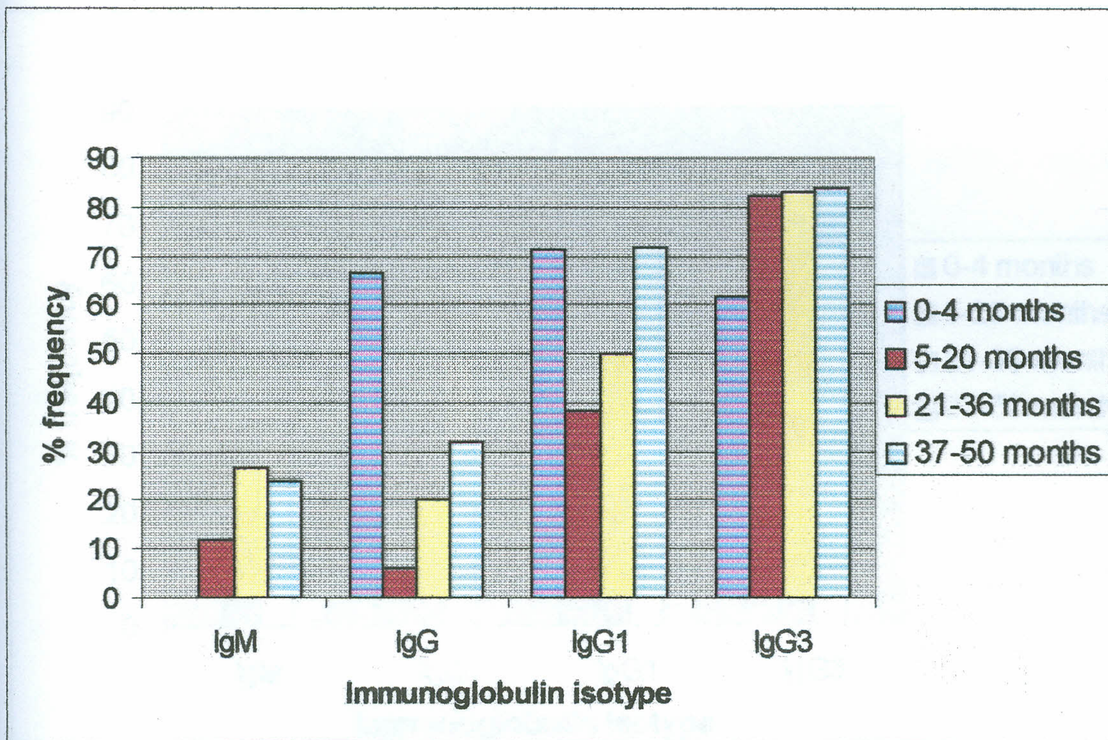


Figure 17. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* glycosylated erythrocyte binding antigen-175 in children aged 0-50 months

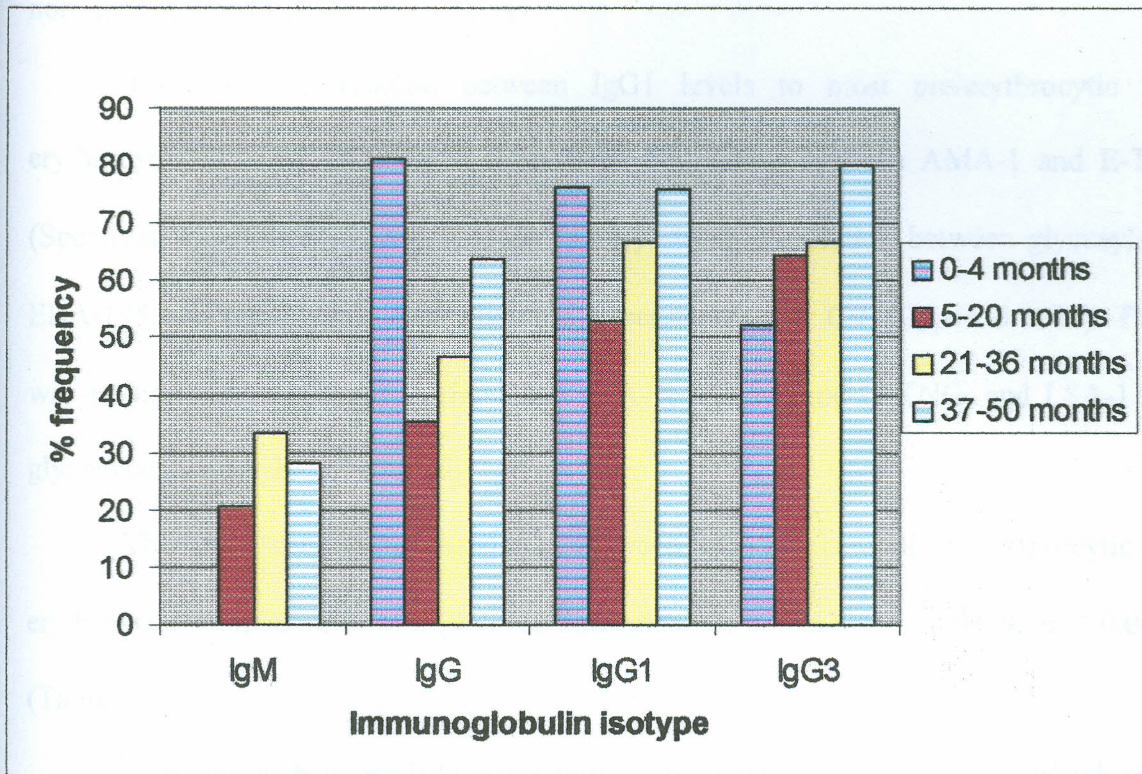


Figure 18. Prevalence of total IgM, IgG and IgG subclasses to *P. falciparum* non-glycosylated erythrocyte binding antigen-175 in children aged 0-4 years

4.7. Correlation between antibody responses to various *P. falciparum* antigens.

The correlation between total IgG levels to most pre-erythrocytic and erythrocytic antigens ranged from weak correlation between glycosylated EBA-175 and Q-TSR (Spearman's $\varphi = 0.1932$, $p = 0.0432$) to very strong correlation between E-KNG and Q-KNG (Spearman's $\varphi = 0.9210$, $p < 0.0001$). However, there was no correlation between CSP and glycosylated EBA-175, and also between LSA-1 and both glycosylated and non- glycosylated EBA-175 (Table 9).

There was correlation between IgG1 levels to most pre-erythrocytic and erythrocytic antigens that ranged from weak correlation between AMA-1 and E-TSR (Spearman's $\varphi = 0.2273$, $p = 0.0169$) to very strong correlation between glycosylated EBA-175 and non-glycosylated EBA-175 (Spearman's $\varphi = 0.9150$, $p < 0.0001$). There was no correlation between AMA-1 and LSA-1, AMA-1 and Q-KNG, and LSA-1 and glycosylated EBA-175 (Table 10).

There was very strong correlation between IgG3 levels to all pre-erythrocytic and erythrocytic antigens that ranged from Spearman's $\varphi = 0.5336$ to 0.9419, $p < 0.0001$ (Table 11).

Correlations between IgM levels to most antigens were very strong although some few weak correlations were observed. The weakest correlation was between AMA-1 and E-TSR (Spearman's $\varphi = 0.2622$, $p = 0.0056$) while the strongest correlation was between CSP and glycosylated EBA-175 (Spearman's $\varphi = 0.9801$, $p <0.0001$).

Table 9. Correlation of total IgG responses between different pre-erythrocytic and erythrocytic antigens[a]

ANTIGEN	TRAP		LSA-1		AMA-1		EBA175-G		EBA175-NG		Q-KNG	
	r^{2b}	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
CSP	0.463	0.0000	0.3218	0.0006	0.2124	0.0259	0.1427	0.1370	0.3051	0.0012	0.4126	0.0000
TRAP			0.4342	0.0000	0.5214	0.0000	0.2816	0.0029	0.4890	0.0000	0.5053	0.0000
LSA-1	0.4342	0.0000			0.2840	0.0026	-0.0199	0.8392	0.4890	0.1369	0.4163	0.0000
AMA-1	0.5214	0.0000	0.2840	0.0026			0.3930	0.0000	0.5990	0.0000	0.4106	0.0000
EBA175-G	0.2816	0.0029	-0.0199	0.8362	0.3930	0.0000			0.7332	0.0000	0.2637	0.0054
EBA175-NG	0.4890	0.0000	0.1427	0.1369	0.5990	0.0000	0.7332	0.0000			0.4675	0.0000
Q-KNG	0.5053	0.0000	0.4163	0.0000	0.4106	0.0000	0.2637	0.0054	0.4675	0.0000		

[a] plasma from 110 individuals were studied for total IgG responses to *P. falciparum* antigens.
[b] spearman rank correlation coefficient.
$p<0.05$ was regarded as significant.

Table 10. Correlation of IgG1 responses between different pre-erythrocytic and erythrocytic antigens[a]

ANTIGEN	TRAP		LSA-1		AMA-1		EBA175-G		EBA175-NG		Q-KNG	
	r^{2b}	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
CSP	0.5621	0.0000	0.3978	0.0000	0.3435	0.0002	0.2684	0.0046	0.3226	0.0006	0. 5223	0.0000
TRAP			0. 4259	0.0000	0. 4117	0.0000	0. 2945	0.0018	0. 3111	0.0009	0. 5332	0.0000
LSA-1	0.4259	0.0000			0.1509	0.1156	0. 1530	0.1105	0.2489	0.0088	0.4182	0.0000
AMA-1	0.4117	0.0000	0.1509	0.1156			0.3896	0.0000	0.3687	0.0001	0.1779	0.0630
EBA175-G	0.2945	0.0018	0.1530	0.1105	0. 3896	0.0000			0.9150	0.0000	0.3424	0.0003
EBA175-NG	0.3111	0.0009	0.2489	0.0088	0. 3687	0.0001	0. 9150	0.0000			0.4212	0.0000
Q-KNG	0.5332	0.0000	0.4182	0.0000	0. 1779	0.0630	0. 3424	0.0003	0. 4212	0.0000		

[a] plasma from 110 individuals were studied for IgG1 responses to *P. falciparum* antigens.
[b] spearman rank correlation coefficient.
$p<0.05$ was regarded as significant.

Table 11. Correlation of IgG3 responses between different pre-erythrocytic and erythrocytic antigens[a]

ANTIGEN	TRAP		LSA-1		AMA-1		EBA175-G		EBA175-NG		Q-KNG	
	r^{2b}	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
CSP	0.7288	0.0000	0.7094	0.0000	0.5769	0.0000	0.5336	0.0000	0.5458	0.0000	0. 7162	0.0000
TRAP			0. 7041	0.0000	0. 6181	0.0000	0. 5532	0.0000	0. 5668	0. 0000	0. 6561	0.0000
LSA-1	0. 7041	0.0000			0.7262	0.0000	0. 6455	0.0000	0.6896	0. 0000	0.7314	0.0000
AMA-1	0. 6181	0.0000	0.7262	0.0000			0.7320	0.0000	0.7986	0.0000	0.7409	0.0000
EBA175-G	0. 5532	0.0000	0.6455	0.0000	0.7320	0. 0000			0.9090	0.0000	0.7853	0.0000
EBA175-NG	0. 5668	0.0000	0.6896	0.0000	0.7986	0.0000	0.9090	0.0000			0.7791	0.0000
Q-KNG	0. 6561	0.0000	0.7314	0.0000	0.7409	0.0000	0. 7853	0.0000	0. 7791	0.0000		

[a] plasma from 110 individuals were studied for IgG3 responses to *P. falciparum* antigens.
[b] spearman rank correlation coefficient.
$p<0.05$ was regarded as significant.

CHAPTER FIVE: DISCUSSION

In malaria endemic areas, it is clear that infants younger than ~6 months of age appear to be protected against severe malaria morbidity, mortality, and high-density asexual parasitaemia compared to older children (Snow *et al.*, 1998; Deloron *et al.*, 1997). However, children younger than 3-4 years suffer the bulk of malaria morbidity and mortality and as age advances, there is gradual decrease of clinical cases of malaria. The mechanism of gradual acquisition of natural immunity to malaria is unclear. Results from this study has shown that young children naturally exposed to malaria have competent T and B cells that produce cytokines and antibodies to mitogens and *P. falciparum* antigenic peptides. There was a gradual increase in the frequency of IFN-γ but not IL-10 responses to MSP-1 and LSA-1 across the age groups. Although young children had antibodies to all antigens tested, the frequencies and levels of IgG antibodies acquired against each antigen varied across the age groups. All pre-erythrocytic and asexual erythrocytic antigens tested except LSA-1 and $MSP\text{-}1_{19}$ variants had higher frequencies and levels of IgG antibodies in age group 0-4 months which decreased in 5-20 months and progressively increased to four years.

Mitogens are known to be polyclonal and nonspecifically stimulate cells to release cytokines hence are used as positive controls in cell culture assays. Varying concentrations of two mitogens, PHA and PMA-I were used to evaluate optimum concentration for IFN-γ and IL-10 production by young children. From this study it was found that 5 μg/ml and 20 ng/ml of PHA and PMA-I respectively gave optimum production of IFN-γ and IL-10 from PBMC of young children. Higher concentration of mitogens required for optimum cytokine production in younger children than older

children and adults may be due to the fact that naïve T cells express fewer receptors with lower affinity and avidity as compared to adult T cells that may be expressing more receptors such as inducible co-stimulator (ICOS) with greater affinity and avidity. Some studies have also demonstrated dose-dependent variability in cytokine production between naïve and memory T cells (Rogers *et al.,* 2000) and at least one study (Pawlec *et al.*, 2000) has shown variability in expression of different receptors between infants and adults, hence more studies are needed in future to clarify these results.

The number of cells cultured per well normally affect the amount of cytokine produced when cells are stimulated with mitogens or antigens. Therefore it was essential to optimize the number of cells required for optimum production of cytokines by infants and young children. Higher cell concentration required for older children and adults requires higher volume of blood, which can be easily obtained by venupuncure. However, it is hard to get venupuncture blood samples from infants and young children yet evaluation of a good number of malaria peptides in PBMC from them is critical since they are the most susceptible to malaria as compared to adults. In order to overcome this problem finger prick blood samples were used to evaluate lower numbers of cells per well ($1x10^5$and $2x10^5$) to see if there was any great variation in IFN-γ and IL-10 production. Although the sample size was small, the frequency, mean and median OD levels of IFN-γ and IL-10 were similar for $1x10^5$ and $2x10^5$ cells per well. Therefore decision was made to use $2x10^5$ per well to evaluate IFN-γ and IL-10 responses to mitogens and *P. falciparum* antigenic peptides, but it is possible that $1x10^5$ cells per well could be used with similar results. Much larger numbers would be needed to validate this possibility. Using this number of cells gave responses in 3-4 year age group that were

similar in frequency and magnitude to those even seen in the adult population from this area (Chandy John, unpublished data). Individuals had similar frequencies and median OD levels of IFN-γ and IL-10 at 72 hours and 120 hours, though there was a strong trend towards higher frequencies at 72 hours, particularly for IFN-γ.

In this study, the frequency and the levels of these two cytokines was evaluated within different age groups in order to understand if there is any variability associated with protection across the age groups. Results showed different patterns for IFN-γ and IL-10 within the age groups. IFN-γ frequencies generally increased progressively with age, for both LSA-1 and MSP-1 peptides. Other studies have also documented an increase of IFN-γ with age and higher prevalence in adults than children (Bucci *et al.*, 2000; John *et al.*, 2001). However, IL-10 responses to both LSA-1 and MSP-1 were similar and generally lower than that of IFN-γ across age groups.

IFN-γ and IL-10 have been associated with *P. falciparum* malaria in several studies (Ho *et al.*, 1995; John *et al.*, 2001). IFN-γ, a dimeric protein produced by CD4, CD8 and B cells, has been shown to be antiviral and antiparasitic (Ibelgaufts, 2002). IFN-g affects all cells except mature erythrocytes as shown by expression of IFN-g receptors by these cells. Its action is thought to be via the release of reactive oxygen and nitrogen intermediates. On the other hand, IL-10 is a homodimeric protein produced by $CD8^+$ and $CD4^+$ after mitogenic or antigenic stimulation (Ibelgaufts, 2002). IL-10 inhibits IFN-γ production indirectly through suppression of nitric oxide and IL-12 synthesis by accessory cells and has been shown to be crucial in the induction of *P. falciparum*-specific antibody production during blood-stage malaria (Taylor-Robinson, 1998; Anstey *et al.*, 1996).

In malaria endemic areas, clinical malaria has been shown to generally decrease with age but the mechanism of this decrease is not clearly understood (Snow *et al.*, 1998). Since some studies have associated IL-10 and IFN-γ with anti-malaria protection, a trend of increase in both IL-10 and IFN-γ responses as age progressed was expected in this study but this was not the case as shown by the results. The results suggested that IFN-γ responses are acquired through frequent exposure to mosquitoe bites and/ or increases with age while IL-10 responses may be acquired at lower frequencies during childhood and remains constant throughout childhood as reported by others (Luty *et al.*, 2001). More studies involving large population samples that compare IFN-γ and IL-10 responses and clinical follow-up in children less than five years with those between five and ten years would possibly shed some light on the trend of these two cytokines.

Frequency and level of parasitaemia in 0-4 months old was lower as compared to uniform frequency and levels of parasitaemia between age group 5 to 50 months. Higher antibody responses in age 0-4 months coincided with lower frequencies of parasitaemia in this group suggesting that these antibodies may be protective against high-density parasitaemia. Other studies have also correlated higher antibody levels with decreased levels of parasitaemia (Deloron et al., 1997).

Several studies have correlated antibody responses to protection against *P. falciparum* infection (Snow *et al.* 1998; Branch *et al.*, 1998; 2000; Egan *et al.*, 1996; Ndungu *et al.*, 2002). These studies included *in vitro* evaluations, serum transfer from immune adults to non-immune and *in vivo* trials involving various stage specific antigens. It is well documented that only maternal IgG antibodies are capable of crossing the placenta during pregnancy (Branch *et al.*, 1998; King *et al.*, 2002). These antibodies enter

infant circulatory system and are thought to protect infants from malaria infections during the first months of their lives (Branch *et al.*, 1998; Deloron *et al.*, 1997). In this study, the frequencies and levels of total IgM, IgG and IgG subclasses to *P. falciparum* pre-erythrocytic and erythrocytic antigens were evaluated to see how they develop with age in infants and young children. The results showed that there was almost complete absence of IgG2 and IgG4 antibody responses to all peptides tested. This IgG subclass pattern has been reported in other malaria endemic regions (Aribot *et al.*, 1996; Deloron *et al.*, 1997).

From this study frequencies and levels of total IgM and IgG, and IgG subclasses to LSA-1 were shown to increase progressively from birth to four years. Since the frequencies of all IgG and IgM antibodies in age group 0-4 months were similar (about 10%), and it is also well documented that maternal IgM does not cross the placenta, this results suggests then that antibodies to LSA-1 may not be passively acquired from the mother but acquired progressively by infants with age. This LSA-1 pattern is consistent with Asembo Bay cohort study done by Zhou *et al* (2002), which reported very strong LSA-1 antibody responses among children in their first year of life.

Responses of total IgG to TRAP and CSP showed very high frequencies (80% and 71.4% respectively) in age 0-4 months then decreased in 5-20 months and thereafter increased progressively. This suggests that maternal IgG to TRAP and CSP crosses placenta at a very high frequency and wanes within four months in infants. This is consistent with the randomized trial on efficacy of RTS,S (a CSP vaccine) which showed that even in adults, antibodies against RTS,S wanes to 0% level by 14 weeks (~3.5months) (Bojang *et al.*, 2001). After four months, it appears that infants start to produce their own IgG to TRAP and CSP as age advances. Subclass IgG antibodies to

TRAP and CSP showed different patterns from that of total IgG i.e. high frequencies (71.4% and 57.7%-76.2% respectively) at 0-4 months that was maintained at these levels across the age groups. Although this results suggest passive acquisition of antibodies to only CSP and TRAP but not LSA-1, Zhou *et al.* (2002) reported that infants passively acquired detectable antibodies to both CSP and LSA-1 from their mothers.

Clinical malaria is mainly due to asexual blood stage *Plasmodium* infections that invade the erythrocytes leading to erythrocyte destruction and eventual release of toxic substances from these cells. During erythrocytic infection, various *Plasmodium* proteins are expressed on the surface of erythrocytes and act as targets of anti-*Plasmodium* antibodies (Rowe *et al*., 2002; Diallo *et al*., 2002). Frequency and levels of total IgG and IgG1 to AMA-1, glycosylated and non-glycosylated EBA-175 showed similar trend across the age groups. These three antigens had very high frequencies (66.7%-90.5%) and levels in age 0-4 months, which decreased markedly in 5-20 months then increased progressively up to four years. These results put together suggest that these antibodies are passively acquired from the mother during pregnancy at very high frequencies and levels and within a wanes very short time (less than four months) in childhood, though a prospective cohort study would be required to definitely document this. Children then start producing their own IgG and IgG1 from the age of four months at an increasing frequency and quantity as age progresses. IgG3 responses to the same antigens were generally high and similar across age groups suggesting that this isotype is passively acquired from the maternal pool and maintained at high level for at least the first four years of life. The difference in patterns suggests that total IgG responses to blood stage antigens relates primarily to IgG1, but not IgG3 in children. The different patterns of

IgG1 and IgG3 suggest that both antibodies evolve independently over time and is consistent with a study on anti-MSP-1_{19} antibodies in Senegalese adults (Diallo et al., 2002).

MSP-1 is a leading vaccine candidate antigen and several investigators have documented its role in malaria immunity (Al-Yamen *et al.*, 1996; Zhang *et al.*, 2002; Kawabata *et al.*, 2002). The pattern of total IgG and IgG subclasses response to MSP-1 alleles was generally different from that of other erythrocytic antigens tested. Frequencies and levels of IgG, IgG1 and IgG3 (only Q-KNG) to MSP-1 alleles were generally similar across the age groups except IgG1 levels to Q-KNG, which was significantly low in age group 0-4 months. Total IgG, IgG1 and IgG3 frequencies possibly acquired from the mother ranged from 25%-54.2%, 51.3%-69.20% and 41.7%-54.4% respectively suggesting that antibodies to MSP-1_{19} alleles may be passively acquired from the mother at lower frequency as compared to other erythrocytic antigens. These frequencies and levels were slightly increased in age group 5-20 months and thereafter maintained up to 36-50 months for all alleles except Q-KNG, which had significant increase in 5-20 months age group. In contrast to these results, a study done on 2-74 years olds in the same region showed that the prevalence and levels of IgG1 and IgG3 responses to MSP-1_{19} alleles increased with age (Shi et al. 1996).

The apparent lack of decrease in anti-MSP-1_{19} antibodies, as compared to the marked decline in antibodies to AMA-1 and EBA-175, at age group 5-20 months may reflect a longer duration of maternal antibodies to this antigen, or, more likely, a preferential early active acquisition of antibodies to this antigen with exposure to *P. falciparum* infection. Since children aged 5-20 months old are the most susceptible to

clinical malaria, antiMSP-1 increase in this age group may also suggest that these antibodies are produced quantitatively during acute *Plasmodium* infections hence may act as markers of acute *Plasmodium* infection. This latter explanation supports earlier infant studies done in Asembo Bay, Western Kenya (another area not far from the present study area) by Branch *et al.* (1998), which showed that IgG to MSP-1 increased during acute infection. These suggestions need to be confirmed in future studies by differentiating maternal antibodies from those of infants and also by evaluating the quantity of these antibodies in different transmission seasons.

IgM antibodies do not cross the placenta but are actively produced by infants depending on age and intensity of exposure. Patterns of IgG antigen differed according to antigen, but IgM antibodies increased with age for all antigens, leveling off at age group 37-50 months. These results are consistent with those from a malaria endemic area of southwestern Nigeria (Achidi *et al.*, 1995). Only LSA-1, TRAP and MSP-1 (Q-TSR and E-TSR) had detectable IgM frequencies in age group 0-4 months, but these were so infrequent (all<10%) that the difference between antigens was not significant. Frequencies of IgM antibodies at older ages differed, ranging from 20% to 48% for Q-KNG and LSA-1 antigens respectively, suggesting that IgM antibodies to specific antigens may be more sensitive markers of recent infection.

Recently, it was shown that there is prenatal sensitization of the fetus to asexual blood stage antigens and that fetus have competent immune cells that are able to produce IgG antibodies against *P. falciparum* asexual blood stage antigens (King *et al.*, 2002). However, there is no report on prenatal sensitization against pre-erythrocytic antigens. The present work reports similar patterns of IgG and IgG1 responses to asexual

erythrocytic antigens (EBA-175 and AMA-1) and pre-erythrocytic antigens (CSP and TRAP) but not MSP-1_{19} and LSA-1 antigens. Similar pattern for pre-erythrocytic and asexual erythrocytic antigens can be explained by the fact that CSP and TRAP are both partly present in the circulating blood and liver stage while LSA-1 is expressed exclusively in the liver stage. Therefore, this suggest that CSP and TRAP antigens circulating in the blood may sensitize the fetus as reported for blood stage antigens (King *et al.*, 2002) leading to production of protein specific antibodies by the fetal and infant immune cells. However, maternal LSA-1 antigens may not be circulated in the blood pool but rather confined to the liver stage hence may not sensitize fetal immune cells.

All these results put together suggest that higher frequency and levels of total IgG and IgG subclass antibodies to erythrocytic and some pre-erythrocytic antigens in the age group 0-4 months may be a combination of maternal and fetal or infant specific IgG antibodies. While lower antibody responses to LSA-1 in 0-4 months may be due to the fact that these antibodies are of maternal origin and infants have not produced any specific antibodies to LSA-1 at this time, however, as infants get exposed to this antigen after birth, their immune cells are primed and start producing anti-LSA-1 antibodies that increases with age and/ or intensity of exposure. To be certain on the origin of these early antibody responses, and probably understand the mechanism of early antibody protection, assays that differentiate maternal from fetal or infant antibodies should be developed in future.

CHAPTER SIX: CONCLUSION.

Although, this study involved a small sample size, it was apparent that infants and young children from this region had competent T and B cells that produced cytokines and antibodies against *P. falciparum* that varied by antigen and age. The frequency of IFN-γ but not IL-10 responders to MSP-1 and LSA-1 antigens progressively increased with age during childhood. However, there were differential patterns of antibody acquisition to *P. falciparum* CSP, LSA-1, TRAP, AMA-1, glycosylated and non-glycosylated EBA-175 and $MSP\text{-}1_{19}$ (alleles). Since some studies have shown that immunity to malaria is not only species specific but also strain specific, it was important to evaluate immune responses to different antigens in this endemic region in order to identify protective antigens that may be included in future studies in malaria vaccine design and assessment in young children from malaria endemic areas.

CHAPTER SEVEN: RECOMMENDATIONS FOR FUTURE WORK.

1. Since the present results were based on a single cross-sectional study with a small sample, future prospective longitudinal studies in different malaria endemic settings involving cytokine and antibody profiles to different *P. falciparum* antigens on a cohort from birth to five years and correlation of the results with parasite density and clinical malaria would possibly give conclusive information on how antimalarial immunity is developed with age.
2. Maternal antibodies have been demonstrated in various studies to protect infants from clinical malaria but the mechanism of protection is not clearly understood. Since this study has shown that infants passively acquire maternal or actively mount their own antibodies to various *P. falciparum* antigens at different rates, it will be important to document whether antibodies to specific antigens correlate with protection from infection and disease. In addition, studies on the functional aspects of maternal verses infant –acquired antibodies may clarify the differences not evident with simple measurement of antibody levels.

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