CHARACTERISATION OF NATURAL IMMUNE RESPONSES TO FAL VAC-1 OF *PLASMODIUM FALCIPARUM* IN CHILDREN AND ADULTS FROM A HOLOENDEMIC AREA OF WESTERN KENYA

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DECLARATION

I, *Tom Were*, do hereby declare that this thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

This thesis is dedicated to my lovely wife Jascinta for whom I have much love and respect, which cannot be expressed with words. Without her unending love, support and encouragement I could not have found the strength to climb this mountain. By believing in me, she has instilled in me confidence and ambition; she has brought to me the realization that hopes and dreams need not stay out of reach. She has taught me by example the value of honesty, dedication and pureness of heart.

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ABSTRACT

Previous studies indicate that FAL VAC-1, a recombinant multistage and multicomponent *Plasmodium falciparum* candidate vaccine containing 12 B cell and 9 T cell epitopes from 9 different antigens of different life cycle stages is immunogenic in animal models and that FAL VAC-1-induced antibodies produced significant antiparasite activities against both sporozoite and blood stages of the parasite. In preparation for vaccine trials in humans, a community based cross-sectional study in a malaria holoendemic area of western Kenya was conducted during April-August 1999, to characterise *in vitro* natural humoral and cellular immune responses to this candidate vaccine and their association with clinical protection against malaria in young children < 2 years old (N=180) and their non-pregnant mothers aged 15-48 years (N=139). FAL VAC-1 antigen was used in antibody measurement by ELISA and in lymphoproliferative experiments. Prevalence and level of antibodies were significantly higher in adults than in children when stratified by age groups: 0-6 months; 7-12 months, 13-18 months; 19-24 months; 15-25 years and 26-48 years. Total IgG, IgG1, IgG3 and IgM were the predominant antibodies. IgG2 responses were low and no IgG4 was detected. In children, there were higher IgG1 levels in parasitaemic group than in the aparasitaemic group (F=3.459, p=0.024, t-test). Furthermore, total IgG, IgG1, IgG3 and IgM levels were inversely associated with haemoglobin levels at the time of sampling (total IgG, r=-0.215, p=0.005, IgG1, r=-0.180, p=0.019; IgG3, r=-0.164, p=0.034; IgM, r=-0.216, p=0.001). Parasitaemic children had significantly higher IgG1 levels at a month prior to sampling. In addition, IgG1 was positively correlated to the rate of high density parasitaemia and to episodes of clinical malaria (r=0.218, p=0.029 and r=0.237, p=0.018), respectively. However, in adults, aparasitaemic individuals had high total IgG, IgG1 and IgM

levels than parasitaemic individuals (total IgG, F=3.856, p=0.007; IgG1, F=2.701, p=0.007; IgM, F=5.133, p=0.001, t-test). In addition, IgG2 was inversely associated with haemoglobin levels at the time of sampling. In contrast to antibody responses, lymphoproliferative responses were higher in children than in adults (one-way ANOVA, F=2.392, p=0.038). Generally, lymphocytes from adults responded at lower antigen concentrations, while those from children responded at higher antigen concentrations. There were no associations between lymphoproliferation and malaria infection or haemoglobin level in either young children or adults.

The results of this study therefore indicate that: FAL VAC-1, a multistage multicomponent malaria vaccine candidate is recognised by individuals naturally exposed to malaria. The antibody responses increase whereas lymphoproliferative responses decrease with age. The higher IgG1 levels in children may indicate the presence of a current *P. falciparum* infection, but in adults from the same holoendemic area, IgG1, may be associated with protection against parasitaemia.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.0. INTRODUCTION

Malaria is a major health problem in the world today as evidenced by the 300 million to 500 million clinical cases which result in 2 - 3 million deaths annually. By far most of the mortality and morbidity associated with malaria is caused by *Plasmodium falciparum* primarily among children younger than five years in sub-Saharan Africa (W.H.O, 1999). The world malaria situation has worsened in recent years because of increasing resistance of the anopheline mosquitoes to insecticides, and of the parasites to available antimalarials. An effective malaria vaccine is urgently needed to complement other intervention strategies to control both the transmission of infection and the impact of disease. Inspite of the fact that *in vitro* studies for most of the single stage-specific subunit vaccines have been promising, *in vivo* protection studies in man have been disappointing due to host genetic restriction of immune responses to specific epitopes and short-lived protective immunity induced by some single antigen/epitope-based vaccines. It is however, believed that an effective malaria vaccine will ultimately consist of key antigens and/or epitopes from all the life cycle stages and that induction of both humoral and cellular protective mechanisms is required for optimal efficacy (Hoffman and Miller, 1996).

FAL VAC-1 is a multicomponent candidate *Plasmodium falciparum* malaria vaccine. This vaccine candidate has been shown to be immunogenic in animal models and the vaccine-induced antibodies had significant antiparasite activities against both the sporozoites and the blood stages of the parasite life cycle (Shi *et al.*, 1999a). In preparation for evaluating the efficacy of FAL VAC-1 in human field trials, it is important to investigate the natural immune responses to the candidate vaccine

antigen, and to determine their association with clinical protection against malaria. A cross-sectional study was therefore conducted among children below two years old and adults from Asembo Bay Area in western Kenya so as to further dissect the immune responses to FAL VAC-1. By choosing vulnerable children and a semi-immune adult population, it was hoped that specific cellular and humoral immunological factors that are important in clinical protection against malaria would be identified. The main objective was to test whether individuals naturally exposed to malaria elicit immune responses against FAL VAC-1 and whether such responses were associated with clinical protection against malaria.

1.1. LITERATURE REVIEW

1.1.1. HUMAN MALARIA PARASITES AND THEIR LIFE CYCLE

The aetiological agents of human malaria are the multistage protozoan blood parasites of the genus *Plasmodium*: *Plasmodium falciparum, P. malariae, P. vivax* and *P. ovale. Plasmodium falciparum* is the most virulent and accounts for most deaths as well as clinical manifestations of malaria (Bruce-Chwatt, 1985).

The human *Plasmodium* parasites (mainly *P. falciparum, P. vivax, P. ovale,* and *P. malariae)* have a life cycle, which is split between a human host and a mosquito vector (Bruce-Chwatt, 1985). The basic life cycle of the parasite is shown in figure 1. The *Anopheles* mosquito is the vector for human malaria. Only female mosquitoes are involved, as the males do not feed on blood.

This highly specialised life cycle is initiated in humans when sporozoites from the mosquito salivary glands are inoculated into the human's bloodstream, as the mosquito feeds. Once injected into the human host the extra-cellular sporozoites are present in the bloodstream for less than an hour, (15-45 minutes). They penetrate hepatocytes where they remain for 9-16 days, multiplying (exoerythrocytic

schizogony) within the cells (the hepatic stage). However, *P. falciparum* and *P. malariae* sporozoites trigger immediate schizogony as *P. ovale* and *P. vivax* sporozoites may either trigger immediate schizogony or have a delayed trigger, resulting in dormant hypnozoites. In case of *P. falciparum* a single uninucleate sporozoite develops in five to six days to a mature liver stage schizont.

Asexual development begins as the liver-stage schizont ruptures and releases uninucleate merozoites, each of which invades an erythrocyte. Within the erythrocyte, erythrocytic schizogony occurs to produce either more merozoites or the sexual micro and macro gametocytes (taking 2 to 6 hours).

In *P. falciparum* schizogony lasts 43-48 hours and gametocytosis takes 10-12 days.

Normally a variable number of asexual erythrocytic schizogony occurs before gametocytes are produced. When the infected erythrocyte ruptures it releases parasite derived toxins, merozoites and gametocytes. Only the asexual erythrocytic stage of the life cycle is associated with initiation and maintainance of pathogenesis and clinical disease. The cycle continues for several rounds until terminated by antimalarial treatment.

The gametocytes are taken up by a female mosquito during a blood meal. Within the mosquito midgut, the male gametocyte undergoes a rapid nuclear division, producing 8 flagellated microgametes which fertilize the female macrogamete to form a zygote. The zygote matures into an ookinete, which penetrates the wall of a cell in the midgut, where it develops into an oocyst. Nuclear sub-division within the oocyst produces many sporozoites and when the oocyst ruptures, the sporozoites migrate through the mosquito haemocoele to the salivary glands, ready for injection into another human host during the mosquito's blood meal, thereby completing the life cycle (Bruce-Chwatt, 1985; Peters and Gilles, 1995).

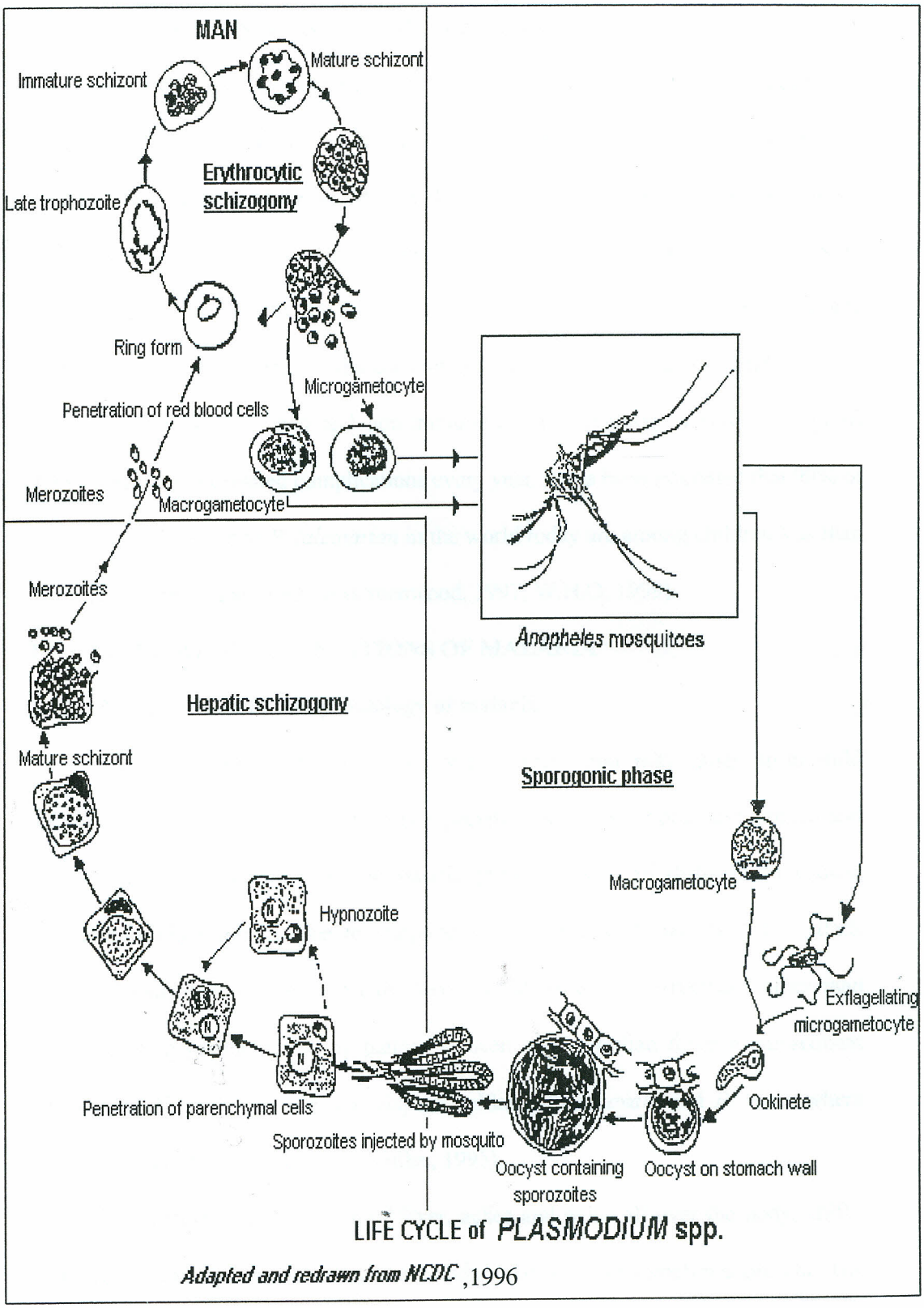


Fig. 1: The life cycle of *Plasmodium spp.*

1.1.2. THE INCIDENCE OF HUMAN MALARIA

Despite efforts to contain the disease malaria remains the most devastating parasitic infection and disease of high morbidity and mortality in the world today. It is found in most regions of the world, namely, Africa, Asia, South and Central America and the Indian subcontinent. The incidence of malaria worldwide is estimated to be in the order of 300 million to 500 million clinical cases annually with two billion people being at risk of contracting the disease and up to three million, mainly children under five years, pregnant women and non-immune adults travelling to endemic regions dying from malaria-related complications every year. It has been estimated that 90% of all deaths attributable to *P. falciparum* in the world today are among children less than five years in sub-Saharan Africa (Greenwood, 1997; W.H.O, 1999).

1.1.3. CLINICAL MANIFESTATIONS OF MALARIA

1.1.3.1 Symptoms and pathophysiology of malaria

Clinical features of malaria are very diverse and may range from mild discomfort to severe disease. Malaria is especially dangerous to pregnant women and young children. Different types of malaria produce fevers of different frequency, depending on how long it takes to complete schizogony in erythrocytes. A first attack of *P. falciparum* is characterised by fever, which is usually irregular, rather than occurring with regular repeating pattern as seen with a tertian fever in subsequent attacks, and there are usually no relapses unlike with *P. ovale* and *P. vivax* where hypnozoites are formed (Peters and Gilles, 1995).

The patient may complain of fever, aches and pains all over the body, chills, malaise, anorexia, nausea, diarrhoea and abdominal pain are sometimes present. The spleen and liver are often palpable on clinical examination. Complications in falciparum malaria include anaemia, cerebral malaria, pulmonary oedema,

hypoglycaemia, coma, generalised convulsions, jaundice, renal failure, fluid, electrolyte and acid-base disturbances, circulatory collapse, shock, disseminated intravascular coagulation, hyperpyrexia, hyperparasitaemia, and malarial haemoglobinuria. These features may occur singly or in combinations (W.H.O, 1990). Severe and complicated malaria is usually caused by delay in treating uncomplicated attack of *P. falciparum.* The clinical manifestations of severe malaria vary with geographic location with different transmission pressure, parasite virulence, host genetic factors and age of the patient (Marsh and Snow, 1999). In nonimmune adults, severe malaria often presents as a multisystem disorder, with features including renal failure, jaundice, acidosis, pulmonary oedema, and shock as well as coma, whereas in African children, cerebral malaria, acidosis and severe anaemia (< 5 g haemoglobin/dl) predominate (Greenwood, 1997). Both age, acquisition of immunity and environmental factors such as transmission pressure of malaria are thought to be important determinants of these differences, although the pathophysiological mechanisms that determine the pattern of complications and the severity of the disease are poorly understood (Marsh and Snow, 1999). In western Kenya anaemia is the major cause of severe disease in children (Bloland *et al.*, 1999b). In endemic areas, the symptoms of malaria may mimic those of other diseases; therefore, presence of parasites on stained blood smears together with symptoms is a good indicator of disease. The clinical manifestations of malaria are influenced by a pre-existing immunity to malaria (Miller *et al.*, 1994). In malaria endemic areas individuals above 5 years may lack clinical symptoms of malaria, even in the presence of high parasite load (McGregor, 1984). However, children below 5 years old are very susceptible to disease, as are individuals with no prior exposure and therefore no immunity to malaria regardless of their age (Brabin, 1991).

Severe anaemia and cerebral malaria are the major cause of mortality especially in young children in absence of treatment. However, severe anaemia is seen only in a small proportion of malaria cases (Greenwood *et al.*, 1991). Several mechanisms e.g. erythrophagocytosis, dyserythropoiesis and immune-mediated haemolysis have been suggested as the cause of anaemia (Turrini *et al.*, 1994). Complications of severe falciparum malaria have been associated with high levels in plasma of the inflammatory cytokines, TNF-α, interleukin (IL)-1β and IL-6, particularly cerebral malaria in African children (Kern *et al.,* 1989; Grau *et al.*, 1989; Playfair *et al.*, 1990; Kwiatkowski and Greenwood, 1989; Aikawa *et al.*, 1990a). Cerebral malaria has been associated with erythrocyte rosetting and adherence to deep vasculature (Carlson, 1993). In *P. falciparum* malaria, the proinflammatory cytokines may have direct systemic effects or adversely affect outcome through up regulation of adhesion molecules, which increase cytoadherence of infected erythrocytes to vascular endothelium (Ho and White, 1999). These factors cause clogging in the capillaries of the brain resulting into impaired blood flow, which may lead to cerebral malaria.

1.1.3.2. Malaria endemicity and clinical manifestations

Manifestations of malaria in people who grow up in endemic areas vary with the degree of endemicity, the age of the patient and the development of immunity. In hypoendemic areas little immunity is acquired, epidemics of malaria are liable to occur and the disease does not differ materially from that in non-immunes. In mesoendemic regions malaria is frequent but only seasonal. Repeated infections lead to anaemia, spleen enlargement and chronic ill health. In these areas of lower endemicity, and especially where malaria is seasonal, cerebral malaria is the most important severe form of the infection. Hyperendemic malaria transmission takes place through out the year but with seasonal increases. Adults develop considerable

immunity although infected individuals may have parasitaemia; malaria causes only occasional short bouts of fever. In holoendemic areas malaria transmission is intense throughout the year and the adults do not suffer from infection although they may support a low parasitaemia. However, in this area of highly endemic malaria severe anaemia is the dominant form of severe malaria and cerebral malaria is uncommon (Brewster and Greenwood, 1993; Snow *et al.*, 1994; 1997).

1.1.4. RESISTANCE AND IMMUNITY TO MALARIA

There are several mechanisms, which contribute significantly towards protection against *P. falciparum* malaria in humans. These include innate non-antigen-specific defence mechanisms as well as defence mechanisms acquired after consecutive malaria attacks.

1.1.4.1. NATURAL RESISTANCE TO MALARIA

Human innate resistance to malaria is based on several genetic features. They may prevent a malaria infection from occurring; or confer significant protection against the severe effects of malaria. Examples of these genetic factors include erythrocyte genetic defects like sickle-cell trait, α and β thelassaemias, glucose-6-phosphate dehydrogenase deficiency (G6PD) and the Duffy blood group antigens (Greenwood *et al.*, 1991). Foetal haemoglobin (HbF), which accords protection against malaria in the first few months of infancy. In addition, immune response gene polymorphisms are associated with distinct disease manifestations in falciparum malaria.

1.1.4.1.1. Sickle-cell trait

The sickle cell trait is a genetic defect resulting from a mutation on the gene encoding the β-chain of haemoglobin. Normal individuals are homozygous for the A*A* gene, heterozygotes have the AS allele while sicklers posses SS genotypes. The heterozygote state of sickle cell anaemia is referred to as sickle cell trait. The

polymorphic frequency of the gene encoding β-haemoglobin is associated with the reduced risk of dying from *P. falciparum* malaria (Allison, 1954; Nagel, 1989). It has been shown that severe complications associated with *P. falciparum* infections are inhibited in sickle-cell gene carriers, but not from acquisition of the disease since the *AS* trait can protect against anaemia and cerebral malaria (Hill *et al.*, 1991; 1992; Greenwood *et al.*, 1991). The mechanisms through which this protection occurs are not clearly defined. However, it is believed that this genetic defect does not favour the optimal growth of parasites in erythrocytes.

1.1.4.1.2. α and β thelassaemias

Alpha-thelassaemia is associated with protection against malaria although the mechanisms involved are unclear (Hill, 1987). Studies have indicated that erythrocytes from individuals with α- and β-thelassaemia as well as sickle cell trait or HbE, form smaller and weaker rosettes than normal (HbAA) red blood cells (Carlson, 1993). This suggests that, impaired rosette formation may be one of the genetic features that constitute the innate resistance to severe *P. falciparum* malaria. Recent studies done to investigate the role of oxidant stress in thelassaemic trait erythrocytes (α and β) have shown that antioxidants improve parasite growth while pro-oxidants have a parasiticidal effect (Senok *et al.*, 1997). This suggests that oxidant stress play a role in mediating the protection against malaria in thelassaemic erythrocytes.

1.1.4.1.3. Duffy antigen

Individuals with erythrocytes lacking the Duffy blood group antigen are protected against *P. vivax* infection (Bruce-Chwatt, 1985). This antigen serves as an anchor for invasion of the erythrocyte and its absence is associated with protection against *P. vivax* infection especially among the indigenous populations of West Africans (Miller *et al.*, 1976).

1.1.4.1.4. Glucose-6-phosphate dehydrogenase (G6PD) deficiency

The genetic deficiency of the enzyme, G6PD is associated with protection against the severe effects of *P. falciparum* infection. It confers a selective advantage, in that G6PD deficient individuals tend to have lower parasitaemia than normal individuals infected with *P. falciparum* (Allison and Clyde, 1961; Gilles *et al.*, 1967), and that ring- infected G6PD deficient erythrocytes are preferentially phagocytosed by macrophages (Giribaldi *et al.*, 1992).

1.1.4.1.5. Foetal haemoglobin (HbF)

Foetal haemoglobin may confer protection against *P. falciparum* especially to neonates and young infants. HbF does not affect the invasion of erythrocytes by *P. falciparum*, but does retard maturation of the parasite within the red blood cell (Pasvol *et al.*, 1976; 1977). Thus the persistence of HbF production in children may confer protection against the severe effects of disease.

1.1.4.1.6. Immune response gene polymorphisms and malaria

There is increasing evidence that host genetic factors play a major role in determining susceptibility to and outcome of many infectious diseases. These include the immune response (Ir) gene which encode the class I and class II major histocompatibilty complex (MHC) antigens known as HLA in humans and non-HLA genes, some of which are encoded in the MHC region, and include the surface antigen receptor of T cells (TcR) and B cells (Ig), cytokine and cytokine receptors, and proteins associated with the complement system and the inflammatory cascade (Riley, 1996).

The generation and nature of an adaptive immune response depend initially upon the genetically controlled recognition of foreign antigenic peptides. This discrimination of self and non-self is mediated by human leukocyte antigen (HLA) class I and class II molecules. HLA class I and II are encoded by genes located in the

major histocompatibility complex (MHC), a complex set of polymorphic genes on the short arm of the human chromosome 6. The polymorphism of MHC genes and their encoded proteins is part of an inherent strategy allowing the presentation of numerous different antigens.

MHC class I genes encode HLA class I molecules (HLA-A, -B, -C). These molecules are expressed on the surface of almost all nucleated cells. Class I molecules present antigens to CD8+ T cells after intracellular synthesis to stimulate these cells. MHC class II genes encode HLA class II (HLA-D) molecules (DR, DQ, DP), which are expressed on antigen presenting cells (APC) and on thymic cortical epithelial cells. HLA class II molecules present fragmented peptides to CD4+ T cells after internalisation and processing of foreign antigens by APC.

HLA elements have been shown to be associated with distinct disease manifestations in *P. falciparum* malaria. HLA-B53 was shown to be associated with protection from severe anaemia and cerebral malaria, and the MHC class II haplotypic combination HLA-DRB1*1302 -DQB1*0501 was found to give significant protection against severe anaemia in children in The Gambia (Hill *et al.*, 1992). HLA-B70, -B50, -Cw2, -DRB1*1302 and -DRB1*1101 have been shown to be associated with elevated levels of TNF-α but not susceptibility to cerebral malaria (Meyer and Kremsner, 1996). A polymorphism within the promoter region of TNF-α gene has been linked to increased risk of death from severe malaria (McGuire *et al.*, 1994). Likewise, TNF-α homozygous individuals were shown to have a higher risk of developing cerebral malaria and death or neurological sequelae (Meyer and Kremsner, 1996).

ICAM-1 is an important receptor for adherence of *P. falciparum* to the endothelium and is associated with the development of cerebral malaria. A polymorphism at the N– terminal domain of this molecule has been associated with

severity of clinical malaria and homozygous individuals have increased susceptibility to cerebral malaria (Fernandez-Reyes *et al.*, 1997).

The complement receptor-1 (CR1) on erythrocytes is involved in rosetting and erythrocytes with a common African CR1 polymorphism (S1a-) have reduced adhesion to the domain of PfEMP1 that binds normal erythrocytes and has been associated with protection against severe malaria (Rowe *et al.*, 1997). In contrast, no association has been reported with polymorphisms in interleukin 1 receptor antagonist (IL-1RA) genes or are contradictory as in inducible NOS2 (Bellamy *et al.*, 1998).

The Fc receptors for IgG (FcγRs) on leukocytes provide an important link between the humoral and cellular arms of the immune response (van de Winkel and Capel, 1993). The binding of these receptors triggers a variety of immune functions, including phagocytosis, antibody-dependent cellular cytotoxicity, release of inflammatory mediators, facilitation of antigen presentation, immune complex clearance, and regulation of antibody production (van de Winkel and Capel, 1993). Among the three classes of FcγR (FcγRI, FcγRII, and FcγRIII), low affinity FcγRII class is the most widely distributed (de Winkel and Capel, 1993; Warmerdam *et al.*, 1991). A polymorphism in the FcγRII in which a point mutation (G to A) resulting in an amino acid change at position 131, arginine (Arg 131) or histidine (His 131), in the second extracellular Ig-like domain of the FcγRIIa subclass is critical for the binding of human IgG2 (Warmerdam *et al.*, 1991). Several *in vitro* studies have shown a role of FcγRII and IgG subclasses in resistance to high-density parasitaemia through ADCI by binding of IgG-merozoite complex to monocytes via the FcγRII receptor, and that ADCI against parasite proliferation correlates with malaria specific IgG1 and/or IgG3, but not IgG2 (Shi *et al.*, 1999b; Bouharoun-Tayoun *et al.*, 1992). Recently it has been

found that FcγRIIa-Arg/Arg131 genotype is associated with protection against high-density *P. falciparum* malaria infection (Shi *et al.*, 2000).

1.1.4.2. ACQUIRED IMMUNITY TO MALARIA

Naturally acquired immunity (anti-parasite and anti-disease) in falciparum malaria refers specifically to a diminished frequency and density of parasitaemia by *P. falciparum* in adults relative to children in areas with hyper- to holoendemic malaria. Individuals repeatedly exposed to infective mosquito bites in endemic areas develop a complex immune response to infection, due to specific malaria parasite antigens from different blood stages of the parasite, modified host erythrocyte antigens and polyclonal B cell activation (Maria, 1997). In their first five years of life, children in malaria endemic areas suffer more episodes of severe malaria attacks with high parasite densities and high morbidity and mortality rates (Greenwood *et al.*, 1991). However, as they grow, they develop naturally acquired clinical and parasitological immunity over several years after repeated exposure to infection (Marsh, 1992). Clinical and epidemiological observations of human *P. falciparum* infections suggest that there exist several states of acquired immunity: **(1.)** The ability to control neurological complications (such as cerebral malaria), which is acquired rapidly, **(2.)** Antidisease or antitoxic immunity, which becomes progressively evident through childhood, **(3.)** Isolate- or strain-specific antiparasite immunity, and **(4.)** after a long time of exposure in hyper- or holoendemic areas, the host-parasite equilibrium characteristic of premunition (Druilhe and Perignon, 1994). Antiparasite immunity seems to be the most effective immune response that humans develop against the pathogenic asexual erythrocytic stage, however, protection against infection is never complete and it is still not known why some infections are mild and some fatal (Marsh, 1992; Trape *et al.*, 1994). Both humoral and cellular mechanisms are involved in acquired

immunity to malaria. This involves actions of a large number of cytokines. These responses are both species- and stage-specific (Baird, 1995).

1.1.4.2.1. HUMORAL IMMUNITY

a) Human *in vivo* studies

Repeated *P. falciparum* infections generally lead to the development of a non-sterile immunity in adults living in malaria-endemic areas. Antibodies mediate part of this immunity. Cohen *et al.* (1961) demonstrated that children were protected against malaria by the passive transfer of immune globulins obtained from immune mothers in The Gambia.

Recurrent malaria induces intense and prolonged production of IgG and IgM in infected individuals who concurrently develop acquired immunity; and although this does not prevent reinfection it significantly decreases the number, duration and severity of subsequent malarial episodes (Maria, 1997).

Congenital malaria is rare because of the placental transfer of specific IgG antibodies from the maternal blood across the placenta. This passively acquired immunity is transient and after 4 - 6 months of life young children have a high parasitaemia and severe clinical illness leading to a high fatality rate (Carlier and Truyens, 1995).

Elevated levels of malaria specific IgE antibodies have been detected in sera of New Guineans, Africans and Asians (Perlmann *et al.*, 1994). The significance of these IgE antibodies in protection is unclear, but raised IgE in children with cerebral malaria in The Gambia has been reported (Perlman *et al.*, 1994).

b) Human *in vitro* studies

Studies carried out in Thai adults indicate that antimalarial antibodies of the IgG_1 and IgG_3 subclasses are associated with protection and a predominance of IgG_2

and IgG_4 or IgM, or low levels of antibodies, with susceptibility (Bouharoun-Tayoun and Druilhe, 1992). The cytophilic antibody IgG_1 and IgG_3 subclasses show extensive complement fixing ability, promote phagocytosis; antibody dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular inhibition (ADCI); this is not the case for IgG_2 or IgG_4 antibodies, which are not cytophilic or complement fixing, and may even affect these mechanisms by competing with IgG_1 and IGg_3 for antigen (Bouharoun-Tayoun and Druilhe, 1992; Ferrante *et al.*, 1991). Further studies have shown that IgG_1 and IgG_3 antibodies are elevated in individuals residing in malaria endemic areas (Chizollini *et al.*, 1988; Riley *et al.*, 1992).

Premunition is characterised by equilibrium in the host parasite-response where the parasite is not eliminated but persists at low densities and confers some protection against subsequent infections (Allison, 1988; Druilhe and Perignon, 1994). Premunition has been reported to be IgG dependent, with the IgG proposed to mediating ADCI. ADCI requires cytophilic antibodies like IgG_1, and appears to be independent of transmission levels (Druilhe and Perignon, 1994).

Studies have been undertaken to define the role of antibody against the various antigens of *P. falciparum* in protective immunity. These seroepidemiological studies have found a correlation between the presence of antibodies to malarial antigens such as merozoite surface protein-1 (MSP-1), merozoite surface protein-2 (MSP-2), circumsporozoite protein (CSP) antigen, ring infected erythrocyte surface antigen (RESA), (Troye-Blomberg and Perlman, 1989); and *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) (Marsh, 1992), among others.

Children repeatedly exposed to malaria infection in endemic areas develop acquired immunity (Marsh, 1992). Studies suggest that this immunity may develop through acquisition of a repertoire of specific protective antibodies directed against

polymorphic target antigens (Marsh, 1992; Molyneux, 1996). The pressure exerted by established anti-PfEMP1 antibodies on infective parasites affords this variant specific protection. Therefore, the polymorphic parasite antigen, PfEMP1 on the surface of infected erythrocytes is a target of naturally acquired immunity to malaria.

Studies from malaria immune adults from The Gambia show that MSP-2 is immunogenic and induces IgG antibodies with opsonizing and complement fixing properties (Taylor *et al.*, 1995), which suggests a protective role against intraerythrocytic parasites.

c) Animal models

Experiments in animal models have also elucidated the protective role of antibodies. Antibodies against the major antigenic repeats of CS-protein have been demonstrated to prevent malaria infection in both rodent and non-primate models (Blackman *et al.*, 1990). Antibodies specific for MSP-1 of rodent malaria parasite has been shown to effectively neutralise malaria parasites in *P. yoelii* models (Troye-Blomberg and Perlmann, 1988).

MSP-2 may also play a role in protective immunity. It has been shown that monoclonal antibodies to MSP-2 inhibit parasite growth *in vitro* (Clark *et al.*, 1989; Epping *et al.*, 1988). MSP-2 is among the antigens recognised by antibodies that inhibit merozoite dispersal (Thomas *et al.*, 1990) and mice immunised with peptides identical to conserved regions of MSP-2 of *P. falciparum* are protected against *P. chabaudi* (Saul *et al.*, 1992).

1.1.4.2.2. CELLULAR IMMUNITY

1.1.4.2.2.1. Neutrophils and macrophages

The major effector cells for parasite neutralisation in blood-stage infection appear to be mononuclear phagocytes, and polymorphonuclear leukocytes. Phagocytes

can mediate protection through phagocytosis of *P. falciparum* merozoites (Roberts and Weidanz, 1978), infected erythrocytes, schizonts (Waters *et al.*, 1987) and gametocytes. Polymorphonuclear neutrophils have also been reported to inhibit the asexual multiplication of *P. falciparum in vitro* (Brown and Greenwood, 1985; Kharazmi and Jepsen, 1994).

Human neutrophil-mediated killing of *P. falciparum* asexual blood forms, the stage that is responsible for clinical symptoms, was demonstrated when it was shown that antiparasite activity of neutrophils is increased three-fold by fatty acids (Kumaratilake, 1997). Polyenoic fatty acids have been shown to prime human neutrophils for increased respiratory burst and degranulation (Bates *et al.*, 1993; Hardy *et al.*, 1991), the functions that have been linked with *P. falciparum* killing by phagocytic cells (Ferrante, 1994). Neutrophil-mediated phagocytosis and killing of erythrocytic *P. falciparum* is increased by cytokines, notably TNF-α, IFN-γ and lymphotoxin (TNF-β), especially in the presence of antimalarial antibody (Kumaratilake *et al.*, 1990; 1991), although, monocyte-derived macrophages (Jones *et al.*, 1989), and polymorphonuclear leukocytes (Nnalue and Friedman, 1988) are able to kill late stages of the intraerythrocytic parasites in the absence of antibodies, through phagocytosis.

The importance of macrophages in protection against malaria infection has been reported. During malaria attacks there is an increase, of monocytes in circulation and accumulation of macrophages in spleen and liver (Brown and Greenwood, 1985). All these contribute to the clearance of infected red blood cells by phagocytosis.

1.1.4.2.2.2. CD4+T cells

CD4+ T cells and CD8+ T cells are the major lineages of T cells. CD4+ T cells recognise antigenic fragments in association with MHC II antigens, which are

expressed by mammalian cells such as macrophages, B-lymphocytes, dentritic cells and endothelial cells. These cells participate in protective immunity by providing cognate help to B cells to secrete antibody or secrete cytokines, which act directly on the parasite or activate phagocytic cells such as macrophages.

The role of CD4+ T cells in cell-mediated immunity against intracellular bacteria, fungi, and protozoa is generally accepted to be via the production of lymphokines that activate macrophages to express powerful antimicrobial activity. This mechanism has been hypothesised for the elimination of plasmodium species, such as *P. chabaudi AS* (Stevenson, 1989). CD4+ T cells may also provide help to expand effector CD8+ T cell populations via production of IL-2. IL-2 produced by CD4+ T cells has been demonstrated not only to enhance the effectiveness of cytotoxic T cells but also the production of IFN-γ by these cells (Farrar, 1981; Stevenson, 1982).

The participation of CD4+ T cells in arresting liver stage parasites has been demonstrated in rodents (Troye-Blomberg and Perlmann, 1994). Resolution of an acute infection with *P. chabaudi adami* was obtained in athymic nude mice after transfer of a CD4+ T cell clone specific for an epitope of a soluble parasite antigen (Brake *et al.*, 1988). In resistant mice, CD4+ T cells of the IFN-γ - IL-2 producing type (T_H1) play a crucial role during the initial phase of protective immune response against *P. chabaudi chabaudi*, while IL-4 - IL-5 producing CD4+ T cells of the helper type (T_H2), together with antibodies, are important during later phases of response (Stevenson and Tam, 1993; Langhorne, 1989; Taylor-Robinson and Phillips, 1993; 1994). In contrast, in susceptible mouse strains, induction of a strong T_H2 response early in infection is associated with a severe and lethal course of malaria (Stevenson and Tam, 1993).

As in mice, CD4+ T cells have a major role in regulating the human immune response to *P. falciparum* blood-stage antigens. The T-dependency of IgG antimalarial

antibodies has been shown directly by assaying antibody secretion after T cell stimulation with antigen in T cell - B-cell co-operation system *in vitro* (Kabilan *et al.*, 1987; Chougnet *et al.*, 1991a). However, *in vitro* stimulation of CD4+ T cells also result in proliferation and/or IFN-γ secretion, neither of which is correlated with levels of serum antibodies against the corresponding antigens (Fievet *et al.*, 1995; Rzepczyk *et al.*, 1989; Troye-Blomberg *et al.*, 1989). Stimulation also induces IL-4 expression which in individual donors is not correlated with either proliferation nor IFN-γ release but correlates well with concentrations of the relevant serum antibodies (Riley *et al.*, 1991; Troye-Blomberg *et al.*, 1990). These results indicate that the human response is controlled by distinct CD4+ T cell subsets that correspond to the T_H1 and T_H2 types found in *P. chabaudi*-infected mice (Langhorne, 1989).

1.1.4.2.2.3. CD8+ T cells

The CD8+ T cells recognise antigenic fragments displayed on infected cells in association with class I MHC antigens. These cells are the primary effector cells in the destruction of intracellular infections.

CD8+ T cells inhibit the development of malaria parasites in the liver stage. Adoptive transfer of CD8+ T cells specific to CSP and other liver stage antigens has been shown to confer protection against lethal malaria in rodents (Riley *et al.*, 1989). Furthermore, studies indicate that CD8+ T cell clones isolated from individuals infected with *P. falciparum* respond to specific antigens *in vitro* (Sinigaglia, 1987).

Since erythrocytes do not express MHC class I antigens it is believed that CD8+ T lymphocytes may not affect blood stage parasites. However, it was shown that CD8+ T cells might exert an antiplasmodial effect indirectly via the production of IFN-γ (Ockenhouse *et al.*, 1984). This lymphokine is necessary for activating macrophages to express enhanced antimicrobial activity against both human and murine *Plasmodium*

species *in vitro* and to destroy intraerythrocytic parasite by the production of hydrogen peroxide (Ockenhouse *et al.*, 1984; Ockenhouse and Shear, 1984).

1.1.4.2.2.4. Gamma-delta T-cells (γδT cells)

During acute *P. falciparum* infection there is high levels of TCR γδT-cells in peripheral circulation and spleen (Chang *et al.*, 1992a; Bordessoule *et al.*, 1990). The ability of parasite material to activate γδT-cells *in vitro,* and the localisation of these cells *in vivo,* in the red pulp of the spleen, suggests a role in the killing of blood stage malaria parasites (Langhorne, 1996).

Human γδT-cells from non-immune individuals responding to *P. falciparum* produce IFN-γ, TNF-α and TNF-β, and little or no IL-4, IL-5 and IL-10 (Goodier *et al.*, 1995), and this suggest activation of macrophages and/or cytotoxic killing via TNF-β. Although there are no reports to date on the ability of products of macrophages activated by γδ T-cells to kill *P. falciparum*, these cells inhibit *P. falciparum* growth *in vitro*, and extracellular merozoites are the targets of killing (Elloso *et al.*, 1994).

1.1.4.2.2.5. Natural killer cells (NK-cells)

The role of human NK-cells in protection against malaria has been elucidated *in vitro*. It was demonstrated that NK cells are cytotoxic against erythrocytic schizonts of *P. falciparum*, and cytotoxicity was enhanced in the presence of IFN-γ and/or IL-2 (Orago and Facer, 1991). NK-cells are also induced by IL-12 to produce IFN-γ which stimulate infected hepatocytes to produce nitric oxide that kills developing parasites (Sedegah *et al.*, 1994).

1.1.4.3. CYTOKINES IN MALARIA

Cytokine networks determine the outcome of an infection. Animal model studies for protozoan and helminthic infections indicate that susceptibility and resistance are related to differential activation of two CD4+ T cell subpopulations,

distinguished by the profile of cytokines that each produces (Sher and Coffman, 1992).

The native (naïve) T lymphocytes (T_H0) are the precursors and secrete a combination of IL-2, IFN-γ, IL-4, IL-5, IL-6 and IL-10. Following antigenic stimulation, T_H cells with a more restricted cytokine profile emerge. The T_H1 cells secrete IL-2, IFN-γ and TNF; T_H2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13. These T cell subsets reciprocally regulate one another since IFN-γ a product of T_H1 cells inhibits T_H2 cell proliferation and IL-4 function. On the other hand IL-4 and IL-10, T_H2 products can down regulate T_H1 responses. T_H1 responses promote cell-mediated effector mechanisms and are responsible for immunity to intracellular parasites. A T_H2 responses influences B-cell development, augments antibody production and humoral immunity to extracellular parasites (Cox, 1992).

A distinct T_H1/T_H2 divergence determines resistance, susceptibility and outcome of most infectious diseases (Infante-Duarte and Kamradt, 1999). The role of T-cells and T cell derived cytokines in malaria has been investigated in rodent models (Grau *et al.*, 1987). CD4+ T cell subsets in humans are less clear-cut. IL-10 is produced by T_H0, T_H1 and T_H2 human T cell clones, CD8+ T cells and monocytes and down regulates the functions of both T_H1 and T_H2 cells (DelPrete *et al.*, 1993). Studies done to detect T cell cytokines in patients during acute uncomplicated *P. falciparum* malaria, have yielded evidence suggesting that there is a more pronounced T_H2 driven immune response during acute untreated *P. falciparum* infection with a shift towards T_H1 responsiveness induced by parasite clearance (Winkler *et al.*, 1998). IL-10 has been shown to have a negative feedback action on the production of inflammatory cytokines in acute falciparum malaria (Ho *et al.*, 1998). It abolished TNF-α production in response to malarial antigen and proliferation of peripheral blood mononuclear cells. This has been confirmed by studies which established that higher levels of IL-10 over

TNF-α might prevent development of malaria anaemia in children by controlling excessive inflammatory activities of TNF-α (Othoro *et al.*, 1999). It has also been demonstrated that endogenous IL-10 modulates the activities of proinflammatory cytokines, TNF-α, IL-1β and IL-6, which are implicated in the pathogenesis of severe *P. falciparum* malaria (Grau *et al.*, 1989; Playfair *et al.*, 1990; Kwiatkowski and Greenwood, 1989) and that during acute *P. falciparum* malaria infection there occurs elevated serum levels of IL-10 and IFN-γ which deplete with treatment (Wenisch *et al.*, 1995). These results suggest that stimulatory and/or inhibitory cytokines for activation and/or antibody production (T_H1 and T_H2 type immunoreactions, respectively) are expressed during acute *P. falciparum* infection and stress the multifactorial network between host and parasite in malaria immunology.

The immediate production of the monocyte derived TNF-α to the parasites presence raises body temperature that is thought to constrain the parasite growth. Early T_H1 derived IFN-γ response stimulates monocytes to produce TNF-α and enhances phagocytic activity of neutrophils. T_H1 released IFN-γ may stimulate monocytes and macrophages, which subsequently release nitrogen and oxygen radicals (Hamilton and Adams, 1987). The macrophages and neutrophils stimulated by T cell cytokines can act by phagocytosis or cytotoxic activity against parasitised erythrocytes (Ferrante *et al.*, 1990). The blood stage malaria parasites release soluble antigens known as exoantigens during their rupture from infected erythrocytes (Playfair *et al.*, 1990; Luty *et al.*, 1994; Taverne *et al.*, 1990). These antigens with glycophosphate inositol tail (GPI anchored protein) are known to induce macrophages to release high levels of TNF-α. The high levels of this cytokine are associated with fever. It has been proposed that individuals living in malaria endemic areas slowly acquire antibodies, especially of IgM class, which neutralise these antigens from inducing TNF-α from macrophages (Bouharoun-

Tayoun and Druilhe, 1992). This explains why individuals in endemic areas are asymptomatic in spite of high parasitaemia (Bate and Kwiatkowski, 1994). This form of immunity is known as antidisease immunity.

In vitro studies have shown that recombinant human IFN-γ (rHuIFN-γ) and recombinant human TNF-β inhibited erythrocytic development of *P. falciparum* but was partially reversed by monoclonal anti-IFN-γ antibodies (Orago and Facer, 1993). Similarly, it was further shown that rHuIFN-γ inhibits schizogony in hepatocytes infected with *P. falciparum* sporozoites (Maheshwari *et al.*, 1996). Therefore the inhibitory effect of IFN-γ and IFN-γ-inducer is limited to the exoerythrocytic stage of parasite development. The effector mechanism against these intracellular stages is the cytokine-induced synthesis of NO (nitric oxide) from L-arginine (Maheshwari *et al.*, 1996).

1.1.4.4. CYTOADHERENCE AND MALARIA

Mature *P. falciparum* parasitised erythrocytes (PE) sequester from the circulation by binding to microvascular endothelial cells. PE sequestration is crucial to the development of the parasite but contributes directly to the virulence and severe pathological sequalae of falciparum malaria (Newbold *et al.*, 1999). Parasite-derived or host-induced molecules on the erythrocyte surface mediate cytoadherence (Roberts *et al*, 1992). CD36 is a major host receptor for PE adherence, which is mediated by the variant *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1). In addition, PfEMP-1 molecules bind to a variety of other cell receptors for cytoadherence and rosetting including interstitial cell adhesion molecule 1 (ICAM-1) and thrombospondin, as well as vascular cell adhesion molecule-1 (VCAM-1), endothelial adhesion molecule-1 (ELAM-1), E-selectin and chondroitin sulphate in the placenta (Target, 1998; Baruch *et al.*, 1999). This phenomenon is a target for

vaccine development and adherence-blocking mechanisms that inhibit ligand-receptor interaction may be useful in anti-adhesion therapeutics (Newbold *et al.*, 1999).

1.1.5. CONTROL OF MALARIA

The objective of malaria control is to prevent mortality and reduce morbidity as well as economic loses due to disease (Trigg and Kondrachine, 1998). Control of malaria revolves around attacks on the vector to disrupt transmission and parasite.

Removal of breeding grounds to destroy the mosquito larva and insecticides such as dichlorodiphenyl trichloroethane (DDT) has been used to control mosquitoes (Philips, 1983). However mosquitoes have developed resistance to it. Infected mosquitoes have also, been prevented from feeding on man through use of impregnated bed nets and insect repellent creams. Studies carried out in Tanzania indicate that children sleeping under bed nets impregnated every six months with pyrethroid insecticides resulted into lower levels of parasitaemia and marked improvement in anaemia compared to those not using them (Curtis, 1990; Premji *et al.*, 1995). However, use of bed nets may hinder the development of acquired immunity that requires continuous exposure to develop, hence may not in the long run prevent mortality and morbidity resulting from malaria. However, vector control effectiveness has declined in recent years, due to lack of funds.

Various drugs have been used for chemotherapy and chemoprophylaxis to eliminate the disease both by travellers and people living in endemic areas. Chloroquine is one of the most widely used drugs in endemic areas to treat chloroquine sensitive malaria (Ekanem *et al.*, 1990). Chloroquine resistance has been observed in *P. falciparum* and *P. vivax* infections and resistance to other antimalarials has concomitantly emerged world-wide (Wernsdorfer *et al.*, 1991; Luxemburger *et al.*,

1994). Furthermore, chemoprophylaxis may reduce acquired immunity and because of poor compliance the development of new methods for malaria control are urgently needed (Trigg and Kondrachine, 1998).

1.1.6. MALARIA VACCINE DEVELOPMENT

The widespread and increasing resistance of malarial parasites to antimalarial drugs, development of resistance of *Anopheles* mosquito vectors to commonly used insecticides, an inappropriate infrastructure for delivery of control measures, population growth and movement of non-immune individuals to malarious areas have all yielded to the persistence and worsening malaria situation (Hoffman and Miller, 1996). Therefore, in an effort to develop new methods for the effective prevention and control, vaccine candidate antigens targeted to the various stages of the complex life cycle of plasmodium have been developed. Although there are concerns that a single antigen and/or single stage specific vaccine may not be effective because of sequence variability among different parasite isolates (parasite polymorphism), poorly understood mechanisms involved in acquired immunity to malaria, lack of suitable animal models, lack of suitable adjuvant, host genetic restriction of immune responses to specific epitopes and short-lived protective immunity induced by some single antigen/epitope-based vaccines. The fact that the parasite has a complex life cycle requires a multistage vaccine to induce multi-immune mechanisms for protection.

1.1.6.1. RATIONALE FOR THE DEVELOPMENT OF MALARIA VACCINES

There is a strong belief that an effective malaria vaccine will eventually be developed. The concept that vaccination may be a useful tool to control the disease is based on a number of observations. Irradiated sporozoites conferred protection in volunteers by induction of neutralising antibodies (Nussenzweig and Nussenzweig, 1969) directed against sporozoite surface proteins and cellular effector mechanisms

that destroy liver stages (Nardin and Nussenzweig, 1993), inoculation of live attenuated parasites can protect naïve volunteers against infection (Clyde *et al.*, 1973), passive transfer of immune immunoglobulins from immune adults can protect children by decreasing blood stage parasitaemia (Cohen *et al.*, 1961), immunisations with whole killed organisms can protect in animal models (Michel *et al.*, 1975) and individuals living in endemic areas can develop acquired immunity to malaria due to repeated exposure (Marsh, 1992). All these factors have immensely contributed to the development of *P. falciparum* malaria vaccines.

Malaria parasite's complex life cycle provides potential antigenic targets favourable for vaccine development. Each stage has different antigens that lead to protective immunity, and in many cases these antigens are not expressed at other stages of the life cycle. So far, several stage-specific candidate antigens of *P. falciparum* have been sequenced and immunologically characterised.

1.1.6.2. Pre-erythrocytic vaccines

These are vaccines targeting stages before the asexual erythrocytic parasites, the sporozoite and hepatic stages (or pre-erythrocytic stages). Although immune effector mechanisms and antigens for sporozoites and hepatic stages are different, the grouping of these vaccines under the single term, "pre-erythrocytic" is dependent on the fact that a fully effective pre-erythrocytic vaccine will prevent infection.

The first documented study was on the sporozoite stage, and vaccines directed against sporozoites are expected to prevent invasion of hepatocytes. It was shown that solid protection against malaria infection in rodents, monkeys and humans is achieved by vaccination with irradiated sporozoites (Nussenzweig and Nussenzweig, 1989) and that humans in areas of endemic malaria have serum antibodies against sporozoites (Nardin *et al.*, 1979).

The sterile immunity achieved by repeated immunisation with irradiated sporozoites is mediated by both neutralising antibodies directed against sporozoite surface proteins and cellular effector mechanisms that destroy the liver stages (Nardin and Nussenzweig, 1993). The targets of such vaccines use the repeats of circumsporozoite protein, (CSP) of *P. falciparum*, (NANP). This contains the immunodominant B-cell epitopes of the sporozoites, which induces production of relevant anti-CSP antibodies (Nussenzweig *et al.*, 1969). Studies in human volunteers have shown that only those with high titres of antibodies are protected, although not everyone with high titres of antibodies to the repeats was protected (Sinnis and Nussenzweig, 1996). Recent work in rodent models has shown that cellular, in addition to humoral immunity plays an important role in sporozoite-induced protection. Murine CD8+ and CD4+ T cells were found to inhibit growth of the intracellular hepatic stage of the malaria parasite *in vitro* (Renia *et al.*, 1991). Passive transfer of cytotoxic T cells, specific for the CS-protein, protected naive mice against sporozoite challenge (Rodrigues *et al.*, 1991). T cell derived lymphokines in particular IFN-γ, also block exoerythrocytic parasite development both *in vitro* and *in vivo* (Ferreira *et al.*, 1986; Schofield *et al.*, 1987a; b). Studies in sporozoite-immunised human volunteers showed that polymorphic sequences of *P. falciparum* contain epitopes, which stimulate cytolytic class II-restricted CD4+ T cells (Moreno *et al.*, 1993). However, the actual mechanism for their role in protection remains to be confirmed.

Volunteers vaccinated with irradiated *P. falciparum* sporozoites developed CTLs specific for CS protein (Malik *et al.*, 1991). These CTLs recognised CS protein region, amino acid 368-390, which is identical to that recognised by mouse CTLs. CD8+ CTL specific for the same sequence have been identified in Kenyan residents residing in a malaria endemic area and in Australian volunteers exposed to malaria

(Udhayakumar *et al.*, 1997; Doolan *et al.*, 1993). The response was HLA restricted and the CTL epitope has been mapped to eight amino acid residues, 368-375, which are recognised in association with the HLA-B35 (Hill *et al.*, 1992). This CTL epitope is situated in a highly polymorphic region and amino acid variation abrogates reactivity of CTL (Udhayakumar *et al.*, 1994). Studies done in western Kenya have shown that CTL reactivity to all the variants are present in the population, but at individual host response was found only in some variants of the CS protein (Udhayakumar *et al.*, 1997). Recently a vaccine formulation, RTS,S/SBAS2, which consists of a recombinant DNA-produced CS-based antigen together with a novel adjuvant SBAS2, has been shown to be highly immunogenic and confers sterile protection against *P. falciparum* sporozoite challenge. It was shown that this antigen induces potent T_H1-type cellular and humoral immune mechanisms and this has important implications for vaccine design (Lalvani *et al.*, 1999).

The liver stage antigen-1 (LSA-1) is another potential candidate vaccine antigen. This protein is a 200-kDa pre-erythrocytic *P. falciparum* antigen expressed throughout schizogony. It is a highly conserved protein localised in the parasitophagous vacuole (Fidock *et al.*, 1994). It is a target of MHC-restricted CD8+ CTL. Studies in The Gambia reported that individuals with HLA-Bw53 had HLA-Bw53 restricted CTL activity against peptide 1s6 from the LSA-1 protein, and this may account for the association between HLA-Bw53 and resistance to severe malaria by decreasing the number of infected mature hepatocytes (Hill *et al.*, 1992).

Further studies have established that the C-terminal, non-repetitive hinge region, and the conserved repetitive region contain major B and T cell determinants. High prevalence and elevated antibody levels to LSA-1 directed primarily although not exclusively, to the repetitive region, were detected in sera of individuals in a

moderately high and in low transmission malaria-endemic areas (Fidock *et al.*, 1994). Secretion of IFN-γ (which is known to inhibit malaria liver stages) and T cell proliferation were detected in 22 to 48% and 6 to 20% respectively in individuals from one of the low transmission areas in response to separate LSA-1 antigens (Fidock *et al.*, 1994). These studies complement findings of conserved CTL epitopes in LSA-1 and support the assertion that immune responses to liver stage antigen are involved in protection against malaria pre-erythrocytic stage (Fidock *et al.*, 1994).

1.1.6.3. ASEXUAL BLOOD STAGE VACCINES

These sets of vaccines act against the asexual erythrocytic stages. Anti-asexual-erythrocytic-stage vaccines are being designed to prevent disease, inhibit invasion, reduce or inhibit growth of the parasite in the blood, inhibit the adherence of infected erythrocytes to endothelial cells and other erythrocytes, and block factors in the parasites, such as toxins, that cause disease (Hoffman and Miller, 1996).

It has been proposed that two alternative strategies for vaccine development can be considered, one leading to a reduction or prevention of parasitaemia (an anti-parasite vaccine) and the second leading to a reduction in the pathological consequences of infection (an anti-disease vaccine). Studies have indicated that plasma levels of the cytokine, TNF-α, are correlated with severity of malaria in African children (Grau *et al.*, 1989; Molyneux *et al.*, 1996). Therefore, these findings suggest that naturally acquired clinical immunity in man may be achieved by maintaining the disease-related cytokines at low levels. Thus, various malarial antigens thought to be involved in the induction of this cytokine and other soluble mediators such as nitric oxide are being investigated so as to develop anti-disease interventions and vaccines. However, the identification of relevant disease causing parasite antigens is hindered by absence of suitable animal models for studying of pathogenesis of clinical disease states of human

malaria. But studies in mouse suggest a possible role of platelets in the late stages of cerebral malaria (Grau *et al.,* 1991). These studies including those that are related to lymphocyte function-associated antigen (LFA) involvement in cytoadherence (Grau *et al.,* 1991) might yield information for development of novel intervention for cerebral malaria in children.

Antigens on the surface of malaria merozoites are of interest as potential targets for vaccine-induced immune responses. Examples of such antigens are merozoite surface protein-1, (MSP-1), merozoite surface antigen-2, (MSA-2) and glutamate- rich protein (GLURP) antigens of *P. falciparum.* Since these antigens may be involved in merozoite adherence to, and invasion of erythrocytes (Holder and Blackman, 1994), antibodies specific for merozoite surface antigens could prevent invasion of erythrocytes and thereby interrupt the asexual cycle of parasite proliferation.

1.1.6.3.1. Merozoite surface antigen-1, (MSA-1)

It is also known as merozoite surface protein-1, (MSP-1). During it's processing only the membrane-anchored C-terminal domain is carried with the invading merozoite into the erythrocytes (Holder, 1988). This domain is highly conserved in *P. falciparum* and was shown to be reactive with monoclonal antibodies inhibiting *P. falciparum* replication in culture (Blackman *et al.*, 1990; 1991; Chang *et al.,* 1992b; Chappel and Holder, 1993; Cooper *et al.*, 1992). These results indicate that a vaccine candidate can be developed from this antigen.

Furthermore, immune responses to proteins from the C-terminus of the MSP-1 molecule and resistance to episodes of fever are associated with high parasitaemia in partially immune Gambian children (Riley *et al.*, 1992); and that the 19-kDa domain of the *P. falciparum* MSP-1 antigen contains T cell proliferative epitopes (Shi *et al.*, 1996).

1.1.6.3.2. Merozoite surface antigen (MSA-2)

This is another candidate vaccine antigen on the surface of *P. falciparum* malaria merozoites, also known as merozoite surface protein-2, MSP-2. Several pieces of evidence suggest that antibodies to MSP-2 may be involved in protective immunity to malaria. Monoclonal antibodies to MSP-2 have been shown to inhibit parasite growth *in vitro* (Clark *et al.*, 1989; Epping *et al.*, 1988). MSP-2 is among the antigens that are recognised by antibodies that inhibit merozoite dispersal (Thomas *et al.*, 1990) and mouse immunised with peptides corresponding with conserved regions of MSP-2 of *P. falciparum* are protected against challenge with the rodent parasite *P. chabaudi* (Saul *et al.*, 1992).

Studies in malaria-immune adults from the Gambia showed that MSP-2 is naturally immunogenic in man, and antibody responses are directed against dimorphic and polymorphic regions of MSP-2, is serogroup specific and is predominantly of the cytophilic and complement fixing subclass IgG_3 (Taylor *et al.*, 1995). Hence, the potential of MSP-2 as a vaccine antigen is dependent on the fact that it is naturally antigenic and that immune responses are directed to dimorphic as well as polymorphic regions of the molecule and that these antibodies are of an appropriate class.

1.1.6.3.3. Pf155/Ring-infected erythrocyte surface antigen, (RESA)

The Pf155/RESA, of *P. falciparum* is considered an important candidate for possible inclusion in a subunit vaccine against malaria. RESA is found on dense bodies of merozoites but after invasion is found associated with the membrane skeleton of the infected erythrocyte (Aikawa *et al.*, 1990b; Culvenor *et al.*, 1991).

Humoral immunity to RESA increases with age, and majority of adults living in areas of endemic malaria have high antibody titres against this antigen, although only IgG_3 were found to be age dependent (Beck *et al.*, 1994). In spite of the fact that

total anti-RESA IgG antibodies were not correlated with protection against malaria, cytophilic antibodies (IgG_1 and IgG_3) were associated with reduced *P. falciparum* prevalence, T-cell proliferation was low but individuals with good humoral and cell mediated immune responses tended to have reduced parasite prevalence (Beck *et al.*, 1994).

1.1.6.3.4. *P. falciparum* erythrocyte membrane protein-1, (PfEMP1)

Antigenic differences on the surface of *P. falciparum* infected erythrocytes arise by antigenic variation of clonal parasite populations at the level of the parasite encoded PfEMP-1 erythrocyte membrane protein. The potential of PfEMP-1 as an important family of target antigens is dependent on the fact that, these proteins are inserted into the red cell surface and are prominently exposed (Magowan *et al.*, 1998), and because they are highly polymorphic and undergo clonal antigenic variation (Iqbal *et al.*, 1993). Furthermore, studies undertaken in Kenyan children showed that anti-PfEMP-1 antibodies agglutinate infected erythrocytes in a variant-specific manner and such responses provide variant-specific protection against disease (Bull *et al.*, 1998).

1.1.6.3.5. Glutamate-rich protein (GLURP)

GLURP is an antigen associated with mature schizont infected erythrocytes and is located on the surface of merozoites. It has four B cell epitopes, which induce antibody production (Borre *et al.*, 1991). It was demonstrated that all anti-GLURP IgG antibodies from adult Senegalese clinically immune to malaria mediated a strong monocyte-dependent parasite growth inhibition through ADCI (Theisen *et al.*, 1998).

Antibody-monocyte co-operation in parasite inhibition is mediated through soluble monocyte-derived substances whose release is triggered by way of interaction with cytophilic antibodies bound to merozoite antigens (Bouharoun-Tayoun *et al.*, 1995). These results were similar to those obtained with antibodies against merozoite

surface antigen-3, MSP3 (Oeuvray *et al*, 1994).

1.1.6.3.6. Rhoptry associated protein-1 (RAP-1)

Proteins localised in rhoptries, specialised organelles of *P. falciparum* merozoite stage known as Rhoptry associated proteins, RAP-1, 2 and 3 are potential vaccine candidates. RAP-1 is an 80-kDa protein. Monoclonal antibodies that bind epitopes in RAP-1 and inhibit invasion have been identified and several of the inhibitory anti-RAP-1 monoclonal antibodies map to the linear sequence at the N-terminus of the molecule (Harnyuttanakorn *et al.*, 1992). The potential role of this antigen in protection is dependent on findings that antibodies from immune monkeys can inhibit *P. falciparum* proliferation *in vitro* in the absence of monocytes or other effector cells (Chulay *et al.*, 1981) and that antibodies produced against RAP-1 epitopes in monkeys resulted into development of antiparasite immunity (Howard *et al.*, 1998).

1.1.6.3.7. Erythrocyte binding antigen-175 (EBA-175)

This is a 175-kDa erythrocytic binding antigen found on merozoites and is one of a number of proteins involved in the invasion of erythrocytes by *P. falciparum* (Dolan *et al.*, 1994). It mediates invasion of erythrocytes in a sialic acid-dependent manner. Studies undertaken show that rEBA-175 fragments are recognised by antibodies in serum from drug-cured malaria immune monkeys and also by antibodies present in sera from individuals residing in endemic areas (Daugherty *et al.*, 1997).

1.1.6.3.8. Apical membrane antigen-1 (AMA-1)

The blood stage apical membrane protein-1 is a leading target vaccine candidate. The AMA-1 of *P. falciparum* is an 83-kDa polypeptide similar to an integral membrane protein (Peterson *et al.*, 1989). It is synthesised during late schizogony and localises in the rhoptries and processed to a 66-kDa protein that is carried on the

surface of mature merozoites, and may be involved in merozoite release and invasion of erythrocytes (Crewther *et al.*, 1990). Studies done with *P. knowlesi* AMA-1 indicate that invasion-inhibiting antibodies can be induced (Deans *et al.*, 1988). Trials in rhesus monkeys indicated effective immunity against *P. knowlesi* challenge and that Fab fragments are more effective than intact IgG in blocking reinvasion of erythrocytes by merozoites *in vitro* (Thomas *et al.*, 1984). This suggests a functional role for AMA-1 protein in invasion. Further studies have shown that *P. falciparum* AMA-1 has T-cell proliferative epitopes localised in the highly conserved regions and may form a basis for development of AMA-1 based vaccine (Lal *et al.*, 1996b).

1.1.6.4. TRANSMISSION BLOCKING VACCINES

Transmission blocking vaccines are distinct from exo-erythrocytic, erythrocytic and antidisease vaccines in that they provide no protection to an individual; rather, in blocking transmission (or sporogonic development) of *P. falciparum,* they serve to interrupt the cycle of transmission by mosquito vectors. These vaccines target the developmental stages of malaria parasites in mosquito midgut. The aim of a transmission blocking (anti-asexual or anti-sporogonic-stage) vaccine is to arrest the development of the parasite in the mosquito and thus reduce or prevent the transmission of the disease in an endemic area (Hoffman and Miller, 1996).

The target antigens of these transmission-blocking antibodies are located on surface of extracellular parasite stages that occur in the mosquito midgut, namely gametocytes, gametes, zygote, ookinete and oocysts. The fact that the extracellular parasites are susceptible to immune effectors such as antibodies and complement, taken up during a blood meal from the vertebrate host (Carter *et al.*, 1988) has facilitated vaccine development. Several candidate antigens have undergone studies.

Recent results from studies conducted on antibody epitopes contained within

surface antigens of *P. falciparum* sexual stages using three potential vaccine candidates Pfs25, Pfs230 and Pfs48/45 suggest that these epitopes show limited diversity. It was demonstrated that anti-Pfs230 monoclonal antibodies of the isotype IgG_{2b} or IgG_{2a} were effective in blocking transmission in the presence of complement (Roeffen *et al.*, 1995). Additionally, it has been shown that anti-Pfs230 antibodies are present in 40 - 85% of the endemic human sera (Graves *et al.*, 1988; Riley, 1994), which has been associated with transmission blocking activity, though not conclusively. Studies in a monkey model have shown that repeats from the Pfs232, AgR45 and Pfs172 induce production of opsonizing antibodies especially in primary type immune responses in semi-immune groups (Bouharoun-Tayoun and Druilhe, 1992).

1.1.6.5. MULTIVALENT MULTISTAGE *P. FALCIPARUM* SUBUNIT VACCINES

Although studies of immunogenicity and the results of *in vitro* protective experiments have been promising for many of the single stage-specific vaccine candidate antigens, *in vivo* protection has not always been satisfactory. In contrast to single antigen-based vaccine, a multivalent, multistage human malaria vaccine would induce multilayer and multiple humoral and cellular protective mechanisms (Hoffman and Miller, 1996), which would provide more efficacious protection against malaria associated with morbidity and mortality. In this way the inability to mount a fully effective immune response to a particular antigenic component of the vaccine or to antigens to a given stage of the life cycle may be compensated for by effective responses to other antigens or life cycle stages, resulting into protective immunity. Furthermore, a multicomponent malaria vaccine would circumvent the problems associated with host genetic restriction and antigenic variability in the case of single antigen-based vaccines.

1.1.6.5.1. Multiple antigenic peptides (MAP) and multiple antigenic constructs (MAC)

Immunisation with combinations of antigen or epitopes derived from single stages of the parasite life cycle has been shown to be more effective in rodent and monkey models of malaria infection than in immunisation with individual antigens or epitopes (Hoffman and Miller, 1996).

A possible approach for producing the malaria vaccine containing multiple parasite-derived B and T-cell epitopes is to include them in multiple antigen peptide systems (MAP) (Tam, 1988). This consists of an oligolysine backbone from which the epitope-containing, 10 and 20 amino acids long branch out. In mice, a high degree of protection against challenge with P. *berghei* sporozoites was obtained by immunisation with MAP containing multiple copies of the immunodominant B cell epitope and a selected T_H epitope of the corresponding CS protein (Tam *et al.*, 1990). The problem of genetic restriction could be overcome by the inclusion of universal T cell epitopes in the vaccine (Calvo-Calle *et al.*, 1993).

Another approach was the incorporation of different epitope sequences in one construct of immunogens that are linear oligopeptides or polymers known as multiple antigenic constructs, (MAC). Studies by Lal (1996a) in rodent malaria models showed that mice immunised with *P. berghei* and *P. yoelii* CS MAC vaccine induced sterile immunity. Further studies demonstrated that MAC containing CS repeat epitopes of *P. falciparum* and *P. vivax*-like human malaria parasite induced long-lasting high titre antibodies in mice against both epitope (Lal, 1996a). In additional studies, MAC for *P. falciparum* antigenic peptides of the CSP, AMA-1, MSP-1, MSP-2, RAP-1, EBA-175, TRAP/SSP2 proteins were prepared with plasmodial T-cell determinants. Immunisation in mice in the presence of potentially human usable non-ionic block

copolymer adjuvant elicited antibodies that blocked growth of two different falciparum strains (Lal, 1996a).

Another form of a multicomponent and multistage vaccine has been described whereby yeast-expressed Pf25, CSP, N-terminal regions of MSP-1, and serine repeat antigen (SERA) were mixed as a vaccine cocktail (Bathurst *et al.*, 1993).

Select epitopes of malaria parasite antigens can be utilised in neutralisation of the infectivity of the human malaria parasite; hence an efficacious multistage malaria vaccine can be developed.

1.1.6.5.2. SPf66

SPf66 was the first chemical synthetic multicomponent *P. falciparum* peptide vaccine. It is an oligomeric construct containing three merozoite-derived epitopes. It combines vaccine candidate antigens from both pre- and intra-erythrocytic stages. It contains the N-terminus of MSP1, and two of other merozoite-specific protein fragments of 35-kDa, 55-kDa and 83-kDa blood stage proteins linked by two copies of the CS-protein repeat NANP all of which had been shown to be protective in a model infection, developed by Pattaroyo *et al.* (1988). This construct induced protection in *Aotus trivirgatus* monkeys against challenge with *P. falciparum* (Pattaroyo *et al.*, 1988). Field trials performed worldwide have been disappointing (D'Alessandro *et al.*, 1995; Nosten *et al.*, 1996; Amador *et al.*, 1992; Pattaroyo *et al.*, 1992; Rocha *et al.*, 1992). Clinical trials in Tanzania, reported only 31% efficacy for protection against clinical malaria episodes, no difference in the parasite prevalence between the vaccinated and control groups, and no influence on the haematocrit values (Allonso *et al.*, 1994). The efficacy result of SPf66 is very difficult to project into practical use since it is far less than that of any other human vaccine in use, furthermore, an insufficient efficacy may prove deleterious since it may just be enough to give rise to

resistant strains (Hogh, 1996).

1.1.6.5.3. NYVAC Pf-7

NYVAC Pf-7 is a vaccinia virus-based multistage *P. falciparum* vaccine, containing 7 stage-specific antigens. The vaccine preparation consists of an attenuated NYVAC vaccina virus strain that expresses genes coding for proteins expressed during the sporozoite (CSP, PfSSP2), liver (LSA1), blood (MSP1, SERA, AMA1), and sexual (Pf25) stages of the parasite's life cycle (Tine *et al.*, 1996). In phase I/IIa trials, cellular immune responses were detected in >90% of the volunteers but antibody responses were poor. Of the 35 individuals challenged, only one was completely protected, although there was a significant delay until the onset of parasitaemia (Ockenhouse *et al.*, 1998).

1.1.6.5.4. 15 CTL epitope vaccine

Recent studies have investigated the protective effects of a multivalent vaccine formulation against the exoerythrocytic stage of the parasite (liver stages), in which 15-plasmodial CTL epitopes and a B-cell epitope were included. Challenge of a rodent model with this construct, induced protective CTL responses (Gilbert *et al.*, 1997).

1.1.6.5.5. FAL VAC-1

FAL VAC-1 is a 41-kDa multi-stage and multi-valent *P. falciparum* vaccine developed using synthetic gene approach (Shi *et al.* 2000). It contains 12 B-cell and 6 T-cell proliferative, and 3 cytotoxic T lymphocyte (CTL) epitopes derived from 9 stage-specific antigens of various stages of parasite, and is expressed in *Baculovirus* expression vector system (BVES) (Shi *et al.* 2000). All antigens included in the vaccine were identified through immunoepidemiological studies in malaria endemic areas, *in vitro* and *in vivo* protection studies in model systems (Table 1).

Immunisation studies in rabbits using different adjuvant showed that

this vaccine is immunogenic, and the vaccine-induced antibodies recognised vaccine construct, linear peptides contained in the vaccine, and all stages of *P. falciparum. In vitro* protective studies demonstrated that the vaccine-induced antibodies inhibit the development of blood stage parasites in the presence of monocytes (ADCI) and inhibit the invasion of sporozoites into liver cells (Shi *et al.*, 1999a).

These observations demonstrate that a multicomponent multistage malaria vaccine can induce immune responses that inhibit parasite development at multiple stages. For this reason it was important to carry out studies to delineate the nature of natural immune responses to this vaccine candidate and association of the immune responses and clinical protection to malaria before human field trials can be carried out.

TABLE 1: ANTIGENS AND EPITOPES IN FAL VAC-1 CONSTRUCT OF *P. FALCIPARUM*

Sequences	Stage	Antigens and epitopes[2]
KPKHKKLKQPGDGNP	Sporozoite	CSP-B
WSPCSVTCG	"	SSP2-B
KPKDELDYENDIEKKICKMEKS	"	CSP-CTL
DIEKKICKMEKCSSVFNVVNS	"	CSP-CS.T3
NSGCFRHLDEREECKCLL	Blood stage	MSP1-B
EDSGSNGKKITCECTKPDS	"	MSP1-B
KPIVQYDNF	Liver stage	LSA1-CTL
3XNANP	Sporozoite	CSP-B
DGNCEDIPHVNEFSAIDL	Blood stage	AMA1-B
GNAEKYDKMDEPQHYGKS	"	AMA1-B
LTPLEELY	"	RAP1-B
KPNDKSLY	Liver stage	LSA1-CTL
QYIKANSKFIGITEL	Tetanus toxoid	P2-T
SNTFINNA	Blood stage	MSP2-B
GQHGHMHG	"	MSP2-B
NEREDERTLTKEYEDIVLK	"	EBA175-B
EFTYMINFGRGQNYWEHPYQKS	"	AMA1-T
DQPKQYEQHLTDYEKIKEG	"	AMA1-T
KPLDKFGNIYDYHYEH	Gametocyte	Pfg27-B
SSPSSTKSSPSNVKSAS	Blood stage	RAP1-T
LATRLMKKFKAEIRDFF	"	RAP1-T
GISYYEKVLAKYKDDLE	"	MSP1-T
Reference	(Shi *et al.*, 2000)	

1: Amino acid sequence of epitopes incorporated into FAL VAC-1 is presented in the first column and indicated by the codes. The second column shows the stage specific antigens used in the construct.**2:** Abbreviations: CSP, circumsporozoite protein; SSP2, sporozoite surface protein-2; LSA-1, liver stage antigen-1; MSP-1, merozoite surface protein-1; MSP-2, merozoite surface protein-2; AMA-1, apical membrane antigen-1; EBA-175, erythrocyte binding antigen-175; RAP-1, Rhoptry associated protein-1; Pfg27, gametocyte 27 kDa antigen, P2, tetanus toxoid universal T cell epitope.

1.1.7. RATIONALE OF THE STUDY

In preparation for evaluating the efficacy of FAL VAC-1 a candidate *P. falciparum* multistage multicomponent vaccine in human field trials, it is essential to investigate the natural immune responses to the candidate vaccine antigen, and to determine the association between the specific immune responses and clinical protection against malaria. This investigation of quality and quantity of lymphocyte proliferative and antibody responses to the multistage and multivalent vaccine, FAL VAC-1 of *P. falciparum* in children less than two years and in adults was meant to delineate and generate information on the characteristics of naturally acquired immunity against the vaccine and the possible relationships between the specific immune responses and clinical protection against malaria. All individuals were tested for cellular and humoral immune responses since both cell-mediated and antibody-dependent immune mechanisms contribute in mediating antimalarial immunity (Marsh, 1994). Since total IgG may not be the most suitable measurement of immunity (Bouharoun-Tayoun and Druilhe, 1992), humoral responses were analysed with respect to IgG isotypes. The information generated from this study will be useful in future malaria vaccine design, development and trials.

1.1.8. OBJECTIVES OF THE STUDY

1.1.8.1. General objective

To characterise the natural immune responses to the candidate vaccine antigen, FAL VAC-1 of *P. falciparum*, and their association with clinical protection against malaria in a holoendemic area of western Kenya.

1.1.8.2. Specific objectives

1.1.8.2.1. To determine lymphocyte proliferative responses to the vaccine candidate.

1.1.8.2.2. To delineate antibody (IgM, total IgG and IgG subclass) responses to the vaccine candidate.

1.1.8.2.3. To determine the association between the natural immune responses to the vaccine candidate and clinical immunity to malaria.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Study area

This cross-sectional study was conducted between April and September 1999 as part of the Centers for Disease Control and Prevention (CDC)/KEMRI, Asembo Bay Cohort Project (ABCP). The major study is designed to address the epidemiology, entomology, parasitology, immunology and molecular biology of malaria in western Kenya (Bloland *et al.*, 1999a). The location of the study site is shown in Figure 2.

2.2. Malaria in the study area

This area is holoendemic for malaria with two peak transmission seasons after the long rainy season from (March to May) and the short rainy season from (October to December). Entomologic inoculation rates as high as 300 infective bites per year have been recorded and *P. falciparum* accounts for about 90% of the malaria infections in the area (Beier *et al.,* 1990). In this high transmission area, children less than 2 years of age experience the greatest morbidity and mortality from malaria, and severe anaemia is the major cause of malaria-related mortality (Bloland *et al.*, 1999b; McElroy *et al.,* 1999).

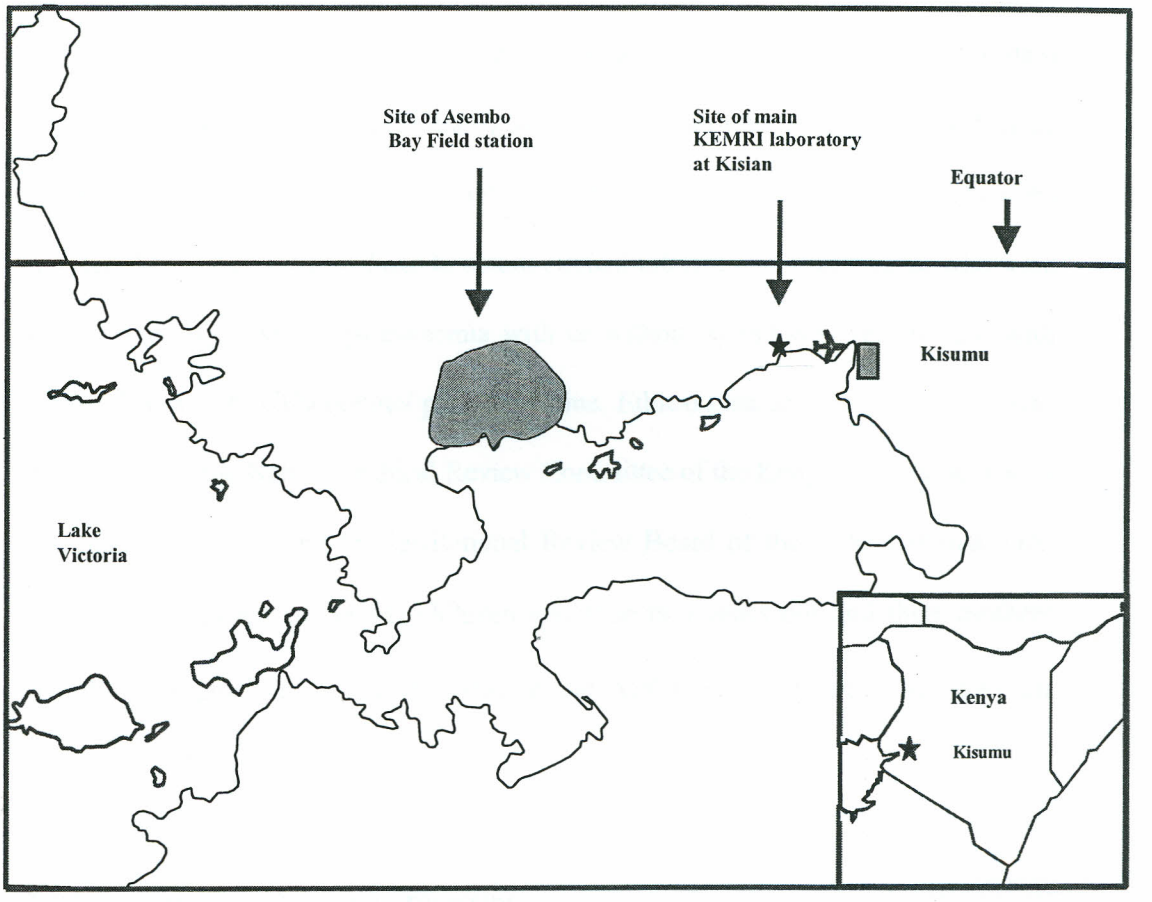


Fig. 2: Location of study site in western Kenya. KEMRI=Kenya Medical Research Institute.

2. 3. Study subjects and ethical clearance

The study area and methods of ABCP have been described in detail elsewhere (Bloland *et al.*, 1999). Ninety-five percent of the population in the Asembo Bay area belongs to the Luo ethnic group. Briefly, the study individuals for the ABCP were identified through a monthly household census, and after obtaining informed written and oral consent (details in appendix 6), women, and children under the age of 5 years were enrolled in the study. The infants were followed-up twice a month from birth through the first five years of age to obtain clinical information. Participants who were febrile with any level of parasitaemia with or without symptoms were treated with standard doses of sulphadoxine/ pyrimethamine. Ethical clearance for the ABCP was obtained from the National Ethical Review Committee of the Kenya Medical Research Institute (K.E.M.RI) and the Institutional Review Board of the CDC (Atlanta, GA, USA). One hundred and eighty children less than two years old and their mothers (N=139, pregnant excluded) enrolled in the ABCP were randomly selected for this cross-sectional study.

2.4. METHODS

2.4.1. Sample collection and processing

Routine blood samples were collected by monthly finger prick or whenever there was a report of a febrile illness (i.e. auxiliary temperature $\geq 37.5^{o}C$). A blood sample was obtained for thick and thin blood smears which were examined for malaria parasites by microscopy, and for haemoglobin determination by haemocue (Angelholm, Sweden). Half a millilitre and 1 ml finger prick blood samples were collected from children and adults respectively, into heparinised tubes. The blood samples were transported at $4^{o}C$ to the Kisian laboratory within 6 hours of collection. The blood samples were spun in a microcentrifuge (Micro 7, Fischer

Scientific, Pittsburgh, U.S.A.) at 7000 r.p.m (g=3.5) for 10 minutes and the plasma obtained was stored in aliquots at -20°C until antibody detection. The remaining pellet was diluted with an equal volume of sterile phosphate buffered saline (PBS, pH 7.20) and PBMCs isolated from it by density gradient centrifugation (Sorvall Centrifuge RT 6000D, Dupont Newton, Connecticut, U.S.A) over Ficol Hypaque at 1400 r.p.m (g=220) for 30 minutes. The PBMCs were then washed twice in PBS by spinning at 1400 r.p.m. for 15 minutes. After the final wash, the cells were resuspended in RPMI 1640 (Gibco-BRL, Grand Island, N.Y., USA) medium supplemented with 5% inactivated human AB+ serum (donors not previously exposed to malaria), 5% Foetal Bovine Serum (Gibco-BRL, Grand Island, N.Y., USA), 2mM L-glutamine, 100 U/ml of penicillin and 100µg/ml of streptomycin (Gibco-BRL, Grand Island, NY, USA). The viability of the cells was determined using Trypan blue dye exclusion test. The cells were stained by Turk's solution and the number of PBMCs enumerated by microscopy using a haemocytometer.

2.4.2. Lymphoproliferative assays

Lymphocyte proliferation was assayed as previously described by Udhayakumar *et al.*, (1995) with minor modifications. Briefly, 5 x 10^4 PBMCs in 200 µl of complete tissue culture medium were set in triplicates in a 96-well round-bottomed microtitre plates (Costar, Cambridge, Mass., USA) and stimulated with FAL VAC-1 recombinant protein antigen at concentrations of 0.001, 0.01, 0.1, 0.5, 1, 2.5 and 5 µg/ml, (Protein Sciences Corp., Meriden, USA). Five µg/ml of phytohaemagglutinin (PHA) a mitogen, which is known to non-specifically activate human T cells (Sigma chemicals Co., St. Louis, Mo., USA), 1 µg/ml of anti-CD3 Mab, anti-CD3 monoclonal antibody that activates human T cells by binding and cross linking through the CD3 molecule, (Pharmingen, San Diego, USA) and 0.05 µg/ml

tetanus toxoid (tt) peptide QYIKANSKFIGITEL, which is a universal T cell epitope for augmenting the immunogenicity of B cell epitopes were used as positive controls and unstimulated cells as a negative control in this *in vitro* cellular assay. The stimulated cells were then cultured at 37° C in humidified incubator (Forma Scientific Inc., Marietta, USA) with 6% CO_2. After 5 days of culture, 150 µl of culture supernatant from each well was collected into tubes and stored at -70° C for cytokine detection. An equal volume of RPMI 1640 was added to replace the supernatant removed and then wells were pulsed with 1 µCi/well of tritiated methyl-thymidine, (Dupont NEN Research Products, Boston, MA.). After 16-18 hours, cells were harvested with a cell harvester (Skatron, Norway) onto filter mats, and radioactive uptake by the cell's nuclei measured using a scintillation counter (Beckman Instruments, LS 5801, California, USA). Stimulation indices were calculated according to the following formula: geometric mean stimulation indices (SI) = geometric mean counts per minute in the triplicate test cultures / geometric mean counts per minute in unstimulated triplicate control cultures.

2.4.3. Antibody ELISA

Dynatech Immulon II plates (IMMULON 2, Dynatech Laboratories, McLean, VA, USA) were coated with 100 µl/well of clinical grade recombinant protein (FAL VAC-1) at a concentration of 0.5 µg/ml in borate buffered saline (BBS), pH 8.0, and incubated overnight at 4°C. The plates were then washed twice by incubating for 3 minutes at each wash with PBS, pH 7.2 containing 0.05% Tween-20 (wash buffer) to remove unbound antigen. The plates were then blocked with 100 µl of blocking buffer (wash buffer containing 0.1% non-fat milk) at room temperature for 1 hour and then washed 3 times with wash buffer. One hundred microlitres of 1:50 diluted positive controls (hyperimmune sera) and plasma samples in dilution buffer (wash buffer +

2.5% non-fat milk) were then added. After incubating at room temperature for 1 hour, the plates were washed 4 times with wash buffer. Goat anti-human IgG HRP conjugate, (Alpha Diagnostics International, USA) and goat anti-human IgM mu chain HRP conjugate (TAGO, Burlingame, CA, USA) diluted at 1:10,000 and 1:6,000, respectively, were added to each well then incubated at room temperature for 1 hour followed by washing 4 times with wash buffer. For IgG isotypes, 100µl primary mouse anti-human IgG subclass monoclonal antibody preparation were added at the following dilutions: IgG1 and IgG2 at 1:6000; IgG3 at 1:50,000; and IgG4 at 1:20,000 (Division of Parasitic Diseases, NCID, CDC, Atlanta, GA, USA) and incubated at room temperature for 1 hour, then washed 3 times with wash buffer. This was followed by addition of 100 µl goat anti-mouse HRP-conjugate (Biosource International, California, USA) at 1:10,000 dilution and incubated at room temperature for 1 hour. After incubation the plates were washed 4 times with wash buffer. One hundred microlitres of substrate i.e.1:1 H_2O_2 and tetra-methylbenzidine, TMB (Kirkegaard and Perry Laboratories, Maryland, USA) were added to each well and incubated for 15 minutes followed by 100 µl of stop solution (1M H_3PO_4). The plates were then read at 450 nm in an ELISA reader (Dynatech, Virginia, USA) and the optical density (O.D.) recorded. The geometric mean O.D. + 2 standard deviations (2SDs) of normal control serum of individuals previously not exposed to malaria from Atlanta, Georgia, U.S.A. were used as the cut-off.

2.4.4. Statistical procedures

Data processing and statistical analyses were done by SPSS for windows version 9.0 (SPSS, Inc., Chicago I11). Parasitaemia, antibody levels and SI were first transformed into natural logarithm (ln) before being used in the analyses. Associations between immunological parameters and haemoglobin levels or clinical endpoints of

malaria (incidences of parasitaemia, high density parasitaemia, fever, episodes of clinical malaria, anaemia and severe anaemia) were correlated by the Pearson's correlation test. The parasite rates were derived by the χ^2 test. One-Way ANOVA was used to test the trend in humoral and cellular responses in the study groups. The antibody levels and proliferation were compared between parasitaemic and aparasitaemic subjects by the Student t-test. All the tests were two-tailed and p values $\leq$ 0.05 considered statistically significant.

CHAPTER THREE: RESULTS

3.1. Study groups

One hundred and eighty children less than two years and their non-pregnant mothers (mean age range 15-48 years) were enrolled into the study. The study subjects were stratified as shown in Table 2. Table 2 also shows the mean age, parasite rate and density, and haemoglobin levels at the time of enrolment for each age group. The age, parasitological and haematological variables were significantly different among the different age groups. In addition, longitudinal clinical follow-up parameters were available for 100 children aged 13-24 months and 79 mothers. These data provided useful information on the functioning of the immune system at different time points. Table 3 shows the mean parasitological and clinical variables for these subjects. The parasitological and clinical variables were significantly different between the groups.

TABLE 2: CHARACTERISTICS OF THE STUDY GROUPS

Age group	*N*	*Parasite rate*	*Parasitaemia*	*Hb levels*
0-6mon	48	0.21	4.37 ± 0.50	10.54 ± 0.35
7-12mon	33	0.39	20.98 ± 0.72	9.51 ± 0.53
13-18mon	44	0.23	4.47 ± 0.49	6.49 ± 0.63
19-24mon	55	0.26	6.39 ± 0.49	7.49 ± 0.56
15-25 years	64	0.14	1.08 ± 0.24	10.10 ± 0.40
26-48 years	75	0.07	0.28 ± 0.11	10.43 ± 0.31
P value	< 0.001	< 0.001	< 0.001	< 0.001

The grouping of the study subjects was based on the age-dependent build-up of immunity in malaria endemic areas (Marsh, 1992). In addition, children under 6 months are protected by passively acquired IgG from the mother (Carlier and Truyens, 1995). Age (months) for young children and (years) for adults. Haemoglobin (Hb) levels in g/dl are given as means ± s.e.m while the parasite density (parasitaemia) is given as geometric mean/µl ± s.e.m. The parasite rate was defined as the number of positive subjects/total number of subjects in the group. The trend in age, parasitaemia, and Hb levels for the study groups was determined by the one-way ANOVA test while variance in the parasite rates was derived from the Chi-square test.

TABLE 3: PARASITOLOGICAL AND CLINICAL CHARACTERISTICS OF SUBJECTS FROM ASEMBO BAY

	Children (n=100)	*Adults (n=79)*	
	Mean ± s.e.m	Mean ± s.e.m	P value
Parasitaemia a month prior to sampling	10.59 ± 0.45	0.73 ± 0.20	< 0.001
Parasitaemia 1 month after sampling	0.86 ± 0.20	0.38 ± 0.14	< 0.001
Incidence of parasitaemia	0.26 ± 0.02	0.11 ± 0.01	< 0.001
Incidence of high density parasitaemia	0.11± 0.01	0.00 ± 0.00	< 0.001
Episodes of clinical malaria	0.06 ± 0.01	0.02 ± 0.00	< 0.001
Incidence of fever	0.10 ± 0.08	0.02 ± 0.00	< 0.001
Hb levels 1 month after sampling	10.27 ± 0.23	11.31 ±0.30	< 0.001
Incidence of anaemia	0.52 ± 0.02	0.43 ± 0.03	< 0.001
Incidence of severe anaemia	0.08 ± 0.01	0.03 ± 0.01	< 0.001

The incidences of malaria clinical indices, which represented an individuals' experience with a malaria infection, were estimated by calculating the ratio between the number of follow-ups and the duration of the follow-up in months, both children and mothers were followed-up after delivery, Hb = haemoglobin, Fever = auxiliary temperature $\geq 37.5^{\circ}C$, Anaemia was defined as a Hb concentration < 11g/dl and severe anaemia as = Hb < 5 g/dl as recommended by C.D.C, 1989 (Atlanta, Georgia, U.S.A.), malaria episode = presence of both parasitaemia and fever, and p value for differences between children and adults derived from Student t test.

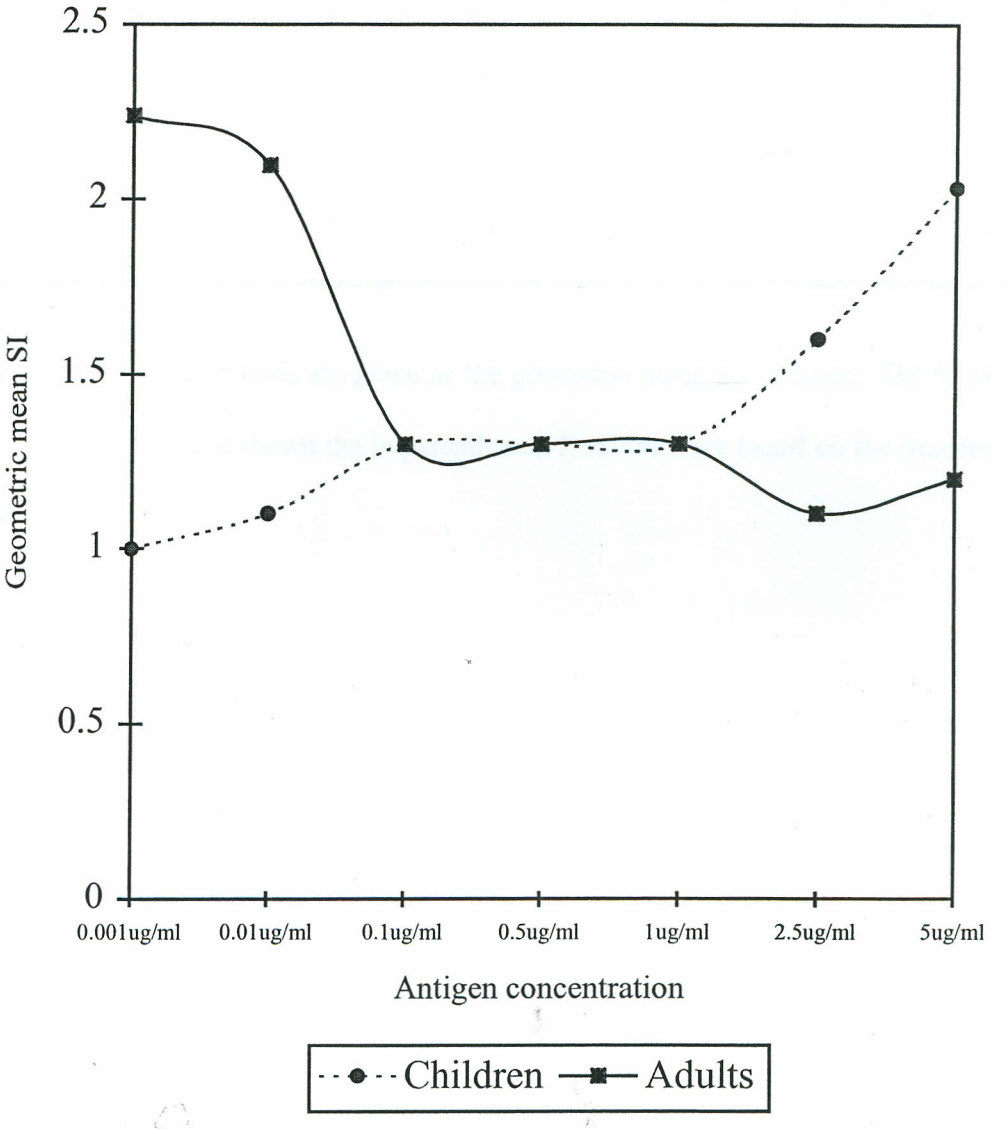
3.2. Lymphoproliferative response to FAL VAC-1

The viability of the cell preparations used in lymphoproliferation assays > 95%.

3.2.1 Antigen concentration dependent lymphoproliferative response

Although both children and adults had similar lymphoproliferative responses at 0.1 μg/ml, 0.5 μg/ml, and 1 μg/ml there was variation in the pattern of response to different concentrations (0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 0.5 μg/ml, 1 μg/ml, 2.5 μg/ml and 5 μg/ml) of FAL VAC-1 antigen in the different age groups (Fig. 3). Peripheral blood lymphocytes isolated from adults responded at lower concentrations, while those from children responded at higher concentrations.

FIGURE 3: ANTIGEN CONCENTRATION DEPENDENT LYMPHOPROLIFERATIVE RESPONSE TO FAL VAC-1



*Five children and adults each were used to plot the lines.

TABLE 4: COMPARISON OF LYMPHOPROLIFERATIVE RESPONSES IN CHILDREN AND ADULTS

Stimulant	Children (Positive responses)	Adults (Positive responses)	P value
PHA	7.91 ± 0.077 **(86)**	12.91 ± 0.094 **(90)**	0.000
Anti-CD3	2.20 ± 0.055 **(30)**	1.98 ± 0.065 **(31)**	0.407
tt	1.78 ± 0.030 **(21)**	1.54 ± 0.040 **(14)**	0.079
FAL VAC-1	2.10 ± 0.027 **(34)**	2.01 ± 0.027 **(30)**	0.396

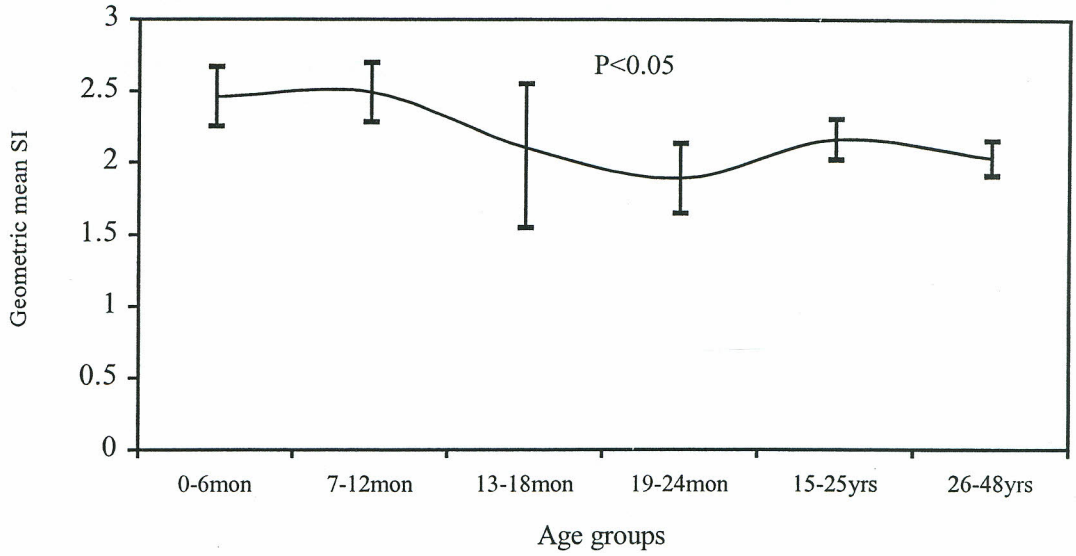
The proliferative responses are given as the geometric mean S.I. ± s.e.m. The % of positive responders is shown the in parentheses. P values were based on the Student t- test.

3.2.2 Prevalence and level of lymphoproliferative response

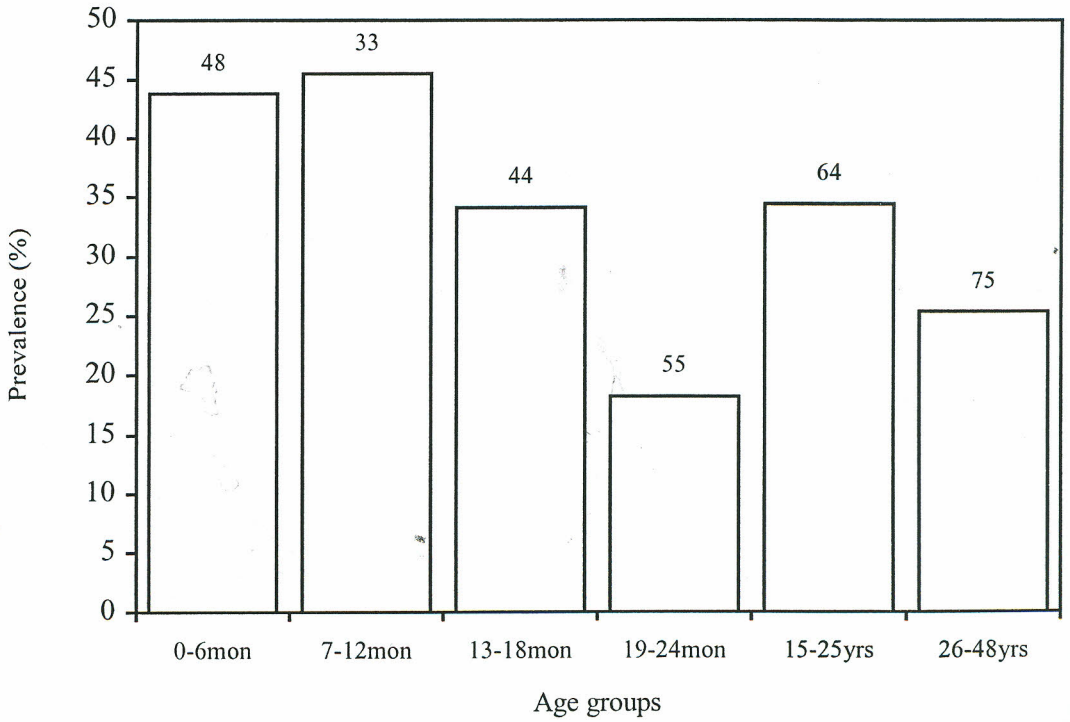
Thirty four percent of children and 30% of adults had positive proliferative responses to FAL VAC-1 antigen. The mean proliferative responses to PHA were significantly higher in adults but proliferative responses to anti-CD3 were similar in both children and adults. On the other hand, the proliferative responses to tt were higher in children than adults. However, when the lymphoproliferative responses were stratified by age, it was found that the levels of lymphoproliferative responses (expressed as the geometric mean SI ± s.e.m) generally decreased with age (one-way ANOVA trend test = $P < 0.05$; Fig. 4A). The geometric mean SI for children ranged from 2.46 ± 0.21 in infants aged 0-6 months, to 1.89 ± 0.24 in children 19-24 months old. Young adults (15-25 year olds) had a geometric mean SI of 2.16 ± 0.14 while older adults (26-48 year olds) had a geometric mean SI of 2.03 ± 0.12 (Fig. 4A). The lymphoproliferative responses varied between 46% in children aged 7-12 months to 25% in adults aged 26-48 years with lowest responses of 18% observed in children aged 19-24 months (Fig. 4B). More than 77 % of the study subjects had positive responses to PHA with a stimulation index greater than 2.5 while less than 37 % of the study individuals had positive proliferative responses to anti-CD3 (Fig. 5A). Most of the study subjects had less than 21% positive proliferative responses to tt except children aged less than 7 months who had 35% positive responses as shown in Figure 5A. The levels to these controls are also shown in figure 5B-D. The lymphoproliferative responses to PHA significantly increased with age (one-way ANOVA trend test = $P < 0.05$; Fig. 5B). There was no variance in lymphoproliferative responses to anti-CD3 monoclonal antibody or tt (Fig. 5C and 5D).

FIGURE 4: LYMPHOPROLIFERATIVE RESPONSES TO FAL VAC-1 ANTIGEN IN CHILDREN AND ADULTS

4.A. Level



4.B. Prevalence

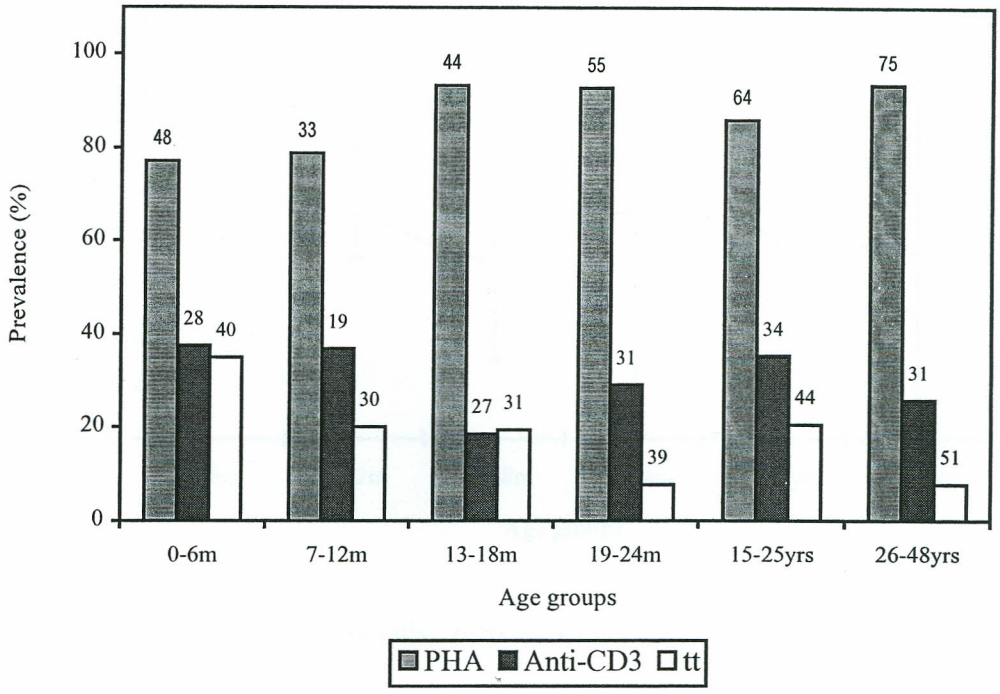


Individuals who showed a stimulation index (SI) > 2.5 for any one of the

concentrations of the antigen were recorded as positive responders. The prevalence of lymphoproliferative responses was expressed as a percentage of positive responses. The number of subjects for each age group is shown on top of bars. The level is given as the geometric mean ± s.e.m while the prevalence is given as the % of positive responses for each age group.

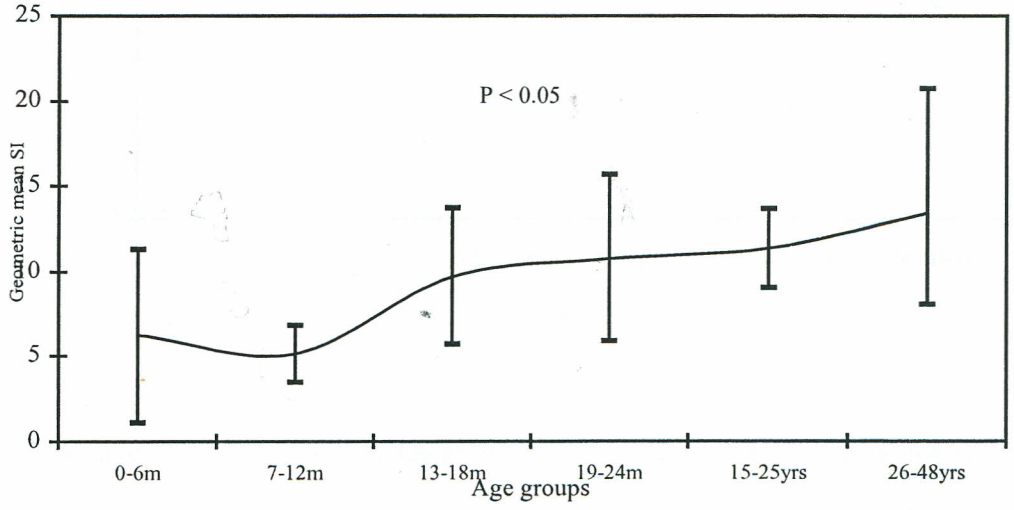
FIGURE 5: LYMPHOPROLIFERATIVE RESPONSES TO PHA, ANTI-CD3 AND TT

5.A. Prevalence of PHA, anti-CD3 and tt positive responses



The number of subjects for each age group for the controls is shown on top of bars for PHA, Anti-CD3 and tt.

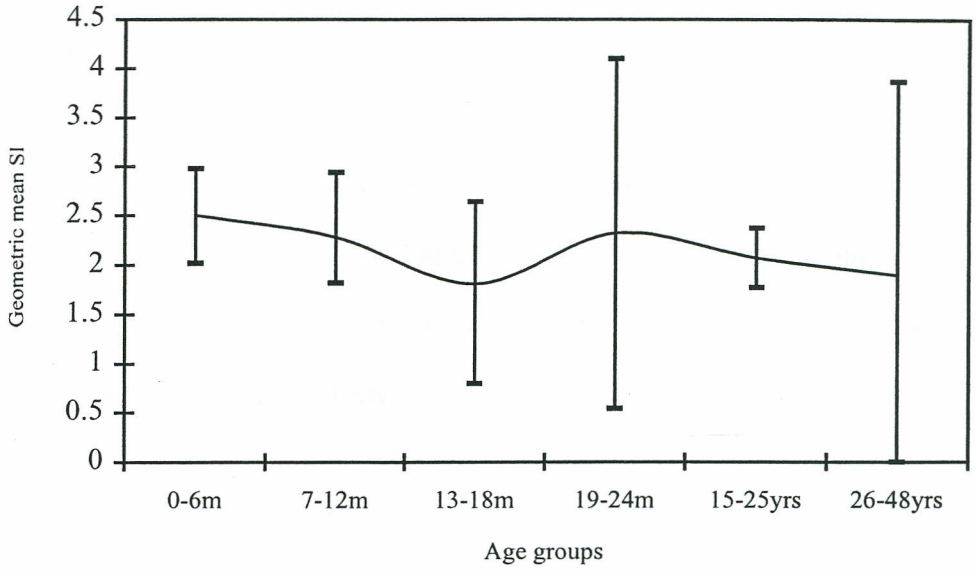
5.B. Level of lymphoproliferative responses to PHA



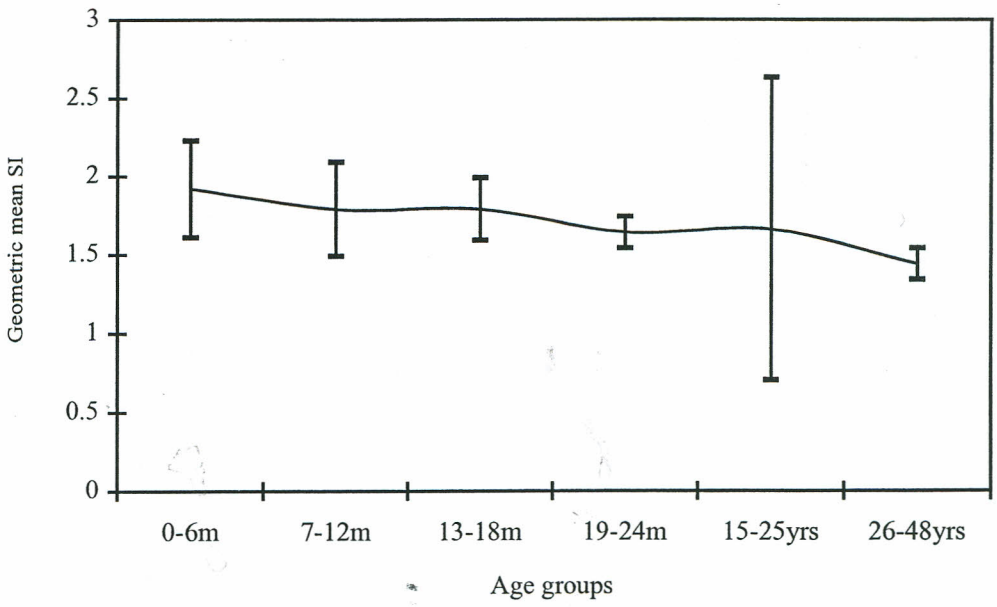
The level lympoproliferative response to PHA is given as the geometric mean SI

with 95% confidence interval for mean for each group.

5.C. Level of lymphoproliferative responses to Anti-CD3



5.D. Level of lymphoproliferative responses to tt



The number of subjects for each age group for the controls is shown for (anti-CD3, tt); 0-6 months (24, 40), 7-12 months (19, 30), 13-18 months (27, 31), 19-24 months

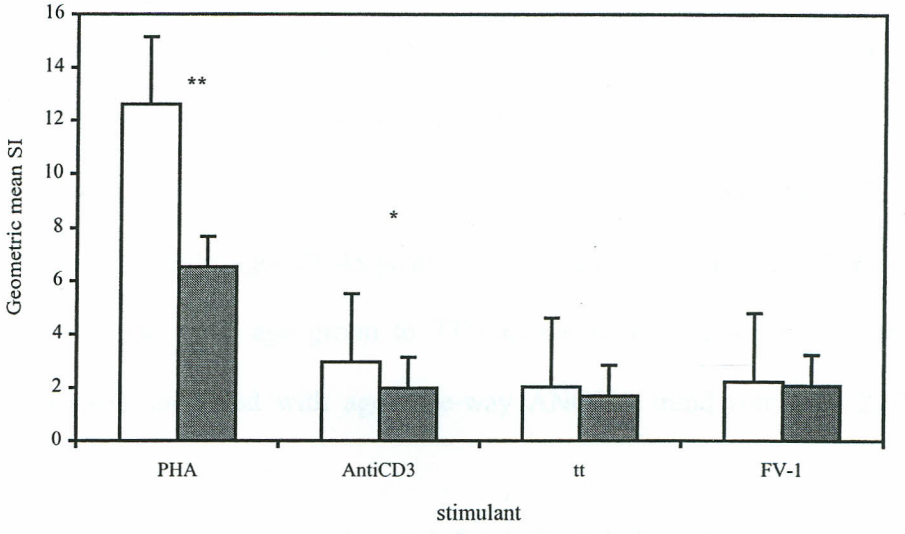
(31, 39), 15-25 years (34, 44) and 26-48 years (31, 51).

3.2.3. Relationship between lymphocyte proliferation and parasitaemia

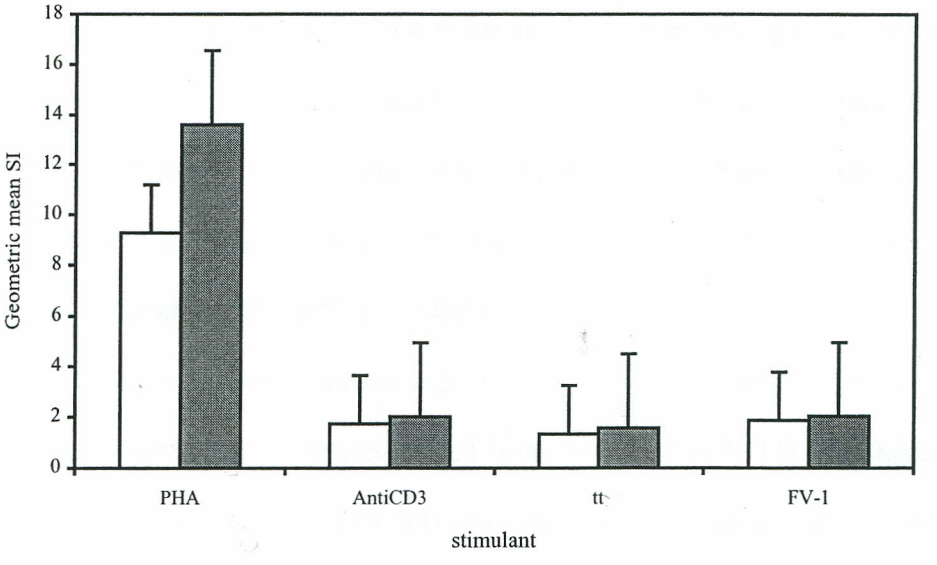
The magnitude of lymphoproliferative responses to FAL VAC-1 was not associated with parasitaemia either in children or in adults from the study area. (Fig. 6). However, lymphoproliferative responses to PHA and anti-CD3 were significantly higher in parasitaemic children than non-parasitaemic children, p=0.002 and p=0.021 respectively (Fig. 6). In adults there was no relationship with respect to lymphoproliferation to any of the stimulants, p=0.279, p=0.569 and p=0.492 for PHA, anti-CD3 and tt respectively.

FIGURE 6: COMPARISON OF LYMPHOPROLIFERATIVE RESPONSES IN PARASITAEMIC AND APARASITAEMIC CHILDREN AND ADULTS

6.A. Children



6.B. Adults



Differences between groups derived from student t-test are given as the geometric mean SI ± s.e.m, □ parasitaemic, ■ aparasitaemic, children (n=177), adults (n=136), children; parasitaemic=46, aparasitaemic=131, adults; parasitaemic=14, aparasitaemic=122, FV-1 = FAL VAC-1, *P<0.05, **P<0.01.

3.3. Antibody responses to FAL VAC-1

The results of the antibody levels (geometric mean O.D.s) and prevalence (%) in children and adults are summarised in Table 5 and Figure 7. These results showed that in both children and adults total IgG, IgG1, IgG3 and IgM were the predominant antibodies. IgG2 subclass levels were low, while IgG4 was not detectable in any of the study samples. The geometric mean total IgG antibody levels varied from 0.040 ± 0.010 O.D.s in children 7-12 months old to 0.203 ± 0.016 O.D.s in older adults aged 26-48 years old while the prevalence ranged from 16% in children of the same age group to 73% in the older adults. The levels of IgG seroreactivity increased with age (one-way ANOVA trend test; $F = 22.35$, $P < 0.001$).

A similar trend was observed for IgG1 subclass response; the geometric mean O.D.s of FAL VAC-1 specific IgG1 ranged from 0.014 ± 0.007 in children less than 7 months old to 0.075 ± 0.008 in older adults whereas the prevalence of IgG1 ranged from 11% in children 13-18 months old to 52% in older adult test plasmas. Analysis for a relationship between the different various age groups showed a significant increase with age with respect to this subclass (one-way ANOVA trend test; $F = 9.59$, $P < 0.001$).

IgG2 subclass responses to FALVAC-1 similarly varied with age (Fig. 7). The geometric mean responses ranged from 0.004 ± 0.003 O.D.s in children 7-12 months of age to 0.045 ± 0.005 O.D.s in older adults. The prevalence of FAL VAC-1 specific IgG2 ranged from 0% in children 7-12 months old to 21% in older adults. These responses were significantly different between the age groups (one-way ANOVA trend test; $F = 14.46$, $P < 0.001$).

The seroreactivity to IgG3 subclass also varied with age (Fig. 7). The

geometric mean O.D.s ranged from 0.010 ± 0.008 in children of age 7-12 months to 0.131 ± 0.022 in the older adults. The prevalence ranged from 13% in the 7-12 months age group to 80% in the older adults. In addition, the seroreactivity to this antibody significantly increased with age (one-way ANOVA trend test; $F = 8.23$, $P < 0.001$). Children aged 0-6 months had slightly higher prevalence and levels of total IgG and IgG3 antibodies due to passively transferred antibodies from the mother (Fig. 7).

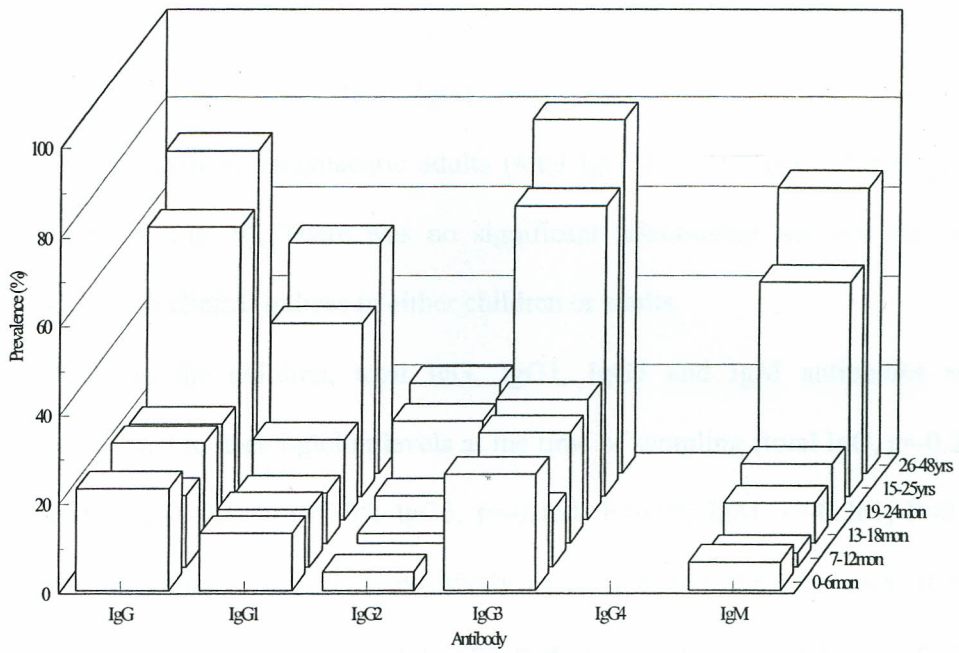
The polyvalent IgM antibody responses to FAL VAC-1 also varied with age in these subjects naturally exposed to malaria (Fig. 7). The geometric mean O.D.s of FAL VAC-1 specific IgM ranged from 0.019 ± 0.004 in children less than 7 months to 0.095 ± 0.006 in older adults residing in this malaria holoendemic area of western Kenya. The prevalence ranged from 3% in children of age 7-12 months to 64% in older adults. Analysis for differences with age indicated that IgM responses significantly increase with age (one-way ANOVA trend test; $F = 40.56$, $P < 0.001$).

TABLE 5: ANTIBODY RESPONSES AMONG CHILDREN AND ADULTS

	Antibody				
Age group (n)	IgG (Positive responders)	IgG1 (Positive responders)	IgG2 (Positive responders)	IgG3 (Positive responders)	IgM (Positive responders)
0-6mon	0.046 ± 0.011	0.014 ± 0.007	0.006± 0.003	0.044 ± 0.016	0.019 ± 0.004
(48)	**(23)**	**(13)**	**(4)**	**(26)**	**(6)**
7-12mon	0.040 ± 0.010	0.019 ± 0.011	0.004± 0.003	0.010 ± 0.008	0.025 ± 0.004
(31)	**(16)**	**(13)**	**(0)**	**(13)**	**(3)**
13-18mon	0.081 ± 0.015	0.039 ± 0.009	0.009 ± 0.006	0.031 ± 0.010	0.048 ± 0.005
(44)	**(23)**	**(11)**	**(2)**	**(25)**	**(9)**
19-24mon	0.078 ± 0.014	0.032 ± 0.005	0.013± 0.006	0.030 ± 0.011	0.049 ± 0.005
(55)	**(22)**	**(18)**	**(6)**	**(27)**	**(13)**
15-25	0.181 ± 0.016	0.066 ± 0.011	0.038 ± 0.005	0.106 ± 0.020	0.088 ± 0.006
years (64)	**(61)**	**(39)**	**(17)**	**(66)**	**(48)**
26-48	0.203 ± 0.016	0.075 ± 0.008	0.045 ± 0.005	0.131 ± 0.022	0.095 ± 0.006
years (73)	**(73)**	**(52)**	**(21)**	**(80)**	**(64)**
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

The antibody responses are given as the geometric mean O.D.s ± s.e.m. Percentage of responders in parentheses. n = number of subjects tested. P values based on one-way ANOVA trend test.

FIGURE 7: PREVALENCE OF ANTIBODIES TO FAL VAC-1 ANTIGEN IN CHILDREN AND ADULTS



The number of subjects for each age group is shown in parenthesis; 0-6 months (48), 7-12 months (31), 13-18 months (44), 19-24 months (55), 15-25 years (64) and 26-48 years (73).

3.4. Association between antibody responses with parasitaemia and haemoglobin levels

Parasitaemic children had higher levels of antibodies, but only IgG1 antibody was significantly higher in parasitaemic than in aparasitaemic children at the time of sampling (IgG1, $P < 0.01$, t-test; Fig. 9). In addition, IgG1 levels were significantly higher in parasitaemic children than non-parasitaemic children at a month prior to sampling (IgG1, $P < 0.05$, t-test; Fig. 8). Though, aparasitaemic adults had higher antibody levels only total IgG, IgG1 and IgM were significantly higher in aparasitaemic than in parasitaemic adults (total IgG, $P<0.01$; IgG1, $P<0.05$; IgM, $P<0.01$; t-test; Fig. 9). There was no significant relationship between the other antibodies with clinical indices in either children or adults.

Among the children, total IgG, IgG1, IgG3 and IgM antibodies were inversely related to haemoglobin levels at the time of sampling (total IgG, $r=-0.215$, $P<0.01$; IgG1, $r=-0.164$, $P<0.05$; IgG3, $r=-0.164$, $P<0.05$; IgM, $r=-0.261$, $P<0.01$; Table 6). In addition, IgG1 was positively associated with the incidence of high density parasitaemia $r=0.218$, $P<0.05$ and episode of clinical malaria in children ($r=0.237$, $P< 0.05$; Table 7). In contrast to children, IgG2 subclass was positively associated with haemoglobin levels at the time of sampling while IgG3 was positively associated with haemoglobin levels a month after sampling in adults (IgG2, $r=0.213$, $P<0.05$; Table 6; IgG3, $r=0.350$, $P<0.014$; Table 8).

TABLE 6: CORRELATION BETWEEN ANTIBODY RESPONSES WITH HAEMOGLOBIN LEVELS IN CHILDREN AND ADULTS AT TIME OF SAMPLING

	Hb levels at sampling time			
	Children (169)		*Adults (131)*	
Antibody	***r***	***P***	***r***	***P***
IgG	-0.215	**0.005****	0.052	0.555
IgG1	-0.180	**0.019***	-0.047	0.599
IgG2	0.015	0.845	0.213	**0.015***
IgG3	-0.164	**0.034***	0.089	0.317
IgM	-0.261	**0.001****	-0.047	0.596

r = Pearson correlation coefficient. (n) = Number of subjects. Differences are significant at P<0.01** or P<0.05*

TABLE 7: CORRELATION BETWEEN ANTIBODY SEROREACTIVITY TO FAL VAC-1 AND LONGITUDINAL MALARIOMETRIC INDICES IN CHILDREN

	Antibody									
	IgG		IgG1		IgG2		IgG3		IgM	
Clinical parameter (n)	***r***	***P***	***r***	***P***	***r***	***P***	***r***	***P***	***r***	***P***
Incidence of parasitaemia (100)	0.155	0.124	0.174	0.084	0.152	0.132	0.171	0.090	0.095	0.347
Incidence of high density parasitaemia (100)	0.141	0.160	0.218	**0.029**	0.138	0.171	0.156	0.121	0.083	0.409
Episodes of clinical malaria (100)	0.155	0.124	0.237	**0.018**	0.079	0.434	0.165	0.101	0.055	0.584
Incidence of fever (100)	0.160	0.112	0.193	0.054	0.097	0.339	0.163	0.106	0.045	0.654
Hb levels a month after sampling (66)	-0.212	0.087	-0.078	0.536	-0.031	0.806	-0.238	0.054	-0.241	0.051
Incidence of anaemia (100)	0.100	0.322	0.032	0.749	0.137	0.175	0.180	0.074	0.023	0.818
Incidence of severe anaemia (100)	-0.012	0.902	-0.052	0.608	-0.100	0.320	0.073	0.473	-0.087	0.458

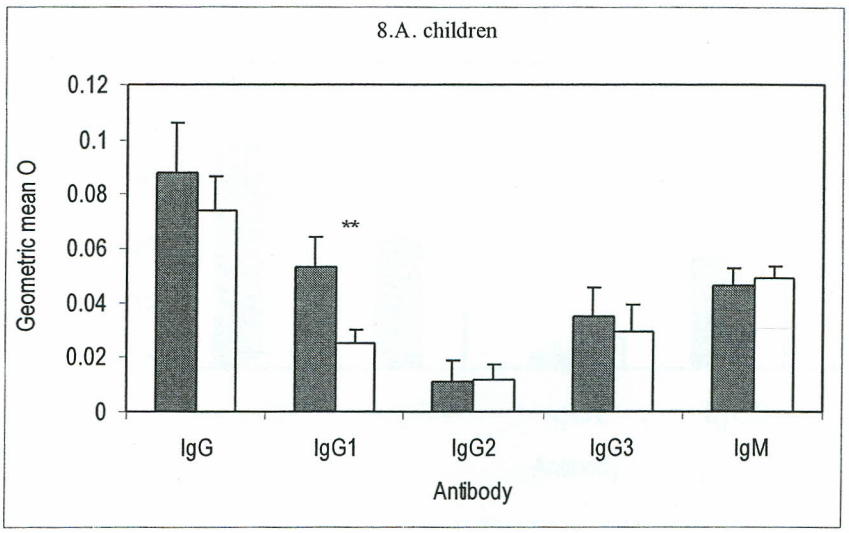
r = Pearson correlation coefficient , differences are significant at $P < 0.05$, (n) = the number of subjects

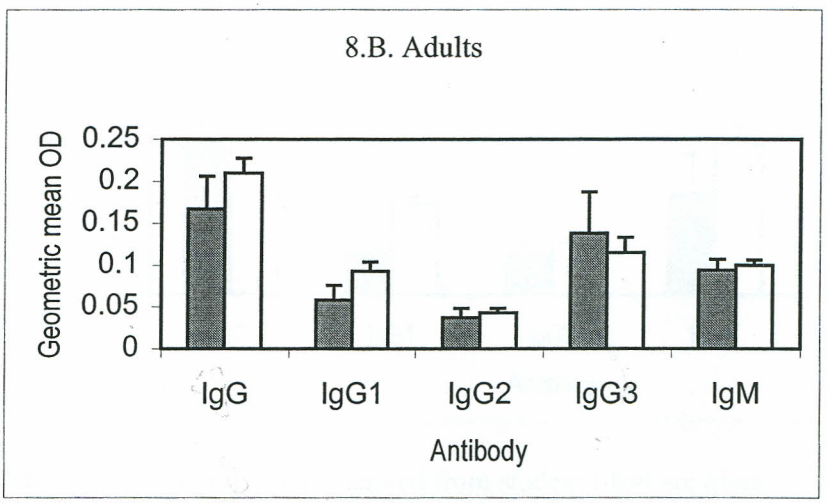
TABLE 8: CORRELATION BETWEEN ANTIBODY SEROREACTIVITY TO FAL VAC-1 AND LONGITUDINAL MALARIOMETRIC INDICES IN ADULTS

	Antibody									
	IgG		IgG1		IgG2		IgG3		IgM	
Clinical parameter (n)	***r***	***P***	***r***	***P***	***r***	***P***	***r***	***P***	***r***	***P***
Incidence of parasitaemia (79)	-0.064	0.578	0.159	0.165	0.116	0.312	-0.162	0.157	-0.130	0.256
Incidence of high density parasitaemia (79)	0.053	0.643	0.128	0.263	0.143	0.213	-0.088	0.445	0.003	0.976
Episodes of clinical malaria (79)	-0.009	0.941	-0.087	0.451	-0.124	0.280	0.097	0.399	-0.074	0.520
Incidence of fever (79)	-0.217	0.056	-0.180	0.114	-0.095	0.410	-0.067	0.562	-0.273	**0.016**
Hb levels a month after sampling (50)	0.231	0.111	-0.088	0.549	0.164	0.262	0.350	**0.014**	0.084	0.566
Incidence of anaemia (79)	0.128	0.265	0.028	0.810	-0.022	0.846	-0.042	0.715	0.200	0.080
Incidence of severe anaemia (79)	0.013	0.907	0.005	0.962	-0.127	0.267	-0.078	0.496	0.165	0.150

r = Pearson correlation coefficient , differences are significant at $P < 0.05$, (n) = number of subjects

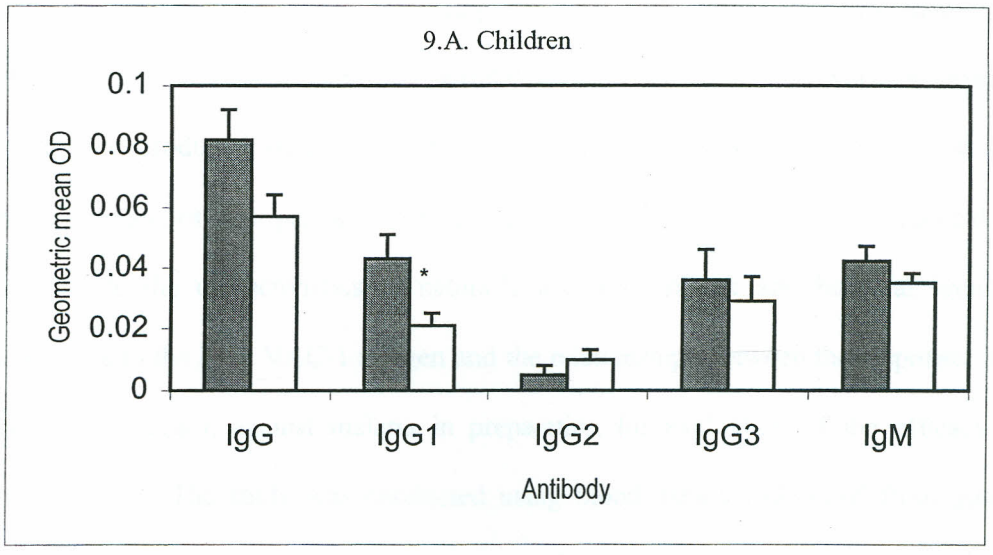
FIGURE 8: COMPARISON OF ANTIBODY SEROREACTIVITY TO FAL VAC-1 BETWEEN PARASITAEMIC AND APARASITAEMIC INDIVIDUALS A MONTH BEFORE SAMPLING

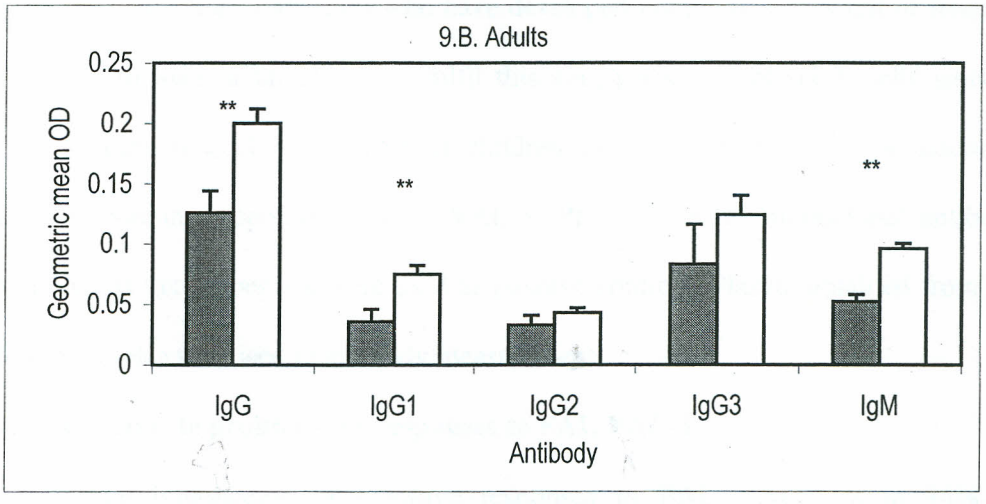




Differences between groups derived from student t-test are given as the geometric mean O.D.s ± s.e.m, ▪ parasitaemic, □ aparasitaemic, children (n=99), adults (n=78), children; parasitaemic=33, aparasitaemic=66, adults; parasitaemic=9, aparasitaemic=69, **P<0.01.

FIGURE 9: COMPARISON OF ANTIBODY SEROREACTIVITY TO FAL VAC-1 BETWEEN PARASITAEMIC AND APARASITAEMIC INDIVIDUALS AT THE TIME OF SAMPLING





Differences between groups derived from student t-test are given as the geometric mean O.D.s ± s.e.m, ▪ parasitaemic, □ aparasitaemic, children (n=177), adults (n=136), children; parasitaemic=46, aparasitaemic=131, adults; parasitaemic=14, aparasitaemic=122, *P<0.05, **P<0.01.

CHAPTER FOUR: DISCUSSION

Previous studies have shown that FAL VAC-1, a recombinant multistage and multicomponent *Plasmodium falciparum* protein candidate malaria vaccine containing 12 B cell and 9 T cell epitopes from 9 different antigens of different life cycle stages is immunogenic in animal models and that the vaccine-induced antibodies produced significant antiparasite activities against both sporozoite and blood stages of the parasite (Shi *et al.*, 1999). Therefore, it was essential to investigate the characteristics of naturally acquired cellular and humoral immune responses to the FAL VAC-1 antigen and the relationships between the responses and clinical protection against malaria in preparation for evaluation of the efficacy in human trials. The study was conducted using blood samples obtained from young children less than two years old, since they are the most vulnerable to the severe effects of malaria infection, and adults who have developed immunity as a result of frequent exposure to malaria infection. To fulfil this aim, peripheral blood T cells isolated from finger prick blood samples of children and adults were used for assessing cellular immune responses to FAL VAC-1. PHA, anti-CD3 monoclonal antibody and tetanus toxoid peptide were used as positive controls. Plasma obtained from the blood samples was used in antibody measurement.

4.1 Lymphocyte proliferative responses to FAL VAC-1

The lymphocyte proliferative responses to FAL VAC-1 were 34% in children and 30% in adults. The lymphoproliferative responses to FAL VAC-1 were not associated with parasitaemia or haemoglobin levels in children or adults. These results are comparable with studies conducted in Madagascar which reported approximately 30% positive proliferative responses of peripheral blood lymphocytes to synthetic peptides representing major epitopes of the merozoite ring-infected

erythrocyte surface antigen (Pf155/RESA) and the circumsporozoite protein (CSP) and the responses were not related to presence of parasitaemia in blood (Chougnet *et al.*, 1991b). On the contrary, lymphoproliferative responses to *P. falciparum* schizont extract in a holoendemic area of Gabon with intense and perennial transmission found that high proliferative responses were predictive of resistance to clinical malaria and that such responses were dependent on antigen presenting cells and CD4+ cells (Mshana *et al.*, 1993). Likewise, it was reported in an endemic area of West Africa that the majority of human lymphocytes in immune donors responding to cloned fragments of PfMSP1 were typically CD4+ and restricted by HLA-DR or–DQ (Riley, 1994).

The results of this study also show that lymphoproliferative responses to FAL VAC-1 decrease with age. In a comparable cross-sectional study in Gabon, it was found that proliferative responses to some regions of PfMSP1 molecule, including some highly conserved sequences, were highest in young children and decreased with age (Riley *et al.*, 1992). The lack of associations between the lymphoproliferative responses and clinical protection in this study suggest that lymphoproliferation may not be a suitable marker for evaluating cellular immunological correlates of protection against malaria and the differences may be dependent on the different levels of exposure to infection whereby adults have had a longer period of exposure and possibly altered immune regulatory mechanisms.

Lymphocyte proliferative responses to PHA increased with age, but no differences were observed in proliferative responses to anti-CD3 or tt epitope in this study. In addition, high lymphoproliferative responses to PHA and anti-CD3 were observed in parasitaemic children than aparasitaemic children. These results are consistent with findings in which lymphoproliferative responses and IFN-γ

production to mitogens or *candida* were not depressed during an acute *P. falciparum* infection (Riley *et al.*, 1988). Likewise, these results are comparable to findings in The Gambia which showed that immune adults had higher lymphoproliferative responses to mitogens and *Candida albicans* than controls or young subjects most of whom were non-responsive (Riley *et al.*, 1988). Therefore, these observations suggest that cellular immune responses in children during *P. falciparum* infection are not depressed and that in fact T lymphocytes may be activated by the presence of parasitaemia.

Although both children and adults had similar responses at three different concentrations of the FAL VAC-1 antigen, the fact that children required higher concentrations in comparison to adults to stimulate proliferative responses suggests that immune activation in adults may be linked to frequent exposure to *P. falciparum* infection in this malaria holoendemic area. These differences may further be explained in part by the constitutional differences in the immune systems of children and adults, which determine the cellular and molecular processes governing naturally acquired immunity to *P. falciparum* malaria (Baird, 1995). The differences may also be due to antigen specific immunosuppression, a feature of acute malaria which has been linked to activation of CD8+ suppressor cells. It has been reported that for some malaria immune individuals, CD8+ T cells activated *in vitro* by exposure to malaria antigen suppress other cellular responses hence obscure the presence of other protective immune mechanisms during *in vitro* proliferative assays (Riley *et al.*, 1989). In immune adults, cell mediated immune responses to malaria antigens are extremely variable when measured *in vitro* and there is no obvious relation between responsiveness and resistance to clinical disease (Riley, 1989). In addition, it could be argued that humans in endemic areas have a pre-existing

population of activated parasite specific T cells which control parasitaemia on activation. However, chronic parasitaemia may induce tolerance of this pre-existing population of T cells, and extensive exposure to malarial antigens in holoendemic areas may also induce tolerance and potentially modify future responses to malaria infections (Good, 1995). This phenomenon may explain in part the low levels of proliferation and unresponsiveness observed in the adults in this study.

The multifactorial and multistage character of protective immunity to *P. falciparum* has been shown in studies carried out in a malaria endemic area of Senegal (Boudin *et al.*, 1994). These Senegalese results, which correspond with findings of this study, showed that children had low humoral responses and high cellular activation whereas high humoral and low T lymphocyte immunity dominated in the adults to the RESA antigen (Boudin *et al.*, 1994). Further explanations for the lower levels of lymphocyte proliferative responses observed in adults in this study could be attributed to induction of anergy or apoptosis in adult lymphocytes on exposure to malaria antigen. One hypothesis put forward to explain the role of apoptosis in disease processes states "that persistent exposure to activation may lead to immune dysfunction and either loss of ability to respond to an antigen (anergy) or induction of an abnormal program of cell death" (Ameisen and Capron, 1991; Groux *et al.*, 1992; Meyaard *et al.*, 1992). Human studies conducted during *P. falciparum* infection indicate that there is increased apoptosis during and following a malaria attack (Toure-Balde *et al.*, 1996). Studies by Toure-Balde *et al.* (1996) confirmed that apoptosis was correlated with exposure to *P. falciparum* which was associated with high levels of surface interleukin receptors (sILRs). These studies also revealed that the parasite antigens were responsible for increased level of *in vitro* apoptosis of PBMCs. Recently it has been observed that levels of

soluble Fas ligand (sFasL) were elevated in the serum of patients with *P. falciparum* infection and may play a role in Fas induced apoptosis in *P. falciparum* malaria (Kern *et al.*, 2000).

In this study there was no relationship observed between antibody responses and the capacity of peripheral blood mononuclear cells to respond to the antigen under the experimental conditions used. This finding is similar to previous results which showed a lack of correlation between antibody responses to MSP-1 antigen and the capacity of peripheral cellular immune effectors to respond to this antigen *in vitro* (Diallo *et al.*, 1999). This suggests that in *P. falciparum* infection, a differential regulation of antibody isotypes and cellular immune mechanisms occurs depending on individual parasite exposure and may determine the outcome of immune responses against malaria. The differences in results of various studies on lymphoproliferative responses of human lymphocytes in malaria infection may be dependent on the type of *P. falciparum* antigen preparation, the endemicity patterns of malaria in an area and the host genetic factors.

4.2 Antibody responses to FAL VAC-1

In addition to cell mediated immune mechanisms, antibody dependent immune mechanisms can contribute in mediating antimalarial immunity (Marsh, 1994). In this study, it was found that the levels and prevalence of antibody responses increased with age and total IgG, IgG1, IgG3 and IgM were the predominant antibodies, with very low levels of IgG2 and no IgG4 detected. In a similar study in a holoendemic area of western Kenya it was found that IgG1 and IgG3 antibody responses to a 19kDa C-terminal domain of MSP-1 increased with age (Shi *et al.*, 1996). The differences in antibody responses in children and adults living in this malaria holoendemic area may be due to differences in the levels and

duration of exposure. In this study it was also observed that children aged 0-6 months had slightly higher levels of total IgG and IgG3 which may be attributed to passively transferred antibodies from the mother as previously reported by Carlier and Truyens (1995). However, low levels of IgG1 subclass in children aged 0-6 months may be linked to a lack of antiparasite protection observed in this group.

The results of this study also showed that parasitaemic children had significantly higher levels of IgG1 than aparasitaemic children at the time of sampling and at a month prior to sampling (Fig. 8). In contrast, non-parasitaemic adults had higher levels of total IgG, IgG1 and IgM. Total IgG, IgG1 and IgM antibody levels were inversely associated with haemoglobin levels in children but no difference in antibody responses in relation to the haemoglobin levels in adults was observed. These findings are similar to studies which reported that levels of antibodies to CSP and Pf155/RESA in children were indicative of exposure to, rather than protection from malaria infection in areas of high transmission where children are at an increased risk of infection once innate protection decreased (Hogh *et al.*, 1996). The results of this study suggest that the presence of total IgG, IgG1 and IgM antibodies in children may indicate a current *P. falciparum* infection while in adults from the same holoendemic area IgG1 may play a role in protective immunity to parasitaemia resulting from malaria.

Only low levels of IgG2 and no IgG4 were detected in this study. It was also observed that in adults, IgG2 was positively associated with haemoglobin levels suggesting a possible role of this antibody in controlling anaemia. Most studies have detected low levels or only trace amounts of parasite specific IgG2 and IgG4 in malaria infection (Sarthou *et al.*, 1997). The role of IgG2 and IgG4 in malaria infection is not well understood. It has been proposed that these antibodies may

inhibit antibody dependent cell-mediated mechanisms by competing for antigen with the cytophilic IgG1 and IgG3 subclasses (Day and Marsh, 1991). However, it has recently been observed that there is a high correlation between GLURP specific IgG2 levels and parasitaemia and age-related exposure in a malaria endemic area of Senegal (Oeuvray *et al.*, 2000). In another study, (Aucan *et al.*, 2000) it was observed that high levels of IgG2 can bind FcγRIIa in individuals with H131allele to RESA and MSP-2 antigens and is associated with low risk of infection while high levels of IgG4 were associated with enhanced risk of infection and a high risk of a malaria attack. More recently, studies in the Asembo Bay area of western Kenya reported that FcγRIIa-Arg/Arg 131 genotype is associated with protection against high density *P. falciparum* malaria infection in infants (Shi *et al.*, 2000). These conflicting results can be attributed to the differences in the infection rates of the study areas.

In this study, it was found that adults had high levels of IgG3, which were not associated with parasitaemia, or haemoglobin levels. This result is comparable to observations in western Kenya which showed that IgG3 was not associated with parasite outcome in malaria infection (Shi *et al.*, 1996). In contrast, it has been shown that a predominance of cytophilic IgG1 and IgG3 antimalarial antibodies is associated with protection (Bouharoun-Tayoun and Druilhe, 1992). It has also been reported that high-avidity cytophilic antibodies predominate in humoral immune response of the anti-P. *falciparum* in clinically immune Senegalese subjects and that the avidity of IgG1 was higher than all the other subclasses (Ferreira *et al.*, 1996). Recently it has been observed in a malaria endemic area of Senegal that high levels of GLURP specific IgG3 were correlated with protection against parasitaemia and age related exposure (Oeuvray *et al.*, 2000). IgG3 response has been described as

one of the factors, which can decrease the effectiveness of the immune response to malaria parasites apart from antigenic polymorphisms, cross-reactive epitopes and poor affinity maturation (Day and Marsh, 1991). Therefore, the lack of association between IgG3 response and protection in this study may be related to its low half-life and low avidity, which means that it may not last long enough to generate its full antiparasite potential.

In this study it was found that IgM was inversely associated with haemoglobin levels in children. This finding may point to a role of IgM in the pathogenesis of anaemia in children. The role of IgM in *P. falciparum* malaria is multifactorial. The seroreactivity of this antibody has been associated with exposure to malaria infection (Branch *et al.*, 1998). Anaemia in children has been attributed to haemolysis of parasitised erythrocytes, dyserythropoiesis, autoimmune mechanisms, and excessive phagocytosis of both infected and uninfected cells in malaria patients (Turrini *et al.*, 1994). In Gabon, Scholander *et al.*, 1998 observed that moderately anaemic children had high IgG and IgM immunoglobulin binding of some parasite isolates and anaemic children had higher rosseting rates than non-anaemic children. It has also been reported that high IgM levels were present in children with parasitaemia, while IgG3, and particularly IgG1 were associated with lower parasitaemia (Luty *et al.*, 1994). In the present study it was also observed that non-parasitaemic adults had high IgM levels than parasitaemic adults and this may point to a role of this antibody in protection against parasitaemia. This observation is comparable to findings in an area of Burkina Faso with hyperendemic malaria, which showed that IgM might play a role in acquisition of immunity against parasitaemia in malaria infection (Boudin *et al.*, 1993).

The results of this study therefore suggests that protective immunity to

malaria passes through different stages and depends on the level and duration of the experience of malaria infections hence there is need to use different concentrations during human trials as children and adults have different levels of exposure.

CHAPTER FIVE: A SUMMARY OF CONCLUSIONS

1. Individuals naturally exposed to *P. falciparum* malaria recognise FAL VAC-I, a multistage multicomponent malaria vaccine candidate by induction of both humoral and cellular immune responses.
2. Although there were no correlations between the antibody and lymphoproliferative responses to FAL VAC-1, antibody responses increased with age whereas lymphoproliferative responses decrease with age in individuals from the study area.
3. Lymphoproliferative responses were not associated with clinical protection in either children or adults.
4. The presence of antibodies (total IgG, IgG1 and IgM) in children was associated with a current *P. falciparum* infection, whereas in adults from the same malaria holoendemic area IgG1 subclass and IgM antibody were associated with antiparasite immunity against *P. falciparum* malaria.
5. The requirement of low antigen concentrations to induce lymphoproliferative responses in adults living in this holoendemic area was associated with immune activation induced by frequent exposure to malaria.

CHAPTER SIX: SUGGESTIONS FOR FUTURE WORK

1. The pattern of morbidity and mortality of malaria depends on transmission intensity, the more intense the transmission; the earlier and more confined the age range of symptomatic malaria. The asymptomatic carrier status is common in highly endemic areas where 60-80% of children have *P. falciparum* parasitaemia at any given time (Hogh, 1996). For this reason it will be important to conduct longitudinal follow up studies of natural immune responses to this antigen in various other endemic areas.
2. Since the burden of malaria infection occurs in pregnant women and children less than five years it will be desirable to carry out a follow up paired study of children and their mothers for a period of five years so as to assess fully the development of natural immune responses to *Plasmodium falciparum* infection.
3. Although the results of this study suggest that lymphoproliferative responses decrease with age and antibody responses increase with age, further studies in different age groups under different malaria infection rates need to be conducted before making generalised conclusions.
4. A significant proportion of individuals in this study either did not show or had low lymphoproliferative or antibody responses to FAL VAC-1. It will be important to carry out immunoactivation, apoptosis and memory studies in order to understand the dynamics of immune responses to *P. falciparum* infection.
5. Cytokines play a crucial role in the dichotomy of an immune response. It would be important to carry out studies on the induction of TH1/TH2 cytokines and protective cytokines (IFN-γ and IL-12) by FAL VAC-1 and their roles in protective immunity to *P. falciparum* malaria.

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APPENDICES

Appendix 1: Recipe for Reagents

1. Antigen

FAL VAC-1 was made by protein Sciences Corporation and supplied in 10ml vials at a concentration of 250μg/ml in PBS pH 7.20. The pyrogen content was < 250EU/ml. This antigen was > 90 % pure and was used without further purification.

2. Phosphate Buffered Saline (PBS), pH 7.2

0.28g monobasic $Na(H_2PO_4)$ anhydrous 1.9mM, 1.15g dibasic $Na_2(HPO_4)$ anhydrous 8.1mM and 9 g NaCl 154mM were added to 1000ml sterile filtered distilled water, pH adjusted to 7.2 then filter sterilised before use.

3. Borate Buffered Saline (BBS)

7.010g NaCl, 3.090g Boric acid and 0.960g NaOH were added to 1000ml double distilled water and pH adjusted to 8.0, then filter sterilized before use.

4. Ficol-Hypaque

64g of polysucrose and 0.7g of sodium chloride were dissolved in 600ml of distilled water then 99g of sodium ditrizoate added. 400ml of distilled water were added to the preparation to make to a litre. This was then filter sterilized before use.

5. Blocking Buffer

Non-fat milk and Tween-20 were dissolved in PBS to make a final volume of 0.1% milk and 0.05% Tween-20. This was prepared by dissolving 0.1g of non-fat milk and 50μl of Tween-20 in 100ml of PBS.

6. Wash Buffer

PBS containing 0.05% Tween-20 washing solution was prepared by adding 500μl of Tween-20 detergent to one litre of PBS.

7. Sample dilution buffer

Wash buffer containing 2.5% non-fat milk and 0.1% NP-40 was prepared by adding 2.5g of milk and 0.1g of NP-40 in 100ml of wash buffer.

Appendix 2: Properties of amino acids

Amino acid	3-letter code	1-letter code	Mol. Wt.
Alanine	Ala	A	89.1
Arginine	Arg	R	174.2
Asparagine	Asn	N	132.1
Aspartic acid	Asp	D	133.1
Asparagine or Aspartic acid	Asx	B	-
Cystein	Cys	C	121.2
Glutamine	Gln	Q	146.1
Glutamic acid	Glu	E	147.1
Glutamine or Glutamic acid	Glx	Z	-
Glycine	Gly	G	75.1
Histidine	His	H	155.2
Isoleucine	Ile	I	131.2
Leucine	Leu	L	131.2
Lysine	Lys	K	146.2
Methionine	Met	M	149.2
Phenylalanine	Phe	F	165.2
Proline	Pro	P	115.1
Serine	Ser	S	105.1
Threonine	Thr	T	119.1
Tryptophan	Trp	W	204.2
Tyrosine	Tyr	Y	181.2
Valine	Val	V	117.1
Unknown or 'other'	-	X	-

Appendix 3: SI for lymphoproliferative responses

Study number	Date of proliferation	PHA	Anti-CD3	tt	FAL VAC-1						
					5 µg/ml	2.5 µg/ml	1 µg/ml	0.5 µg/ml	0.1 µg/ml	0.01 µg/ml	0.001 µg/ml
CHILDREN											
20396	20.04.99	5.1	.	1.1	.	.	0.9	0.9	.	.	.
20489	29.04.99	4.2	.	5.8	.	2.3	2.9	1.5	.	.	.
20497	03.05.99	16.5	.	0.6	.	2.3	3.7	1	.	.	.
20508	15.04.99	3.2	.	1.2	.	1	1.1	.	.	.	.
20525	20.04.99	8.4	4.5	1.4	1.8	1.9	2.8	.	2.8	2.9	4
20527	13.04.99	2.6	2.1	2.5	0.6	2	2.2	.	2.1	2.3	1.4
20571	15.04.99	1.7	.	1.9	1.8	4	1.7	.	.	.	.
20581	20.04.99	1.7	1	1.7	0.7	0.9	3.1	.	3.2	6.5	2.3
20588	07.04.99	1.2	.	1.2	.	.	2.8	.	.	.	.
20616	26.04.99	15.6	.	2.1	.	0.6	2	1.3	.	.	.
20617	26.04.99	17.1	13.8	2	.	.	2.7	.	.	.	.
20618	22.04.99	4.3	.	2.3	.	1	1.4	1.5	.	.	.
20620	19.04.99	9.3	3.7	1.6	.	1.5	3.1	3.5	.	.	.
20634	19.04.99	9.9	.	0.8	.	1	3	1.9	.	.	.
20647	13.04.99	0.7	.	2.3	.	.	1.5	.	2.2	1	1.1
20656	28.04.99	3.7	.	.	.	.	.	1.4	.	.	.
20659	07.04.99	1.5	2.4	3	2.3	1.7	2.7	.	2.6	2.6	2.8
20692	19.04.99	2.8	1.3	3.1	.	2.3	1.3	1.5	.	.	.
20701	22.04.99	1.3	1.3	3.2	.	3.5	3.9	.	1.6	1.4	.
20719	27.04.99	37.8	3.4	3.2	4	.	2	2	.	.	.
20732	19.04.99	4.1	.	1	.	2.7	1.9	4	.	.	.
20739	19.04.99	10.9	1.6	1.2	.	0.5	0.5	0.9	.	.	.
20739	03.05.99	27.5	4.8	1.7	.	1.9	1.9	2	.	.	.
20752	08.04.99	5.5	3.3	2.1	0.8	.	2.4	1.2	.	.	.
20752	19.04.99	9.8	3	2	.	1	3	2	.	.	.
20753	19.04.99	3	1.8	1.7	.	0.7	0.9	2	1	1	1
20764	15.04.99	1.6	1	0.7	1	1.1	.	1.8	.	.	.
20768	13.04.99	3.2	.	0.9	1.8	.	1.7	.	.	.	.
20773	29.04.99	6.6	.	1.5	.	3.3	1.1	.	.	.	.
20792	22.04.99	2	.	1.1	.	1.4	1.6	1.6	.	.	.
20793	08.04.99	3.4	.	0.9	.	.	.	.	1.6	.	.
20809	26.04.99	24.8	.	2.6	.	2.3	1.6	.	.	.	.
20810	14.04.99	3.5	1.7	1.3	1.4	1.4	1	.	.	.	.
20826	24.05.99	91.2	5	3.6	.	2.4	2.6	5.7	.	.	.

20827	20.04.99	8.7	.	1.2	.	2	2.1	.	.	.	.
20832	14.04.99	5	.	.	.	2.5	.	.	.	.	.
20833	28.05.99	6.1	1.4	1	.	2.1	2.2	2.1	.	.	.
20842	07.04.99	1.6	1.6	0.8	1.5	.	1.9	1.4	.	.	.
20856	11.05.99	2.7	2	.	.	1.2	1.5	3.2	.	.	.
20859	08.04.99	3.7	.	2.3	.	.	.	.	.	1.4	.
20881	29.04.99	10	.	.	.	.	2.6	.	.	.	.
20885	14.04.99	17.6	1.3	2.8	1.3	.	2.7	2.4	.	.	.
20893	26.04.99	40.2	1.5	1.7	1	1.7	1.7	1.3	1	.	.
20899	22.04.99	3	.	1	.	1.5	1.6	.	.	.	.
20900	08.04.99	2.2	2	.	0.8	.	1.6	2.3	.	.	.
20900	06.05.99	3.8	2	1.5	.	2	2.6	2.1	.	.	.
20914	14.04.99	6.1	.	2.1	0.6	0.6	1.7	2.3	2.3	.	.
20919	13.04.99	8.2	1.3	2.8	2.7	2.9	1.9	1	1	1	1
20920	14.04.99	5.5	.	2.3	0.6	0.6	1.1	.	.	.	.
20924	03.05.99	11.8	4.8	8.9	.	3.3	5	.	.	.	.
20925	07.04.99	2.5	1.1	1	.	2	.	1.7	1	.	.
20928	27.04.99	71	.	1.1	.	1.3	1.3	1.4	.	.	.
20940	21.04.99	3.7	.	6.2	.	3.1	.	.	.	.	.
20941	05.05.99	8.1	2.7	2.8	.	6.5	5.3	7.1	.	.	.
20944	20.04.99	1.5	1	0.8	.	1.6	1	.	.	.	.
20945	29.04.99	2.7	2.4	1.8	.	2.4	2.8	3.4	.	.	.
20948	08.04.99	1.4	.	.	.	.	.	.	1.3	.	.
20950	28.04.99	216	.	.	.	.	0.6	0.6	.	.	.
20959	27.04.99	5.7	2	1.2	.	0.6	1.3	1	.	.	.
20961	18.05.99	73.6	2.5	7.3	.	2.3	2.7	4.1	.	.	.
20972	08.04.99	5.4	.	1.2	1.2	1.1	1.4	1	.	.	.
20975	25.05.99	41.2	7.1	3.8	.	2.1	5.9	3.8	.	.	.
20977	08.04.99	42.7	1.9	.	3.5	1.6	.	.	.	.	.
20977	05.05.99	2.9	.	2.8	.	.	1.6	.	.	.	.
21013	07.04.99	3.7	1.8	.	.	.	2.4	.	.	.	.
21046	19.04.99	3.5	.	1.5	.	1.3	2.2	1.4	.	.	.
21065	19.04.99	4.5	.	1.5	.	1.1	1.7	3.1	.	.	.
21072	02.06.99	10.7	11.3	9.9	.	3.4	5.2	6.7	.	.	.
21081	08.04.99	1.7	1.5	1.6	1.2	1.8	1.5	1.3	.	.	.
21104	28.04.99	2.4	3	3.1	.	1.3	2.1	.	1.3	1.3	.
21109	29.04.99	7.5	.	1.6	.	0.5	0.9	.	.	.	.
21148	03.05.99	6	3.6	2.9	.	.	3.9	2.9	.	.	.
21200	07.04.99	1.4	2.6	2	2.6	2.9	4	.	2	1.2	1
21213	21.04.99	2.1	.	3.6	.	.	3.6	.	.	.	.
21231	07.04.99	2.3	2.1	1.3	1.5	.	.	.	.	.	.

21247	04.05.99	5.5	.	.	.	2.3	4.4	4.3	.	.	.
21264	27.04.99	16.2	.	1.2	.	1.6	1.7	2	.	.	.
21295	23.04.99	9.4	5.8	.	.	4.2	1.8	1.2	.	.	.
21302	07.04.99	5.3	.	6.1	.	.	3.4	.	.	.	.
21362	19.04.99	8.7	.	0.9	.	1.7	2	1.4	.	.	.
20012	16.08.99	19.4	.	.	.	.	1.6	1.4	.	.	.
20054	12.07.99	9.4	1.9	1.7	.	1.5	1.5	1.1	.	.	.
20056	21.07.99	14.6	.	.	.	0.7	0.9	2.9	.	.	.
20070	19.07.99	8.5	2.1	.	.	1.6	2.5	2.6	.	.	.
20079	20.07.99	5.7	1.2	1.8	.	1.2	1.2	1	.	.	.
20086	06.07.99	2.7	.	0.8	.	1	1.2	1.3	.	.	.
20090	01.07.99	10.9	.	2	.	1.1	1.7	2	.	.	.
20095	24.08.99	6	1.7	1.7	.	1.4	1.5	1.1	.	.	.
20109	07.07.99	91.7	.	.	.	1.3	1.5	.	.	.	.
20111	30.07.99	16.2	.	2.2	.	1	1.5	.	.	.	.
20115	20.07.99	5.6	.	1.8	.	1.3	1.2	1.1	.	.	.
20118	09.08.99	15.7	2.5	.	.	1.5	1.1	1	.	.	.
20128	12.08.99	13.1	.	.	.	.	0.9	2.5	.	.	.
20143	04.08.99	27.7	7.3	2.5	.	2.1	2.5	3	.	.	.
20148	21.07.99	4.2	.	.	.	.	1	.	.	.	.
20161	26.07.99	8.5	1.3	.	.	1.4	1.8	1	.	.	.
20164	19.07.99	4.4	.	.	.	1.4	1.5	1.4	.	.	.
20170	02.07.99	11.6	.	1	.	0.1	0.2	0.1	.	.	.
20184	06.07.99	4.7	.	0.9	.	1	1.3	2	.	.	.
20185	02.08.99	30.3	.	2.5	.	1	1.1	1.1	.	.	.
20197	20.07.99	19.8	2.8	2.5	.	3.4	1.7	1.3	.	.	.
20208	17.08.99	24.1	.	2.6	.	1.3	1.6	2.6	.	.	.
20214	22.07.99	12.9	2.6	.	.	1.4	1.8	1	.	.	.
20224	02.08.99	16.9	1.8	1.8	.	1.6	1.6	1.6	.	.	.
20227	01.07.99	187.3	.	2.2	.	.	1.4	1.2	.	.	.
20232	06.07.99	4.1	2.5	1.2	.	1.1	2.1	1.5	.	.	.
20234	16.07.99	8.2	2.1	1.3	.	1.7	1.6	1.8	.	.	.
20237	07.07.99	22.7	1.1	1.8	.	3	1.4	.	.	.	.
20251	30.07.99	4.1	1	1.5	.	1.2	1.5	1.7	.	.	.
20252	15.07.99	2	1.3	1.4	.	1.3	1.2	1.1	.	.	.
20254	08.07.99	2.8	1.5	1.4	.	1.3	1.6	1.1	.	.	.
20256	28.07.99	2.5	.	2.5	.	1.5	1.8	2.6	.	.	.
20266	16.08.99	41	13.6	1.2	.	1.6	1.8	11.5	.	.	.
20269	10.08.99	51.6	7.8	1.6	.	1.1	1.2	3.6	.	.	.
20274	16.08.99	1.5	1.5	1.5	.	.	1.6	1.4	.	.	.
20275	13.07.99	1.2	0.8	0.9	.	0.8	0.6	0.4	.	.	.

20277	08.07.99	3.8	.	.	.	.	1.1	.	.	.	.
20280	09.07.99	6.5	1.3	1.7	.	.	1	1.1	.	.	.
20288	22.07.99	11.6	.	.	.	1.5	4	.	.	.	.
20294	27.07.99	6	1.4	.	.	1.4	2.5	.	.	.	.
20309	12.07.99	5.3	.	0.8	.	0.9	0.9	0.9	.	.	.
20315	09.07.99	2.8	.	.	.	1	.	1.1	.	.	.
20325	09.07.99	63.9	2.6	1.7	.	1.8	2.5	1.7	.	.	.
20329	19.08.99	44.1	2.1	.	.	0.7	1	1.4	.	.	.
20332	14.07.99	2.5	1.1	1.1	.	1.1	1.3	1	.	.	.
20336	27.07.99	27.2	3.2	2.9	.	2	1.3	1.2	.	.	.
20338	10.08.99	5.4	3	1.4	.	1	1	1.6	.	.	.
20343	20.08.99	20.8	.	.	.	2.5	1.3	1.1	.	.	.
20344	05.07.99	10.5	.	1.1	.	.	1.9	2.2	.	.	.
20347	27.08.99	172.9	55.9	.	.	1.1	1.2	1	.	.	.
20352	01.07.99	81.7	.	2.1	.	2.2	2.5	2.5	.	.	.
20354	06.07.99	33.8	1.1	1.2	.	1.1	1	1	.	.	.
20369	20.07.99	14.6	2.1	2.5	.	1.4	1.6	2.5	.	.	.
20374	19.07.99	4.2	2	1.7	.	2	1.9	2.6	.	.	.
20377	12.07.99	6.3	1.5	1.9	.	1.8	2.5	1	.	.	.
20377	12.07.99	6.3	1.5	1.9	.	1.8	2.5	1	.	.	.
20381	18.08.99	53.2	23.3	2	.	1.3	1.6	2.5	.	.	.
20387	06.07.99	23	.	1.1	.	1	1	1.1	.	.	.
20388	12.07.99	2.7	1.3	1.6	.	0.6	1	1.6	.	.	.
20390	01.07.99	16.1	.	.	.	3	.	.	.	.	.
20391	21.07.99	4	1.8	1.1	.	0.9	0.8	1.1	.	.	.
20395	16.07.99	12.6	2.3	1.6	.	1.6	1.7	1.7	.	.	.
20413	27.07.99	13.6	.	2.9	.	1.3	1.2	1.5	.	.	.
20419	26.07.99	87.5	.	1.6	.	1.1	1.4	1	.	.	.
20423	05.07.99	7.4	.	1.2	.	0.8	1.3	2.5	.	.	.
20427	09.08.99	5.1	2.1	2.5	.	1.8	1.9	2.5	.	.	.
20441	17.08.99	5.5	0.8	.	.	.	.	0.5	.	.	.
20447	01.07.99	8.6	.	1.5	.	1.3	2.1	1.4	.	.	.
20453	20.08.99	44.1	3.5	4.9	.	2.5	4.5	4	.	.	.
20456	28.07.99	144.8	2.2	.	.	2.1	2.8	2.6	.	.	.
20457	14.07.99	5.2	1.6	2.9	.	0.8	1.2	1.1	.	.	.
20458	02.08.99	14	.	.	.	.	1.1	.	.	.	.
20460	24.08.99	3.2	2.6	.	.	1.4	1.3	1.6	.	.	.
20461	30.06.99	3.3	.	1.4	.	2	2.5	1.4	.	.	.
20468	20.07.99	3.8	1.3	2	.	3.1	2.7	2.5	.	.	.
20469	19.08.99	13.9	1.2	.	.	1.2	2.6	1.2	.	.	.
20473	13.07.99	4.2	7.2	2.6	.	3.1	2.2	1	.	.	.

20474	04.08.99	16.3	1.5	1.3	.	2.5	1.8	1.6	.	.	.
20477	19.07.99	4.6	.	.	.	1.1	.	.	.	.	.
20488	08.07.99	2.8	1.3	2.1	.	1.3	1.2	1.2	.	.	.
20494	25.08.99	28.2	0.7	2.1	.	1.9	2	2.7	.	.	.
20498	12.07.99	9	2	1.3	.	1.5	1.4	1.1	.	.	.
20503	05.07.99	37.2	1.6	0.9	.	0.9	0.9	0.8	.	.	.
20504	12.07.99	22.4	.	5.6	.	1.8	2.6	2.9	.	.	.
20514	14.07.99	3.6	1.3	1.7	.	1	2.1	2.6	.	.	.
20519	08.07.99	2.7	1.1	1.4	.	2.2	3.3	2	.	.	.
20528	17.08.99	25.3	2.9	.	.	2.8	3.3	1.9	.	.	.
20535	19.07.99	2.1	.	.	.	.	1.5	1.1	.	.	.
20540	07.07.99	5.6	.	1.7	.	1.3	1.3	2.5	.	.	.
20544	02.07.99	5.7	.	1.3	.	.	1.1	1	.	.	.
20545	30.08.99	3.3	1.1	.	.	1	1.4	1.2	.	.	.
20558	04.08.99	3.1	.	.	.	1.7	.	2.6	.	.	.
20579	13.07.99	3.3	2.5	1.5	.	2	2.5	1.3	.	.	.
20580	23.08.99	36.1	1.8	2.7	.	21	1.6	1.5	.	.	.
20587	13.07.99	38.3	.	0.9	.	0.9	0.9	2.5	.	.	.
20605	27.07.99	1.1	.	.	.	1.3	1.3	.	.	.	.
20608	06.07.99	22	.	1.5	.	1	1.5	.	.	.	.
20625	24.08.99	54.2	2.3	1.5	.	1.5	1.4	2.5	.	.	.
20646	28.07.99	5.1	.	4.2	.	3.2	3.3	3.5	.	.	.
20745	15.07.99	5.5	1.4	2.4	.	1.8	2.5	1.5	.	.	.
ADULTS											
10054	12.07.99	17.5	2.9	1.6	.	.	.	.	1.6	1.1	1
10056	21.07.99	9.4	.	.	.	.	.	.	0.7	.	.
10070	19.07.99	30.6	.	.	.	.	.	.	2.5	.	.
10086	06.07.99	34.5	.	.	.	.	.	.	2.1	.	.
10095	27.07.99	15	1.5	1.4	.	.	.	.	1.1	1	1.1
10109	07.07.99	371.7	.	1.7	.	.	.	.	1.2	1.5	1.3
10136	07.07.99	84.7	.	.	.	.	.	.	1.1	1.1	1.2
10148	21.07.99	7.1	1.7	1.3	.	.	.	.	2.5	1.4	1
10161	26.07.99	6.8	.	1.4	.	.	.	.	1.1	1.4	1.7
10164	19.07.99	13.5	1.1	1.6	.	.	.	.	1.2	1.6	2.5
10170	02.07.99	199.4	.	2.1	.	.	.	.	2.5	2	1.1
10234	16.07.99	15.3	1.5	.	.	.	.	.	1.2	1	1.1
10237	07.07.99	160.2	.	1.7	.	.	.	.	1.3	1.3	.
10239	26.07.99	1.3	.	.	.	.	.	.	0.8	2.5	.
10252	15.07.99	2.9	.	.	.	.	.	.	.	.	1
10254	08.07.99	2.3	.	.	.	.	.	.	1.2	.	.
10269	10.08.99	22.9	1.3	1.4	.	.	.	.	1.4	1.6	1.7

10274	20.07.99	23	1.1	.	.	.	.	.	1.3	2.5	1.2
10275	13.07.99	1.7	.	1.1	.	.	.	.	1.7	2	2.5
10297	21.07.99	17.4	0.7	.	.	.	.	.	0.9	1.3	1.7
10309	12.07.99	73	5.4	2.5	.	.	.	.	2.5	1.9	1.3
10315	09.07.99	22.2	2.9	.	.	.	.	.	1.5	1.7	1.6
10325	09.07.99	3.1	.	0.9	.	.	.	.	1.4	1.5	.
10326	06.07.99	7.8	1.8	1.2	.	.	.	.	2.5	1.7	1.6
10327	30.06.99	138.6	.	2.1	.	.	.	.	2.7	2	1.9
10331	05.07.99	118.3	.	0.7	.	.	.	.	1.2	2.5	1.7
10332	14.07.99	16.9	2.2	2.9	.	.	.	.	1	1.3	1.4
10336	27.07.99	29.4	.	.	.	.	.	.	1.5	1.2	.
10338	12.07.99	27.3	.	.	.	.	.	.	2.5	2	1.3
10352	01.07.99	6	.	1.7	.	.	.	.	2.6	1.3	1.1
10354	06.07.99	37.8	.	1.5	.	.	.	.	1.5	2.5	1.3
10365	15.07.99	7.4	.	.	.	.	.	.	.	1.1	.
10374	19.07.99	11.5	.	1.1	.	.	.	.	.	1.5	.
10381	21.07.99	56.1	.	.	.	.	.	.	1.1	1.4	2.7
10389	22.07.99	32.9	1.6	4.3	.	.	.	.	1.4	1.9	3
10390	01.07.99	19.2	.	1.3	.	.	.	.	1	1.1	1.2
10399	13.07.99	53.5	1.7	.	.	.	.	.	2.5	3.5	2.3
10402	22.07.99	20.9	3.2	2	.	.	.	.	1.6	2.2	2.5
10413	27.08.99	64.5	3.6	.	.	.	.	.	3.3	2.5	2.5
10420	26.07.99	35.7	.	2.9	.	.	.	.	1.6	2.5	1.1
10430	09.07.99	17.1	3.5	2.9	.	.	.	.	1.4	1.6	2.5
10441	17.08.99	15.9	3	.	.	.	.	.	2.5	2.2	1.7
10442	28.07.99	74.7	63.1	.	.	.	.	.	2.5	1.3	1.1
10447	01.07.99	8.4	.	1.6	.	.	.	.	1.4	1.8	2.1
10459	26.07.99	16.4	.	.	.	.	.	.	0.9	1.2	1
10460	24.08.99	19.5	.	.	.	.	.	.	1.8	2.9	2.1
10461	30.06.99	14.8	.	1.3	.	.	.	.	2.5	1.2	1.2
10468	20.07.99	4.3	0.9	.	.	.	.	.	2.5	.	.
10473	13.07.99	7.6	2.2	.	.	.	.	.	2.5	2.8	2.5
10474	04.08.99	3	2.5	.	.	.	.	.	2.5	2.1	1.9
10477	19.07.99	12.7	1.3	2.2	.	.	.	.	0.9	1	1.2
10486	30.06.99	1.1	.	1	.	.	.	.	0.8	0.9	1.1
10488	08.07.99	4	.	0.7	.	.	.	.	1	1.3	.
10496	15.07.99	10.7	.	1.5	.	.	.	.	2	1.8	1.7
10498	12.07.99	7.3	.	1.2	.	.	.	.	1	2.5	1.6
10503	05.07.99	53.7	.	0.9	.	.	.	.	1	1.4	.
10514	14.07.99	16.3	4	2.2	.	.	.	.	1.6	2.7	1.2
10519	15.07.99	9.9	2.3	3.8	.	.	.	.	1.2	1.2	2.5

10528	20.07.99	29.4	.	.	.	.	.	.	.	1	.
10535	19.07.99	7.2	1.2	2.9	.	.	.	.	1	1.6	1.6
10540	07.07.99	69.6	.	.	.	.	.	.	1	1.1	1.5
10544	02.07.99	35	.	1	.	.	.	.	1.7	1.7	1.5
10545	30.08.99	4.5	2	.	.	.	.	.	1.5	1.5	2
10555	22.07.99	16	.	.	.	.	.	.	1.4	.	.
10558	04.08.99	6.6	.	1.2	.	.	.	.	1.1	1.5	2.7
10575	28.07.99	54.3	1	.	.	.	.	.	1	1.3	2.5
10579	13.07.99	33.1	.	1	.	.	.	.	1.1	1.1	.
10580	23.08.99	54.2	2	.	.	.	.	.	1.1	1.7	2.6
10587	10.08.99	12.8	.	.	.	.	.	.	0.7	0.6	.
10591	26.07.99	50.7	.	.	.	.	.	.	1.2	.	1
10605	27.07.99	26.2	.	.	.	.	.	.	1	1.4	.
10612	13.07.99	8.3	.	.	.	.	.	.	1.1	1	.
10626	15.07.99	7.4	2.6	1.6	.	.	.	.	1.1	1.4	1.8
10641	12.08.99	6.7	.	.	.	.	.	.	1.1	1.4	.
10646	28.07.99	20.3	1.7	2.2	.	.	.	.	2	2.5	1.3
10695	18.08.99	39.4	10.8	44.2	.	.	.	.	4	4	6
10699	03.08.99	6.1	1.3	1.4	.	.	.	.	1.3	2	1.2
11090	16.08.99	70.6	3.6	2.7	.	.	.	.	1.4	1	1
11180	12.08.99	30.8	.	.	.	.	.	.	1.4	1.2	.
10396	20.04.99	6.6	0.9	1.2	.	.	.	.	0.6	0.7	1
10497	03.05.99	55.1	2.2	.	.	.	.	.	.	2.2	2.4
10508	28.05.99	51.8	2.7	2.9	.	.	.	.	1	2	4.3
10542	20.04.99	2.3	.	0.8	.	.	.	.	1.3	1.2	1.6
10557	03.06.99	32	1.1	1.1	.	.	.	.	1	1	1
10571	15.04.99	2.1	.	1.2	.	.	1.6	.	.	.	.
10581	20.04.99	6.6	.	1	.	.	.	.	0.6	.	.
10586	26.04.99	2.8	.	1.6	.	.	.	.	0.7	.	.
10588	07.04.99	4.9	2	1.6	2.1	1.9	1	1	1.4	1	1
10616	26.04.99	30.2	2.3	1.6	.	.	.	.	1.8	1	1
10617	26.04.99	6	.	0.5	.	.	.	.	.	2	.
10637	27.04.99	53.8	2.7	2.6	.	.	.	.	2.2	1.7	1.5
10659	07.04.99	2.9	1.5	2.3	0.6	2.9	2	.	.	1.4	1.3
10681	28.04.99	10.7	.	1.9	.	.	.	.	3.2	.	.
10692	19.04.99	25.2	.	2.6	.	.	.	.	1.8	2.1	3.9
10701	29.04.99	16.3	0.5	1.1	.	.	.	.	2.2	2	1.4
10719	27.04.99	93.4	1.6	1.2	.	.	.	.	1.2	1	3.1
10732	04.05.99	28.5	.	0.5	.	.	.	.	3	1.2	.
10739	19.04.99	8.4	1.1	1	0.9	.	0.8	1.4	1.2	3	1.9
10746	20.04.99	10.2	1.8	1.6	.	3.6	.	2.2	2.4	1.8	1.2

10764	15.04.99	7	1.2	1	.	.	2.8	1.4	1	1	1
10792	22.04.99	3.2	.	1.5	.	.	.	.	1.4	1.8	2.8
10794	14.04.99	54.5	2.5	2.2	.	.	2.3	2.1	1.7	1.1	1.7
10809	26.04.99	37.1	.	1.2	.	.	.	.	2.5	3.2	1.1
10810	14.04.99	23.8	1.3	2.4	.	.	2.6	3.1	.	2.1	1.7
10819	20.04.99	2.4	1.1	1	.	.	.	.	1.3	1	1
10827	18.05.99	75	1.8	1.5	.	.	.	.	1.5	2.6	3.3
10833	28.05.99	36.5	2.1	1.1	.	.	.	.	1.1	1.4	.
10842	07.04.99	3.7	3	1.2	.	.	.	.	2.1	3.2	4.2
10856	11.05.99	2.8	.	.	.	.	.	.	1.6	2.3	1.5
10859	08.04.99	2.6	.	1.1	.	.	.	.	.	.	3.4
10859	06.05.99	16.7	4.9	2.4	.	.	.	.	0.6	1.6	1.4
10876	29.04.99	8.8	.	1.1	.	.	.	.	1.2	.	.
10881	29.04.99	14.1	.	1.8	.	.	.	.	2.1	.	1.9
10885	14.04.99	4.4	.	1.7	.	.	1.2	.	1.5	2.3	2
10893	26.04.99	31.1	1.1	1	.	.	.	.	1.1	0.9	0.9
10899	22.04.99	3.6	0.7	1	.	.	.	.	1.5	3.3	2.4
10900	06.05.99	3.9	.	.	.	.	.	.	2.9	.	1.1
10910	28.04.99	4.2	.	2.2	.	.	.	.	2.9	.	.
10919	13.04.99	12.2	.	1.5	2.8	2.6	2.8	1	.	.	.
10925	07.04.99	2.8	3.4	2	1.2	.	1.2	.	1.3	2.8	6.6
10933	13.04.99	2.8	0.6	0.7	1.4	1	2	1	1.3	1	1
10940	21.04.99	2.5	.	1	.	.	.	.	2.5	2.9	2.9
10950	28.04.99	2	.	.	.	2.2	3.5	.	.	.	.
10959	27.04.99	61.7	.	1.1	.	.	.	.	1.5	1.8	1.8
10966	06.05.99	17	2.8	.	.	.	.	.	1	1.5	4.2
11002	15.04.99	1.3	.	2.2	.	.	.	.	0.6	0.5	1
11013	07.04.99	1.7	2	1.8	.	.	1.5	.	.	1.4	1.7
11060	26.04.99	21.9	2.5	2.2	.	.	.	.	4.8	2.5	1.1
11065	19.04.99	7.8	.	1.9	.	.	2.1	.	1.3	1.3	.
11099	07.04.99	1.4	2.6	2.1	.	.	0.9	.	.	.	.
11104	28.04.99	14.5	.	1.4	.	.	.	.	0.6	1.2	1.9
11143	22.04.99	4.4	.	.	.	.	.	.	1.1	.	.
11200	07.04.99	2.1	2	0.8	1.7	.	0.6	2	3	1.6	0.8
11231	07.04.99	1.3	1.1	0.6	1	0.7	0.8	.	0.7	0.8	0.5
11247	04.05.99	23.9	.	.	.	.	.	.	0.6	0.6	.
11263	04.05.99	36.1	.	.	.	.	.	.	3.1	.	.
11295	23.04.99	9.5	.	1	.	.	.	.	1.5	2.6	5.9
11302	07.04.99	2.6	.	2.7	.	.	.	.	2.1	6	2.4
11362	19.04.99	4	3.7	3	.	.	.	.	2.7	3.7	1.1

Appendix 4: O.D.s for antibody responses

Study number	Date of ELISA	IgG	IgG1	IgG2	IgG3	IgG4	IgM
CHILDREN							
20396	22.01.00	0.024	0.022	0.002	0	-0.02	0.027
20489	21.01.00	0.121	0.113	0.006	-0.005	-0.004	0.044
20497	22.01.00	0.258	0.314	0	-0.001	-0.02	0.106
20508	19.01.00	-0.02	-0.026	-0.002	-0.007	0.001	0.01
20525	22.01.00	0.061	0.027	0.037	0.025	-0.021	0.028
20527	.	.	.	.	.	.	.
20571	19.01.00	0.125	-0.008	0.042	-0.005	-0.002	0.038
20581	22.01.00	0.01	0.008	0.002	-0.005	-0.021	0.031
20588	19.01.00	0.031	-0.002	0.001	0.008	0.001	0.029
20616	19.01.00	0.055	0.003	-0.003	0.002	-0.001	0.019
20617	19.01.00	0.055	-0.005	0.026	0.019	-0.001	0.04
20618	21.01.00	0.019	0.012	0.001	0.003	-0.004	0.012
20620	27.01.00	0.004	-0.003	-0.009	-0.005	-0.007	0.012
20634	28.01.00	0.015	0.006	0.001	0.013	0.002	0.029
20647	21.01.00	0.001	-0.001	0.003	-0.002	-0.006	0.005
20656	22.01.00	0.031	0.031	-0.001	-0.007	-0.021	0.044
20659	19.01.00	-0.002	-0.024	-0.004	-0.008	-0.002	0
20692	28.01.00	0.067	0.073	0	0.016	-0.002	0.062
20701	21.01.00	0.016	0.01	0.001	0.01	-0.003	0.01
20719	22.01.00	0.116	0.153	0.001	0	-0.02	0.057
20732	21.01.00	0.022	0.023	0	-0.002	-0.005	0.003
20739	27.01.00	0.007	-0.009	-0.013	0.001	-0.013	0.001
20739	21.01.00	0.022	0.016	0.005	0.018	-0.002	0.009
20752	19.01.00	-0.01	-0.024	-0.003	-0.009	-0.001	0.005
20752	21.01.00	0.005	-0.004	-0.004	-0.008	-0.009	0.001
20753	27.01.00	0.015	-0.002	-0.009	0.006	-0.01	0.007
20764	27.01.00	0.017	0.008	0.047	0	-0.007	0.04
20768	21.01.00	0.009	0.002	0	-0.002	-0.006	0.006
20773	21.01.00	0.013	0.017	0.001	-0.006	-0.004	0.014
20792	21.01.00	0.144	0.106	0.071	0.003	0.001	0.046
20793	21.01.00	0.012	0.008	-0.003	-0.004	-0.01	0.006
20809	19.01.00	-0.001	-0.035	-0.006	-0.013	-0.002	-0.005
20810	19.01.00	-0.021	-0.037	-0.008	-0.013	-0.004	-0.004
20826	21.01.00	0.001	0.002	0.001	0	-0.003	0.005
20827	22.01.00	0.018	0.022	0.001	0.003	-0.019	0.029
20832	19.01.00	0.01	-0.026	-0.005	-0.009	-0.002	0.009
20833	28.01.00	0.001	-0.001	-0.001	0.007	-0.004	0.011
20842	19.01.00	-0.004	-0.029	-0.006	-0.011	-0.002	0.007
20856	21.01.00	0	-0.002	0.002	0.017	-0.004	-0.003
20859	.	.	.	.	.	.	.
20881	21.01.00	0.323	0.002	0.004	0.582	0.001	0.058
20885	19.01.00	-0.005	-0.029	0.002	-0.005	-0.002	0.009
20893	19.01.00	0.122	-0.027	-0.005	0	-0.002	0.012
20899	21.01.00	0.022	0.064	0.004	0.019	-0.005	0.017
20900	21.01.00	0.005	-0.003	0.004	-0.006	-0.006	0.003
20900	21.01.00	0.033	0.013	0.015	0.028	-0.008	0.026
20914	19.01.00	0.006	-0.016	-0.005	-0.011	0.002	-0.001
20919	21.01.00	0.006	0.001	0.002	-0.004	-0.006	0.009
20920	19.01.00	-0.011	-0.034	-0.005	-0.012	-0.002	-0.006
20924	21.01.00	0.096	0.03	0.002	0.293	-0.002	0.039
20925	19.01.00	-0.009	-0.027	-0.006	0.02	-0.002	0.006
20928	28.01.00	0.004	0	-0.001	-0.001	-0.001	0.008
20940	22.01.00	0.02	0.01	0.006	-0.005	-0.022	0.007
20941	19.01.00	0.114	0.027	0.006	0.086	0.001	0.025
20944	22.01.00	0.01	0.009	-0.001	0.002	-0.021	0.01
20945	28.01.00	0.136	0.058	0.018	0.342	-0.001	0.09
20948	21.01.00	0.33	0.068	0.149	0.631	-0.006	0.056
20950	01.02.00	0.016	0.006	0.001	0.005	0	0.004
20959	22.01.00	0.006	0.008	0	0.012	-0.02	0.007

20961	26.01.00	0.085	0.025	-0.001	0.017	0	0.038
20972	21.01.00	0.016	0	0	0.014	-0.007	-0.004
20975	26.01.00	0.176	0.065	-0.003	0.043	0.001	0.154
20977	19.01.00	0.216	0.03	0.032	0.165	-0.002	0.06
20977	21.01.00	0.175	0.192	0.001	-0.005	-0.009	0.044
21013	19.01.00	-0.001	-0.022	-0.006	-0.011	-0.003	-0.003
21046	28.01.00	0.006	0.002	-0.001	0.003	-0.002	0.005
21065	27.01.00	0.005	0	-0.005	0.006	0.006	0.002
21072	21.01.00	-0.006	-0.007	-0.003	-0.009	-0.011	-0.008
21081	21.01.00	-0.002	-0.003	-0.001	-0.003	-0.007	-0.005
21104	22.01.00	0.146	0.214	0.002	0	-0.019	0.088
21109	21.01.00	0.12	0.013	0.017	0.153	-0.005	0.061
21148	22.01.00	0.002	0.006	0	-0.003	-0.017	0.002
21200	19.01.00	-0.002	-0.03	0.003	-0.005	-0.001	0.004
21213	22.01.00	0.062	0.033	0.003	0.1	-0.022	0.021
21231	19.01.00	0.002	-0.02	0.006	-0.009	0.002	-0.003
21247	21.01.00	0.003	0.004	0.009	-0.007	-0.005	-0.001
21264	22.01.00	0.001	0.001	0	-0.006	-0.019	-0.002
21295	28.01.00	0.02	0.016	0.008	0.008	-0.003	0.012
21302	19.01.00	-0.001	-0.031	0.001	-0.007	-0.001	0
21362	28.01.00	0.15	0.01	0.013	0.341	-0.002	0.07
20012	26.01.00	0.036	0.008	-0.006	-0.002	-0.001	0.051
20054	25.01.00	0.039	0.016	0.036	0.006	0.001	0.023
20056	25.01.00	0.084	0.074	0.013	0.023	0.004	0.03
20070	26.01.00	0.075	0.034	0	-0.001	-0.004	0.061
20079	24.01.00	0.083	0.057	-0.009	-0.005	-0.004	0.038
20086	26.01.00	0.03	0.01	-0.006	-0.003	-0.003	0.041
20090	27.01.00	0.028	0.005	0.001	0.001	-0.003	0.019
20095	27.01.00	0.121	0.04	-0.007	0.091	-0.01	0.049
20109	26.01.00	0.64	0.154	0.329	0.117	-0.003	0.225
20111	24.01.00	0.186	0.038	0.122	0.053	-0.005	0.098
20115	24.01.00	0.057	0.034	-0.007	0.015	-0.002	0.048
20118	26.01.00	0.115	0.031	0.015	0.004	-0.002	0.068
20128	27.01.00	0.024	-0.004	0.006	0.005	-0.003	0.045
20143	24.01.00	0.02	0	-0.004	-0.008	-0.007	0.036
20148	25.01.00	0.042	0.038	0.014	0.013	0.01	0.061
20161	24.01.00	0.424	0.181	0.019	0.184	-0.006	0.169
20164	25.01.00	0.009	0.007	0.006	0.003	0.001	0.025
20170	22.01.00	0.022	0.018	0.012	0.013	-0.019	0.04
20184	26.01.00	0.645	0.061	0.112	0.519	-0.004	0.152
20185	26.01.00	0.035	0.009	-0.005	0.002	0.001	0.011
20197	24.01.00	0.013	0.002	-0.008	-0.004	-0.004	0.031
20208	26.01.00	0.019	0.005	-0.007	-0.001	-0.001	0.047
20214	24.01.00	0.042	0.031	-0.002	0.003	-0.004	0.035
20224	26.01.00	0.023	0.006	-0.005	-0.001	-0.003	0.038
20227	27.01.00	0.072	0.036	0	0.019	0.001	0.042
20232	26.01.00	0.034	0.008	-0.005	-0.003	-0.003	0.079
20234	27.01.00	0.027	-0.001	0.001	-0.006	-0.007	0.03
20237	26.01.00	0.075	0.011	0.037	-0.003	-0.003	0.075
20251	24.01.00	0.118	0.07	-0.004	0.056	-0.004	0.069
20252	25.01.00	0.012	0.01	0.005	-0.001	-0.003	0.034
20254	25.01.00	0.047	0.038	0.007	0.029	0	0.032
20256	22.01.00	0.01	0.017	0.002	-0.005	-0.019	0.044
20266	26.01.00	0.053	0.016	0.003	0.009	-0.003	0.06
20269	24.01.00	0.107	0.071	-0.008	0.019	-0.004	0.062
20274	26.01.00	0.111	0.008	0.054	0	0.003	0.048
20275	24.01.00	0.009	0.004	-0.002	-0.005	-0.003	0.01
20277	25.01.00	0.025	0.023	0.017	0.011	0.005	0.033
20280	25.01.00	0.232	0.191	0.008	0.17	-0.005	0.059
20288	24.01.00	0.023	0.015	-0.006	0	0.001	0.02
20294	22.01.00	0.046	0.013	0.001	0.005	-0.015	0.033
20309	25.01.00	0.079	0.069	0.014	0.005	0.001	0.034
20315	25.01.00	0.186	0.124	0.02	0.008	-0.004	0.045
20325	25.01.00	0.05	0.028	0.008	0.041	-0.002	0.034

20329	27.01.00	0.088	0.074	0.001	0.008	-0.007	0.055
20332	24.01.00	0.039	0.028	-0.002	-0.006	-0.004	0.027
20336	22.01.00	0.023	0.017	0	0.021	-0.014	0.045
20338	24.01.00	0.052	-0.002	0.033	-0.006	-0.003	0.045
20343	26.01.00	0.041	0.016	0.011	0.012	-0.002	0.044
20344	21.01.00	0	-0.002	-0.001	-0.005	-0.008	-0.005
20347	27.01.00	0.005	-0.011	-0.012	-0.005	-0.011	0.013
20352	28.01.00	0.032	0.016	0	0.009	-0.004	0.043
20354	26.01.00	0.073	0.023	-0.005	0.008	-0.002	0.047
20369	24.01.00	0.022	0.016	-0.007	-0.003	-0.006	0.031
20374	26.01.00	0.054	0.013	-0.001	0.014	-0.002	0.043
20377	25.01.00	0.025	0.031	0.006	0.002	-0.002	0.026
20377	28.01.00	0.005	0.002	0	0.001	-0.002	0.017
20381	27.01.00	0.041	0.015	-0.003	0.005	-0.007	0.029
20387	26.01.00	0.548	0.227	0.009	0.157	-0.005	0.15
20388	25.01.00	0.023	0.022	0.009	-0.001	-0.002	0.033
20390	28.01.00	0.038	0.026	0.002	0.01	-0.003	0.052
20391	25.01.00	0.085	0.047	0.01	0.106	0.005	0.033
20395	28.01.00	0.169	0.021	0.002	0.45	0	0.121
20413	22.01.00	0.033	0.023	0.006	0.009	-0.014	0.037
20419	24.01.00	0.036	0.006	0	0.009	-0.006	0.059
20423	27.01.00	0.018	-0.003	-0.009	-0.007	-0.009	0.016
20427	26.01.00	0.015	0.003	-0.006	0.003	0	0.029
20441	26.01.00	0.021	0.008	-0.006	0.001	-0.002	0.022
20447	28.01.00	0.063	0.042	0.006	0.032	-0.002	0.055
20453	26.01.00	0.03	0.006	-0.003	0.005	0.001	0.037
20456	22.01.00	0.13	0.056	0.001	0.181	-0.02	0.041
20457	24.01.00	0.058	0.04	-0.006	0.012	-0.002	0.045
20458	26.01.00	0.064	0.018	0.006	0.003	0.001	0.044
20460	27.01.00	0.127	0.107	-0.008	0.002	-0.012	0.05
20461	28.01.00	0.018	0.018	0.003	0.004	0.001	0.022
20468	24.01.00	0.02	0.008	0.02	0.001	-0.002	0.022
20469	27.01.00	0.019	-0.004	-0.006	0.003	-0.01	0.021
20473	25.01.00	0.02	0.016	0.004	-0.001	-0.001	0.048
20474	24.01.00	0.041	0.019	-0.008	0.019	-0.003	0.032
20477	26.01.00	0.132	0.05	0	0.009	-0.005	0.059
20488	25.01.00	0.048	0.01	0.048	-0.006	-0.005	0.039
20494	26.01.00	0.03	0.01	0	0	0	0.049
20498	25.01.00	0.065	0.044	0.018	0.028	-0.004	0.028
20503	27.01.00	0.031	-0.005	-0.007	0.002	-0.011	0.043
20504	25.01.00	0.031	0.019	0.027	0	-0.002	0.029
20514	24.01.00	0.005	0.001	-0.005	-0.006	-0.005	0.03
20519	25.01.00	0.126	0.114	0.009	0.013	-0.004	0.059
20528	26.01.00	0.075	0.019	-0.002	0	-0.002	0.04
20535	26.01.00	0.109	0.041	-0.003	0.032	-0.004	0.041
20540	26.01.00	0.026	0.004	-0.001	0.006	-0.002	0.066
20544	22.01.00	0.027	0.029	0.001	0.005	-0.02	0.026
20545	24.01.00	0.151	0.052	0.033	0.044	-0.005	0.063
20558	24.01.00	0.029	0.012	-0.01	0.006	-0.006	0.022
20579	25.01.00	0.006	0.012	0.002	0.008	0	0.033
20580	26.01.00	0.029	0.007	0	0.001	-0.004	0.062
20587	25.01.00	0.578	0.364	0.289	0.292	0.001	0.202
20605	22.01.00	0.008	0.004	0.001	-0.003	-0.01	0.026
20608	26.01.00	0.308	0.018	0.01	0.37	-0.003	0.093
20625	27.01.00	0.275	0.193	-0.007	0.079	-0.007	0.11
20646	22.01.00	0.056	0.017	0.007	0.05	-0.019	0.026
20745	25.01.00	0.049	0.038	0	-0.001	-0.005	0.047
ADULTS							
10054	25.01.00	0.137	0.067	0.135	0.044	0.001	0.06
10056	25.01.00	0.106	0.061	0.048	0.069	0.003	0.055
10070	26.01.00	0.136	0.012	0.02	0.148	-0.001	0.153
10086	26.01.00	0.431	0.146	0.012	0.288	-0.004	0.157
10095	22.01.00	0.061	0.025	0.016	0.051	-0.019	0.086
10109	26.01.00	0.239	0.05	0.054	0.1	-0.003	0.101

10136	26.01.00	0.086	0.011	0.012	0.002	0	0.093
10148	25.01.00	0.056	0.041	0.027	0.007	0	0.061
10161	24.01.00	0.229	0.159	0.001	0.012	-0.006	0.127
10164	25.01.00	0.155	0.119	0.07	0.019	0.001	0.071
10170	22.01.00	0.047	0.024	0.028	0.013	-0.018	0.045
10234	27.01.00	0.252	0.147	0.012	0.05	-0.007	0.122
10237	26.01.00	0.332	0.128	0.008	0.018	0	0.115
10239	24.01.00	0.011	0.01	-0.005	-0.005	-0.006	0.049
10252	25.01.00	0.123	0.095	0.062	0.023	0.008	0.094
10254	25.01.00	0.478	0.432	0.164	0.022	-0.001	0.134
10269	24.01.00	0.08	0.021	0.01	0.009	-0.006	0.073
10274	24.01.00	0.136	0.036	0.066	0.025	-0.002	0.078
10275	24.01.00	0.135	0.062	0.002	0.096	-0.003	0.049
10297	25.01.00	0.211	0.055	0.027	0.57	0.004	0.068
10309	25.01.00	0.121	0.072	0.071	0.023	-0.001	0.048
10315	25.01.00	0.195	0.084	0.058	0.39	0	0.07
10325	25.01.00	0.197	0.179	0.027	0.045	-0.001	0.087
10326	26.01.00	0.098	0.047	0.003	0.006	0	0.074
10327	27.01.00	0.391	-0.003	0.025	0.496	-0.007	0.1
10331	27.01.00	0.436	0.062	0.018	0.526	-0.01	0.14
10332	24.01.00	0.08	0.03	0.012	0.007	-0.005	0.059
10336	22.01.00	0.046	0.027	0.013	0.014	-0.019	0.049
10338	25.01.00	0.16	0.15	0.029	0.041	0.004	0.133
10352	27.01.00	0.203	0.071	0.02	0.006	-0.009	0.105
10354	26.01.00	0.357	0.21	0.025	0.032	-0.003	0.141
10365	25.01.00	0.339	0.246	0.037	0.386	0.005	0.089
10374	26.01.00	0.95	0.09	0.038	1.074	0.003	0.234
10381	25.01.00	0.095	0.028	0.056	0.103	0	0.063
10389	24.01.00	0.1	0.041	0.026	0.022	-0.003	0.052
10390	27.01.00	0.135	0.016	0.003	0.317	-0.007	0.06
10399	24.01.00	0.104	0.062	0.018	0.019	-0.003	0.06
10402	24.01.00	0.737	0.175	0.022	0.851	-0.002	0.213
10413	22.01.00	0.094	0.055	0.056	0.031	-0.017	0.076
10420	24.01.00	0.789	0.616	0.046	0.076	-0.002	0.261
10430	25.01.00	0.508	0.371	0.127	0.231	-0.003	0.128
10441	26.01.00	0.063	0.017	-0.002	0.022	-0.003	0.05
10442	22.01.00	0.202	0.194	0.106	0.092	-0.019	0.176
10447	27.01.00	0.093	0.011	0.04	0.018	-0.005	0.061
10459	24.01.00	0.271	0.097	0.039	0.141	0.001	0.108
10460	27.01.00	0.467	0.128	0.022	0.603	-0.011	0.18
10461	27.01.00	0.282	0.17	0.037	0.028	-0.009	0.119
10468	24.01.00	0.025	0.005	0.004	-0.005	-0.003	0.013
10473	24.01.00	0.043	0.013	0.007	0.026	-0.005	0.046
10474	24.01.00	0.322	0.207	0.045	0.037	-0.005	0.18
10477	25.01.00	0.169	0.083	0.152	0.084	0.001	0.08
10486	27.01.00	0.029	0.009	-0.007	0.001	-0.012	0.042
10488	25.01.00	0.325	0.267	0.048	0.182	0.001	0.116
10496	25.01.00	0.218	0.104	0.128	0.104	0.001	0.087
10498	.	.	.	.	.	.	.
10503	27.01.00	0.052	0.012	-0.005	0.038	-0.005	0.07
10514	24.01.00	0.026	0.011	0.001	-0.002	-0.007	0.053
10519	25.01.00	0.243	0.079	0.112	0.069	-0.003	0.116
10528	24.01.00	0.185	0.077	0.05	0.029	-0.003	0.139
10535	25.01.00	0.264	0.113	0.032	0.379	0.003	0.107
10540	26.01.00	0.309	0.212	0.015	0.024	-0.003	0.131
10544	22.01.00	0.022	0.026	0.004	0.007	-0.016	0.034
10545	24.01.00	0.226	0.087	0.023	0.138	-0.002	0.1
10555	24.01.00	0.223	0.06	0.071	0.112	-0.004	0.119
10558	24.01.00	0.258	0.105	0.034	0.109	-0.006	0.094
10575	22.01.00	0.091	0.059	0.035	0.125	-0.018	0.065
10579	24.01.00	0.109	0.04	0.038	0.035	0.001	0.119
10580	26.01.00	0.444	0.071	0.179	0.067	0.003	0.172
10587	24.01.00	0.582	0.224	0.138	0.155	-0.007	0.234
10591	24.01.00	0.113	0.048	0.021	0.026	-0.001	0.066

10605	22.01.00	0.08	0.071	0.036	0.12	-0.015	0.078
10612	25.01.00	0.188	0.06	0.043	0.331	0.001	0.068
10626	25.01.00	0.174	0.056	0.113	0.171	0	0.115
10641	27.01.00	0.42	0.026	0.059	0.277	-0.007	0.219
10646	22.01.00	0.057	0.026	0.053	0.036	-0.018	0.066
10695	27.01.00	0.246	0.041	0.165	0.014	-0.009	0.1
10699	22.01.00	0.042	0.038	0.031	-0.002	-0.019	0.058
11090	26.01.00	0.082	0.018	0.007	-0.001	-0.002	0.109
11180	27.01.00	0.357	0.034	-0.012	0.385	-0.012	0.137
10396	27.01.00	0.312	0.057	0.09	0.386	-0.007	0.096
10497	21.01.00	0.302	0.141	0.007	0.408	-0.005	0.077
10508	27.01.00	0.061	0.035	-0.006	-0.006	-0.012	0.044
10542	22.01.00	0.123	0.081	0.013	0.091	-0.02	0.101
10557	27.01.00	0.218	0.076	0.033	0.014	-0.007	0.101
10571	19.01.00	0.033	-0.014	0.021	0.004	0	0.037
10581	27.01.00	0.076	0.003	0.005	0.055	-0.011	0.066
10586	19.01.00	0.158	0.026	0.18	0.028	-0.002	0.109
10588	19.01.00	0.093	-0.015	0.014	0.135	-0.001	0.049
10616	19.01.00	0.771	-0.014	0.02	0.926	0	0.159
10617	19.01.00	0.233	-0.022	0.195	0.045	-0.002	0.076
10637	22.01.00	0.107	0.054	0.014	0.104	-0.02	0.116
10659	19.01.00	0.242	0.047	0.06	-0.006	0	0.092
10681	22.01.00	0.088	0.135	0.016	0.03	-0.017	0.156
10692	27.01.00	0.173	0.045	0.023	0.166	-0.008	0.076
10701	21.01.00	0.051	0.018	0.039	0.02	-0.006	0.032
10719	22.01.00	0.869	0.035	0.118	1.341	-0.018	0.265
10732	21.01.00	0.03	0.009	0.045	0.023	-0.002	0.02
10739	27.01.00	0.07	0.025	0.016	-0.003	-0.009	0.04
10746	22.01.00	0.415	0.09	0.107	0.633	-0.019	0.215
10764	27.01.00	0.031	-0.002	0.014	-0.002	-0.004	0.031
10792	.	.	.	.	.	.	.
10794	19.01.00	0.127	0.018	0.023	0.02	-0.002	0.07
10809	19.01.00	0.053	-0.018	0.028	-0.004	0	0.047
10810	19.01.00	0.266	0.012	0	0.339	-0.001	0.073
10819	22.01.00	0.109	0.091	0.044	0.013	-0.015	0.103
10827	26.01.00	0.331	0.094	0.045	0.052	0.002	0.191
10833	27.01.00	0.086	0.028	0.034	-0.004	-0.006	0.075
10842	19.01.00	0.089	-0.007	0.026	0.016	0	0.048
10856	21.01.00	0.061	0.069	0.013	0.013	0.006	0.027
10859	21.01.00	0.139	0.128	0.004	0.002	-0.008	0.065
10859	21.01.00	0.116	0.115	0.006	0.001	-0.009	0.055
10876	21.01.00	0.143	0.051	0.056	0.094	-0.003	0.047
10881	21.01.00	0.635	0.013	0.029	1.183	-0.002	0.157
10885	19.01.00	0.016	-0.018	0.008	-0.004	-0.001	0.004
10893	19.01.00	0.196	0.039	0.034	0.018	-0.001	0.096
10899	21.01.00	0.081	0.051	0.052	0.006	-0.005	0.035
10900	21.01.00	0.218	0.075	0.066	0.243	-0.006	0.08
10910	22.01.00	0.318	0.319	0.096	0.073	-0.02	0.172
10919	21.01.00	0.171	0.128	0.026	0.051	-0.002	0.061
10925	19.01.00	0.174	0.024	0.014	0.089	0.002	0.067
10933	21.01.00	0.215	0.053	0.191	0.031	0	0.1
10940	22.01.00	0.066	0.032	0.038	0.011	-0.02	0.085
10950	27.01.00	0.27	0.063	0.047	0.078	-0.007	0.099
10959	22.01.00	0.122	0.061	0.028	0.071	-0.02	0.078
10966	21.01.00	0.207	0.007	0.014	0.511	-0.007	0.061
11002	19.01.00	0.048	0.007	0.01	0.009	-0.001	0.017
11013	19.01.00	0.355	0.134	0.03	0.022	-0.001	0.112
11060	19.01.00	0.118	-0.027	0.019	0.102	-0.003	0.051
11065	27.01.00	0.045	0.006	0	0.053	-0.006	0.059
11099	19.01.00	0.212	-0.004	0.13	0.022	-0.001	0.076
11104	22.01.00	0.169	0.077	0.078	0.123	-0.019	0.095
11143	21.01.00	0.213	0.172	0.039	0.077	0.002	0.069
11200	19.01.00	0.076	-0.017	0.013	0.016	0	0.066
11231	19.01.00	0.091	0.022	0	-0.006	-0.002	0.066

11247	21.01.00	0.137	0.148	0.016	-0.003	-0.006	0.048
11263	21.01.00	0.045	0.016	0.017	0.021	-0.006	0.015
11295	27.01.00	0.148	0.06	0.016	0.138	-0.007	0.066
11302	19.01.00	0.226	0.028	0.049	0.039	-0.003	0.065
11362	27.01.00	0.563	0.007	0.108	0.778	-0.009	0.181

Appendix 5: Clinical data

Study number	Parasitaemia a month before sampling	Parasitaemia at time of sampling	Parasitaemia a month after sampling	Hb at time of sampling	Hb a month after sampling	Rate of parasitaemia	Rate high density parasitaemia	Rate of fever	Clinical malaria episode	Rate of severe anaemia (Hb<5g/dl)	Rate of anaemia (Hb<11g/dl)
CHILDREN											
20396	.	0	.	8.3	.	.	.	.	.	.	.
20489	.	800	.	7.8	.	.	.	.	.	.	.
20497	.	0	.	7.3	.	.	.	.	.	.	.
20508	.	0	.	10.7	.	.	.	.	.	.	.
20525	.	5921	.	8.3	.	.	.	.	.	.	.
20527	.	0	.	9	.	.	.	.	.	.	.
20571	.	45606	.	7	.	.	.	.	.	.	.
20581	.	0	.	11.7	.	.	.	.	.	.	.
20588	.	0	.	10.3	.	.	.	.	.	.	.
20616	.	12028	.	3.2	.	.	.	.	.	.	.
20617	.	0	.	10.8	.	.	.	.	.	.	.
20618	.	0	.	.	.	.	.	.	.	.	.
20620	.	0	.	10.3	.	.	.	.	.	.	.
20634	.	0	.	9.3	.	.	.	.	.	.	.
20647	.	0	.	10.3	.	.	.	.	.	.	.
20656	.	0	.	10.8	.	.	.	.	.	.	.
20659	.	0	.	10.7	.	.	.	.	.	.	.
20692	.	77636	.	18	.	.	.	.	.	.	.
20701	.	453	.	9.2	.	.	.	.	.	.	.
20719	.	1120	.	8.3	.	.	.	.	.	.	.
20732	.	320	.	9.3	.	.	.	.	.	.	.
20739	.	1334	.	10.3	.	.	.	.	.	.	.
20739	.	560	.	7.4	.	.	.	.	.	.	.
20752	.	1547	.	11.3	.	.	.	.	.	.	.
20752	.	480	.	12.7	.	.	.	.	.	.	.
20753	.	22723	.	8.7	.	.	.	.	.	.	.
20764	.	0	.	11.3	.	.	.	.	.	.	.
20768	.	0	.	11.7	.	.	.	.	.	.	.
20773	.	533	.	7	.	.	.	.	.	.	.

20792	.	0	.	11.2	.	.	.	.	.	.	.
20793	.	0	.	.	.	.	.	.	.	.	.
20809	.	0	.	11	.	.	.	.	.	.	.
20810	.	0	.	12.7	.	.	.	.	.	.	.
20826	.	3574	.	10.1	.	.	.	.	.	.	.
20827	.	110254	.	11.7	.	.	.	.	.	.	.
20832	.	0	.	8.3	.	.	.	.	.	.	.
20833	.	0	.	9.7	.	.	.	.	.	.	.
20842	.	0	.	9.7	.	.	.	.	.	.	.
20856	.	0	.	10.6	.	.	.	.	.	.	.
20859	.	5387	.	.	.	.	.	.	.	.	.
20881	.	0	.	9.5	.	.	.	.	.	.	.
20885	.	0	.	9	.	.	.	.	.	.	.
20893	.	0	.	7.8	.	.	.	.	.	.	.
20899	.	0	.	11	.	.	.	.	.	.	.
20900	.	0	.	.	.	.	.	.	.	.	.
20900	.	0	.	.	.	.	.	.	.	.	.
20914	.	0	.	12.7	.	.	.	.	.	.	.
20919	.	0	.	12	.	.	.	.	.	.	.
20920	.	0	.	12	.	.	.	.	.	.	.
20924	.	53	.	3.2	.	.	.	.	.	.	.
20925	.	80	.	11	.	.	.	.	.	.	.
20928	.	16535	.	6.5	.	.	.	.	.	.	.
20940	.	0	.	14.7	.	.	.	.	.	.	.
20941	.	0	.	11.2	.	.	.	.	.	.	.
20944	.	0	.	11	.	.	.	.	.	.	.
20945	.	0	.	6.9	.	.	.	.	.	.	.
20948	.	0	.	10.3	.	.	.	.	.	.	.
20950	.	.	.	.	.	.	.	.	.	.	.
20959	.	0	.	8.1	.	.	.	.	.	.	.
20961	.	8721	.	7.5	.	.	.	.	.	.	.
20972	.	0	.	16	.	.	.	.	.	.	.
20975	.	1467	.	8.8	.	.	.	.	.	.	.
20977	.	0	.	8.3	.	.	.	.	.	.	.
20977	.	0	.	7.2	.	.	.	.	.	.	.

21013	.	0	.	10.7	.	.	.	.	.	.	.
21046	.	0	.	11.3	.	.	.	.	.	.	.
21065	.	0	.	12.7	.	.	.	.	.	.	.
21072	.	0	.	10.4	.	.	.	.	.	.	.
21081	.	0	.	11	.	.	.	.	.	.	.
21104	.	35311	.	8.6	.	.	.	.	.	.	.
21109	.	0	.	11.3	.	.	.	.	.	.	.
21148	.	0	.	11.3	.	.	.	.	.	.	.
21200	.	0	.	11	.	.	.	.	.	.	.
21213	.	0	.	13	.	.	.	.	.	.	.
21231	.	0	.	14.7	.	.	.	.	.	.	.
21247	.	0	.	13.5	.	.	.	.	.	.	.
21264	.	0	.	3.2	.	.	.	.	.	.	.
21295	.	0	.	11.7	.	.	.	.	.	.	.
21302	.	0	.	11.7	.	.	.	.	.	.	.
21362	.	0	.	13.3	.	.	.	.	.	.	.
20012	0	0	.	.	.	0.3	0.27	0.14	0.11	0.04	0.42
20054	0	0	0	12.4	12.1	0.13	0.03	0.2	0.07	0.03	0.47
20056	160	1200	80	10.8	10.6	0.36	0.14	0.12	0.12	0.04	0.68
20070	0	35151	0	3	10.3	0.13	0.09	0.09	0.04	0.05	0.47
20079	33551	0	0	10.1	10.6	0.23	0.13	0.14	0.11	0.04	0.64
20086	17362	0	0	10.8	4.3	0.44	0.19	0.13	0.07	0.07	0.53
20090	587	0	0	11.3	12.2	0.5	0.25	0.3	0.26	0.03	0.76
20095	1067	0	.	3.2	.	0.3	0.15	0	0	0.08	0.32
20109	0	0	0	10.4	.	0.41	0.14	0.15	0.08	0	0.6
20111	0	1227	.	10.9	.	0.55	0.1	0.05	0	0.05	0.65
20115	0	5707	.	3	.	0.38	0.13	0.13	0.07	0.14	0.5
20118	0	0	.	3.1	.	0.5	0.18	0.13	0.08	0.1	0.81
20128	0	0	.	10.8	.	0.15	0.15	0.04	0	0.04	0.64
20143	107	0	.	3	.	0.19	0.1	0.08	0.04	0.21	0.84
20148	17336	0	6774	3.3	2.8	0.28	0.16	0.1	0.1	0.13	0.79
20161	0	0	0	11.2	10.6	0	0	0.08	0.04	0	0.2
20164	0	0	0	12.4	11.7	0.04	0	0	0	0.04	0.25
20170	10001	240	267	3.1	2.9	0.67	0.1	0.2	0.2	0.11	0.84
20184	0	0	0	2.7	2.8	0.13	0.09	0.18	0.05	0.17	0.83

20185	0	347	.	10.5	11.1	0.5	0.28	0.11	0.11	0.07	0.72
20197	0	427	.	3.3	.	0.3	0.04	0.1	0.05	0.2	0.6
20208	0	0	.	11.3	.	0.08	0	0.04	0.04	0.09	0.39
20214	107	0	0	.	.	0.06	0	0	0	0	0.5
20224	0	0	0	2.9	3.2	0.1	0	0	0	0.14	0.43
20227	0	10721	0	2.7	.	0.11	0.06	0.17	0.06	0.06	0.18
20232	0	0	0	10.5	10.6	0.14	0.05	0.05	0	0.05	0.6
20234	0	0	0	12.5	13.1	0	0	0.09	0	0.04	0.13
20237	0	0	0	10.6	.	0.3	0.15	0.05	0.05	0.06	0.53
20251	0	12455	0	11.4	11.4	0.12	0.06	0.24	0.06	0	0.24
20252	3120	0	0	2.8	6.2	0.43	0.14	0.05	0.05	0.05	0.75
20254	26003	0	.	2.6	.	0.78	0.44	0.28	0.22	0.06	0.75
20256	0	0	0	12.8	.	0	0	0	0	0	0.12
20266	0	827	.	2.6	.	0.33	0.2	0	0	0.15	0.77
20269	14215	9895	.	6.4	.	0.55	0.2	0.11	0.06	0.11	0.74
20274	0	0	.	3.7	.	0.13	0.04	0.09	0.09	0.05	0.86
20275	0	0	.	11.8	.	0	0	0.06	0	0	0.44
20277	533	24563	3520	3.2	3.1	0.46	0.27	0.04	0.04	0.12	0.81
20280	320	110360	0	2.9	3.2	0.57	0.3	0.32	0.28	0.08	0.8
20288	14535	0	880	3.2	3.1	0.7	0.35	0	0	0.11	0.84
20294	0	0	0	10.2	10.7	0.13	0.09	0.09	0.05	0	0.59
20309	0	0	0	11.4	11.8	0.13	0.04	0.1	0.05	0.05	0.52
20315	5947	0	9975	10.5	2.9	0.46	0.36	0.27	0.27	0.09	0.91
20325	3120	0	773	3.1	2.8	0.44	0.06	0.06	0.06	0.19	0.69
20329	133	2987	.	2.6	.	0.56	0.22	0.22	0.11	0.33	0.89
20332	0	0	0	3.3	10.2	0.38	0.14	0.17	0.11	0.06	0.5
20336	0	0	0	4	10.6	0.37	0.29	0.12	0.08	0.07	0.48
20338	0	0	.	11.1	.	0	0	0	0	0.09	0.64
20343	0	0	.	2.9	.	0.27	0.05	0.18	0.05	0.1	0.67
20344	0	0	0	3.3	2.7	0	0	0.07	0	0.25	0.58
20347	0	0	.	3.3	.	0	0	0	0	0.17	0.67
20352	27	187	80	10.2	3	0.59	0.31	0	0	0.08	0.67
20354	0	0	.	11.2	.	0.56	0.13	0	0	0.07	0.86
20369	0	0	0	10.8	3.3	0	0	0.06	0	0.06	0.47
20374	960	0	0	10.6	11.7	0.24	0.06	0.06	0.06	0.06	0.53

20377	0	0	0	12.3	11.9	0.35	0.23	0.04	0.04	0.08	0.5
20377	0	0	0	12.3	11.9	0.35	0.23	0.1	0.1	0.08	0.5
20381	160	1520	.	11.5	.	0.64	0.27	0	0	0.05	0.38
20387	27	62061	0	3.3	11.3	0.27	0.06	0	0	0.12	0.41
20388	0	0	0	10.1	3	0	0	0	0	0.1	0.9
20390	0	0	2240	2.6	6.4	0.32	0.2	0.14	0.14	0.17	0.78
20391	0	1547	9841	2.7	2.9	0.16	0.05	0.16	0.05	0.19	0.5
20395	0	0	0	2.7	.	0.33	0.22	0.04	0.04	0.05	0.86
20413	800	0	0	5.9	2.6	0.05	0	0.05	0.05	0.05	0.55
20419	0	0	0	12.5	11.1	0.14	0.05	0.1	0.05	0.05	0.25
20423	0	0	0	10.9	11.4	0.25	0.25	0.06	0	0.06	0.88
20427	0	0	.	11.6	.	0	0	0	0	0	0.2
20441	1600	0	.	11.6	.	0.44	0.06	0.06	0.06	0	0.47
20447	27	0	0	11.5	10.8	0.18	0.09	0.14	0.1	0	0.33
20453	0	0	.	3.7	.	0.19	0.13	0.13	0.13	0.06	0.75
20456	2134	3174	187	2.8	.	0.58	0.16	0.12	0.12	0.19	0.69
20457	0	0	.	3.3	11.3	0	0	0.06	0	0.07	0.2
20458	0	0	.	10.8	.	0.5	0.22	0.22	0.17	0	0.59
20460	0	0	0	10.6	.	0.12	0.06	0.13	0.13	0.13	0.31
20461	0	0	0	12.2	15.7	0.22	0.06	0.06	0	0	0.63
20468	0	0	0	12.1	12.1	0	0	0	0	0	0.56
20469	0	0	0	.	.	0.08	0	0.08	0	0	0.5
20473	0	0	0	3.1	3.2	0.13	0.07	0.07	0.07	0.21	0.71
20474	0	0	0	3.1	3.2	0.18	0.06	0.07	0	0.13	0.8
20477	0	933	0	3.3	3.1	0.1	0.05	0.05	0	0.11	0.5
20488	0	0	0	3.1	.	0.05	0.05	0.05	0	0.24	0.47
20494	0	0	.	3.1	.	0	0	0	0	0.08	0.33
20498	0	0	0	11.1	10.3	0.06	0	0.06	0.06	0	0.41
20503	0	0	.	6.6	.	0.4	0.07	0.21	0.14	0	0.81
20504	347	0	0	2.6	2.6	0.42	0.12	0.1	0.05	0.23	0.91
20514	0	0	0	11.5	11.7	0	0	0.06	0	0	0.06
20519	39525	0	0	10.6	11.8	0.55	0.27	0.2	0.15	0.05	0.68
20528	0	0	.	11.1	.	0.29	0.13	0	0	0	0.5
20535	18189	0	14722	2.5	10.1	0.11	0.11	0.05	0.05	0.11	0.53
20540	0	0	0	10.8	.	0	0	0	0	0.09	0.27

20544	4107	0	.	4	.	0.08	0	0.23	0.08	0.08	0.42
20545	0	0	.	3	.	0.53	0.21	0.11	0.11	0.17	0.56
20558	0	3707	0	2.7	2.6	0.1	0	0.14	0	0.15	0.45
20579	0	0	10348	10.4	5.8	0.06	0.06	0.13	0.06	0.13	0.56
20580	0	0	0	2.6	.	0.27	0	0.21	0.21	0	0.47
20587	21043	0	0	.	.	0.53	0.32	0.11	0.11	0	0.69
20605	960	0	0	.	10.6	0.47	0.12	0.06	0	0	0.5
20608	0	160	667	2.7	3.1	0.59	0.14	0.19	0.19	0.14	0.81
20625	0	4747	.	2.7	.	0.06	0	0	0	0.07	0.57
20646	7814	213	.	3.3	.	0.7	0.23	0.24	0.24	0.08	0.88
20745	.	0	240	10.8	.	0.07	0	0	0	0	0.54
ADULTS											
10054	0	0	0	12	11.8	0.07	0	0.08	0	0	0.19
10056	0	80	0	12	12	0.04	0	0.04	0	0	0.08
10070	0	0	0	8.8	8.8	0	0	0.04	0	0	0.68
10086	0	0	0	10.3	.	0.06	0	0.06	0	0	0.38
10095	720	0	0	7.6	10.6	0.29	0	0.04	0	0	0.29
10109	27	0	0	11.5	11.8	0.12	0	0.04	0	0	0.19
10136	0	0	.	9.2	.	0.14	0	0	0	0	0.15
10148	0	0	0	6.8	6.8	0.29	0	0	0	0	0.97
10161	0	0	0	11.8	12.3	0	0	0	0	0	0
10164	0	0	0	12.1	11.9	0.04	0	0	0	0	0.19
10170	187	0	0	10.6	11.7	0.06	0	0.06	0	0	0.22
10234	0	0	0	13.1	13.2	0	0	0	0	0	0.12
10237	0	0	0	9.7	10.3	0.05	0	0	0	0	0.5
10239	0	0	0	9	16.5	0.13	0.09	0	0	0	0.35
10252	0	0	0	10.4	.	0	0	0.1	0	0	0.48
10254	0	0	.	9.9	.	0.69	0.06	0	0	0	0.64
10269	0	0	.	10.7	.	0.11	0	0	0	0	0.33
10274	0	0	0	11.1	11.8	0.09	0	0.09	0	0	0.44
10275	0	0	.	12	.	0.05	0	0	0	0	0.35
10297	0	0	.	12.5	.	0.17	0	0.04	0.04	0	0.18
10309	0	0	0	11.5	12.4	0	0	0.13	0	0	0.21
10315	1200	0	0	11.2	11.4	0.23	0	0	0	0	0.23

10325	0	0	0	11.6	11.2	0.06	0	0.06	0	0	0.53
10326	0	0	0	10.9	10.3	0.12	0.04	0	0	0	0.65
10327	0	0	0	11.1	10.3	0	0	0	0	0	0.71
10331	27	0	0	11.9	14.3	0.13	0	0	0	0	0.26
10332	0	0	0	11	.	0.1	0	0	0	0	0.32
10336	0	0	133	12.5	12.8	0.2	0	0	0	0	0.14
10338	0	0	0	5.5	5.7	0.19	0	0	0	0.04	0.68
10352	0	0	0	8.2	10	0.24	0.04	0	0	0	0.79
10354	0	0	.	3.4	.	0.38	0	0	0	0.14	1
10365	0	0	0	11.6	.	0.09	0.05	0	0	0	0.29
10374	0	0	0	12.3	15	0	0	0.05	0	0	0.26
10381	0	187	0	12.4	13	0.31	0	0	0	0	0.22
10389	0	0	0	11.8	.	0.05	0	0	0	0	0.23
10390	0	0	0	12.4	13.3	0	0	0	0	0	0.23
10399	0	0	0	8.8	9.1	0.04	0	0.09	0	0	0.95
10402	0	0	0	9.3	.	0	0	0	0	0	0.8
10413	0	0	.	11.7	.	0.17	0	0.04	0.04	0	0.25
10420	0	0	0	10.4	.	0	0	0	0	0	0.56
10430	0	0	80	12.6	12.9	0.21	0	0	0	0	0.17
10441	0	0	.	10.5	.	0.42	0	0.18	0.12	0	0.42
10442	0	0	.	12	10.8	0	0	0	0	0	0.21
10447	0	0	0	12.8	14	0.05	0	0	0	0	0.11
10459	0	0	.	11.2	.	0.05	0	0	0	0.05	0.6
10460	0	0	.	11.4	.	0.15	0	0.05	0.05	0.06	0.37
10461	0	0	0	12.5	4.3	0.1	0	0	0	0.06	0.22
10468	0	0	0	3.2	10.4	0.14	0	0	0	0.1	0.81
10473	613	0	0	10.3	10.4	0.25	0	0.05	0	0.06	0.79
10474	80	0	0	2.4	2.5	0.33	0	0	0	0.28	0.95
10477	0	0	0	11.7	4	0	0	0	0	0.05	0.43
10486	0	0	.	3.2	.	0.2	0	0.06	0	0.07	0.67
10488	0	0	0	.	12.4	0.18	0	0.05	0	0	0.15
10496	0	0	0	12.1	12.5	0.09	0	0.05	0	0	0.1
10498	0	0	0	12.8	12.6	0.27	0	0.05	0	0.05	0.23
10503	27	0	.	2.6	.	0.2	0	0	0	0.2	1
10514	0	0	0	11.4	12.2	0.14	0	0.1	0	0	0.1

10519	0	0	.	10.7	.	0.07	0	0	0	0	0.81
10528	0	0	0	3.3	10.8	0	0	0	0	0.13	0.69
10535	0	0	0	10.6	10.2	0	0	0	0	0.05	0.45
10540	0	0	0	12.3	.	0	0	0	0	0	0.31
10544	0	0	.	12.1	.	0	0	0.14	0	0	0.14
10545	0	0	.	12.1	.	0	0	0	0	0	0.35
10555	0	0	0	10.5	3.7	0	0	0	0	0.05	0.45
10558	0	0	0	4.3	13.7	0.22	0	0.04	0	0.05	0.35
10575	0	0	0	12.5	11.9	0.06	0	0	0	0	0.28
10579	0	0	0	3.3	3.3	0	0	0.05	0	0.11	0.53
10580	0	0	.	19.2	.	0.06	0.06	0	0	0	0.53
10587	0	0	.	3.3	.	0.05	0	0	0	0.11	0.79
10591	0	0	0	12.4	14.5	0.1	0	0	0	0	0.05
10605	0	0	0	.	11.5	0	0	0	0	0	0.22
10612	0	0	0	10.7	14.2	0.13	0	0.07	0	0	0.47
10626	27	0	0	11.6	11.6	0.28	0	0	0	0	0.22
10641	0	0	0	10.2	.	0	0	0	0	0	0.42
10646	0	0	0	9	17.6	0.1	0	0	0	0	0.67
10695	0	0	0	.	.	0.17	0	0	0	0	0.8
10699	0	0	0	10.1	10.7	0	0	0	0	0.16	0.77
11090	0	0	0	11.5	.	0	0	0	0	0.11	0.33
11180	0	0	0	3.3	.	0	0	0	0	0.2	1
10396	.	0	.	12	.	.	.	.	.	.	.
10497	.	0	.	7.7	.	.	.	.	.	.	.
10508	.	0	.	11.7	.	.	.	.	.	.	.
10542	.	0	.	10.7	.	.	.	.	.	.	.
10557	.	80	.	.	.	.	.	.	.	.	.
10571	.	0	.	13.3	.	.	.	.	.	.	.
10581	.	0	.	10.7	.	.	.	.	.	.	.
10586	.	0	.	.	.	.	.	.	.	.	.
10588	.	0	.	13	.	.	.	.	.	.	.
10616	.	0	.	11.9	.	.	.	.	.	.	.
10617	.	0	.	13.4	.	.	.	.	.	.	.
10637	.	0	.	12.1	.	.	.	.	.	.	.
10659	.	0	.	9.3	.	.	.	.	.	.	.

10681	.	0	.	9.1	.	.	.	.	.	.	.
10692	.	0	.	10.7	.	.	.	.	.	.	.
10701	.	0	.	10.9	.	.	.	.	.	.	.
10719	.	0	.	10	.	.	.	.	.	.	.
10732	.	53	.	8.6	.	.	.	.	.	.	.
10739	.	907	.	12.7	.	.	.	.	.	.	.
10746	.	0	.	11.7	.	.	.	.	.	.	.
10764	.	533	.	13	.	.	.	.	.	.	.
10792	.	0	.	11.4	.	.	.	.	.	.	.
10794	.	0	.	11.7	.	.	.	.	.	.	.
10809	.	0	.	11.7	.	.	.	.	.	.	.
10810	.	240	.	9.7	.	.	.	.	.	.	.
10819	.	0	.	12	.	.	.	.	.	.	.
10827	.	0	.	10.9	.	.	.	.	.	.	.
10833	.	0	.	8.5	.	.	.	.	.	.	.
10842	.	0	.	9	.	.	.	.	.	.	.
10856	.	27	.	9.9	.	.	.	.	.	.	.
10859	.	0	.	.	.	.	.	.	.	.	.
10859	.	0	.	.	.	.	.	.	.	.	.
10876	.	27	.	12.1	.	.	.	.	.	.	.
10881	.	0	.	10.9	.	.	.	.	.	.	.
10885	.	0	.	16.7	.	.	.	.	.	.	.
10893	.	0	.	11.1	.	.	.	.	.	.	.
10899	.	0	.	5.6	.	.	.	.	.	.	.
10900	.	0	.	11.6	.	.	.	.	.	.	.
10910	.	0	.	12.6	.	.	.	.	.	.	.
10919	.	0	.	11.3	.	.	.	.	.	.	.
10925	.	53	.	10	.	.	.	.	.	.	.
10933	.	0	.	13.3	.	.	.	.	.	.	.
10940	.	0	.	8.7	.	.	.	.	.	.	.
10950	.	.	.	.	.	.	.	.	.	.	.
10959	.	0	.	3.6	.	.	.	.	.	.	.
10966	.	27	.	9.1	.	.	.	.	.	.	.
11002	.	107	.	10.3	.	.	.	.	.	.	.
11013	.	0	.	11.3	.	.	.	.	.	.	.

11060	.	0	.	11.1	.	.	.	.	.	.	.
11065	.	0	.	9	.	.	.	.	.	.	.
11099	.	9335	.	9.7	.	.	.	.	.	.	.
11104	.	0	.	9.9	.	.	.	.	.	.	.
11143	.	0	.	9.7	.	.	.	.	.	.	.
11200	.	0	.	11.3	.	.	.	.	.	.	.
11231	.	0	.	13.3	.	.	.	.	.	.	.
11247	.	27	.	8.2	.	.	.	.	.	.	.
11263	.	0	.	4.3	.	.	.	.	.	.	.
11295	.	0	.	10.7	.	.	.	.	.	.	.
11302	.	0	.	6.7	.	.	.	.	.	.	.
11362	.		.	11.3	.	.	.	.	.	.	.

Appendix 6: Consent for parents or guardian of infants and children participating in malaria vaccine-related immunologic studies.

The Kenya Medical Research Institute and the Centers for Disease Control and Prevention are doing the study to test immune responses to a multi-stage and multi-component malaria vaccine in infants and children less than 5 years old. The results of this study will provide baseline information for future vaccine trials. If you agree for your child to be in the study we will ask you some questions about the health of your child and will take some blood from your child's finger or heel at first visit. Then we will plan to see your child one more time during malaria high transmission season. We will also ask you some questions about your child's health and will also take some blood from your child's finger or heel. The amount of blood we will collect will be very small, so this will not cause any health problems. The blood will be examined for malaria parasites, anaemia, and immune responses to malaria vaccine. We do not expect that there will be any other discomfort to your child and inconvenience to you. The test and examination will be done for free. If your child has malaria and anaemia, we will provide treatment at no cost.

Parent or guardian's name (Print)_____

Identity card number_____

Village_____ House number_____

Parent or guardian's statement: The above study has been explained to me. I understand that this study is completely voluntary. By my signature below, I agree to have my child to participate in this study.

Parent or guardian's signature

*_____**Date**_____

Witness's

signature_____**Date**_____

*: Parent or guardian may sign or provide verbal consent in the presence of a witness who then signs.

Investigator's signature_____

Date_____

Laboratory Tests for vaccine-related immunologic studies

Study number: [_][_]. [_][_][_][_] **Date:** [_][_]/[_][_]/[_][_]

Visit: 1st_____, 2nd_____.

Please check off as samples are collected.

Haemoglobin_____

Malaria smear_____

Plasma (for antibody test)_____

Cell pelet (for immunologic studies)_____

Laboratory Results of samples.

(HAEMO) Haemoglobin [_][_]. [_] 9=Not done [_]

(BLOOD) Blood smear results.....[_]

(PARAS) If positive, parasite count____/300 species_____

(OTHER) Other results, specify

Signature of staff: _____Date: [_][_]/[_][_]/[_][_]

Questionnaire for vaccine-related immunologic studies

Study number [_][_]. [_][_][_][_] Date: [_][_]/[_][_]/[_][_] Visit: 1st____, 2nd____.

Subject name:.....(first name).....(Last name)

Birth date (or age)....., Sex: [_] male [_] female,

Ethnic group:.....

Identity card number....., Village....., House number.....

Body temperature……°C

1. Does your child have cough?……
2. Does your child have running nose?……
3. Does your child have sore throat?……
4. Does your child have diarrhoea?……
5. Has child's mother ever been tested for the AIDS virus?……..

[_] Yes [_] No [_] Don't know

If the answer to # 5 is NO, skip to # 7.

6. What was the AIDS test results of the child's mother?

[_] Positive [_] Negative [_] Don't know

7. Does your child have any known diseases?……..

If yes, indicate which disease……

8. Currently, is your child under any medication?……

If yes, indicate which medication……., for how long……

9. Is your child currently under medication for malaria?

If yes, indicate what drugs: [_] Chloroquine, [_] Fansidar, [_] Quinine, [_] Antipyretics (e.g, aspirin)

[_] Other, [_] Don't know.

Appendix 7: Abstracts for conferences/seminars/workshops

1. **Natural immune responses to FAL VAC-1 of *Plasmodium falciparum* in children and adults from a holoendemic area of western Kenya.**

Were Tom[#$], *Lal AA*[*], *Nahlen BL*[*$], *Ter Kuile FO*[*$], *Orago ASS*[#], *Kariuki SK*[#$], *Ong'echa JM*[#$] *and Shi YP*[*$].

*Division of Parasitic Diseases, National Center for Infectious Diseases, Molecular Vaccine Section, Atlanta, GA. U.S.A. [#]Department of Zoology, Kenyatta University, Nairobi, Kenya. [$]Centre for Vector Biology and Control Research, Kenya Medical Research Institute, Kisumu, Kenya.

21st African Health Sciences Congress 24 – 28 April 2000

KEMRI, Nairobi, Kenya.

FAL VAC-1 is a recombinant multicomponent *Plasmodium falciparum* protein vaccine candidate. It contains 12 B cell and 9 T cell epitopes from 9 different antigens of different life cycle stages. Previous studies have shown that this vaccine candidate is immunogenic in animal models and the vaccine-induced antibodies inhibit the development of blood stage parasites and invasion of liver cells by sporozoites *in vitro*. In this study we investigated natural cellular and humoral immune responses to FAL VAC-1, and their association with clinical protection against malaria in children less than 2 years (N=180) and in adults (N=139) from an area of western Kenya with perennial malaria transmission. The prevalence of antibodies was significantly higher in adults than in children when stratified by age; 0-6 months, 7-12 months, 13-18 months and >18 months for total IgG, IgG1, IgG2, IgG3 and IgM ($P<0.001$). IgG4 was not detected in any of the test plasmas. In children, total IgG and IgG1 antibodies were positively associated with parasitaemia (total IgG, $r=0.148$, $P<0.05$; IgG1, $r=0.236$, $P<0.01$) and inversely with haemoglobin

levels (total IgG, r=-0.215; P<0.01; IgG1, r=-0.180, P<0.05). Compared to children there was no association between antibodies and haemoglobin levels in adults, but IgG1 was inversely associated with parasitaemia (r=-0.169; P<0.05). In this study we also found that there was variation in the pattern of lymphoproliferative responses to different concentrations (0.001μg/ml, 0.01μg/ml, 0.1μg/ml, 0.5μg/ml, 1μg/ml, 2.5μg/ml and 5μg/ml) of the FAL VAC-1 antigen in different age groups. PBMCs from adults required lower concentrations for a response, while those from children responded at higher concentrations. Based on these results 1μg/ml was chosen to test the lymphoproliferative responses in children and adults. We found that lymphoproliferative responses were significantly higher in children (stratified by age) than in adults (F=5.09, P<0.001). Lymphoproliferative responses were neither associated with parasitaemia nor with haemoglobin levels. From these results we conclude that: (**1**) individuals naturally exposed to malaria recognize FAL VAC-1, a multivalent vaccine candidate; (**2**) antibody responses increase with age, whereas lymphoproliferative responses decrease with age; (**3**) the presence of antibodies (total IgG and IgG1) in children indicates the presence of *P. falciparum* infection, while in adults from the same malaria holoendemic area the antibody, IgG1, may be important in protection against falciparum malaria; (**4**) differences in lymphoproliferative response at different concentrations in children and adults indicate immune activation in the latter.

2. **Natural immune responses to the multicomponent FAL VAC-1 of *P. falciparum* in children and adults from a holoendemic area of western Kenya.**

Were Tom (MSc. Project).

Supervisors: Prof. ASS Orago and Dr. Ya Ping Shi (MD).

Annual postgraduate conference August 2000.

Kenyatta University, Nairobi, Kenya.

FAL VA-1 is a recombinant multicomponent *P. falciparum* protein vaccine candidate. It contains 12 B cell and 9 T cell epitopes from 9 different antigens of different life cycle stages. Previous studies have shown that this vaccine candidate is immunogenic in animal models and the vaccine-induced antibodies inhibit the development of blood stage parasites and invasion of liver cells by sporozoites *in vitro*. We investigated natural immune responses to FAL VAC-1, and their association with clinical protection against malaria in children < 2 years (N=180) and in adults (N=139) from an area of western Kenya with perennial malaria transmission. Antibody prevalence was significantly higher in adults than in children for total IgG, IgG1, IgG2, IgG3 and IgM ($P<0.001$). IgG4 was not detected in any of the test plasmas. In children, total IgG and IgG1 antibodies were positively associated with parasitaemia (total IgG, $r=0.148$, $P<0.05$; IgG1, $r=0.236$, $P<0.01$) and inversely with haemoglobin levels (total IgG, $r=-0.215$; $P<0.01$; IgG1, $r=-0.180$, $P<0.05$). Compared to children there was no association between antibodies and haemoglobin levels in adults, but IgG1 was inversely associated with parasitaemia ($r=-0.169$; $P<0.05$). We also found that there was variation in the pattern of lymphoproliferation to different concentrations (0.001μg/ml, 0.01μg/ml, 0.1μg/ml, 0.5μg/ml, 1μg/ml, 2.5μg/ml and 5μg/ml) of the FAL VAC-1 antigen in different age

groups. PBMCs from adults required lower concentrations for a response, while those from children responded at higher concentrations. Based on these results 1μg/ml was chosen to test the lymphoproliferative responses in children and adults. We found that lymphoproliferatiion was significantly higher in children than in adults ($F=5.09$, $P<0.001$). Lymphoproliferation was neither associated with parasitaemia nor with haemoglobin levels. From these results we conclude that: (**1**) individuals naturally exposed to malaria recognize FAL VAC-1 vaccine candidate; (**2**) antibody responses increase with age, whereas lymphoproliferative responses decrease with age; (**3**) the presence of antibodies (total IgG and IgG1) in children indicates the presence of *P. falciparum* infection, while in adults from the same area the antibody, IgG1, may be important in protection to malaria; (**4**) differences in lymphoproliferation at different concentrations in children and adults indicate immune activation in the latter.

3. Natural immune responses to FAL VAC-1 of *Plasmodium falciparum* in young children and adults from a holoendemic area of western Kenya

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Previous studies indicate that FAL VAC-1, a recombinant multistage, multivalent *Plasmodium falciparum* candidate malaria vaccine is immunogenic in animal models and that vaccine-induced antibodies in rabbits have significant anti-parasitic effects against the sporozoite and blood stages of the parasite. In order to characterize natural humoral and cellular immune responses to this candidate vaccine and their association with clinical protection against malaria, we conducted a community based cross-sectional study in children < 2 years (N=180) and their non-pregnant mothers aged 15-48 years (N=139) in a holoendemic area of western Kenya during April-August 1999. FALVAC-1 antigen was used in antibody measurement by ELISA and in lymphocyte proliferative experiments. Prevalence and level of antibodies were significantly higher in adults than in children. In

children, there were higher IgG1 levels in the parasitaemic group than in the aparasitaemic group. Furthermore, IgG1 levels were inversely associated with haemoglobin level. However, in adults, aparasitaemic individuals showed significantly higher IgG1 level than parasitaemic individuals, but there was no association between antibodies and haemoglobin levels. In contrast to antibody responses, lymphoproliferative responses were higher in children than in adults. We also found that lymphocytes from adults responded at lower antigen concentrations, while those from children responded at higher antigen concentrations. There were no associations between lymphoproliferation and malaria infection or haemoglobin level in either young children or adults. The results of this study suggest that: (**1**) the antibody responses increase with age; the higher IgG1 levels in children may indicate the presence of a current *P. falciparum* infection, but in adults from the same holoendemic area, IgG1, may be associated with protection against parasitaemia; (**2**) decreases in the lymphoproliferative responses with age and the lack of associations between lymphoproliferation and clinical protection suggests that lymphoproliferation may not be suitable marker for evaluation of immunological correlates of protection against malaria; (**3**) the requirement of low antigen concentration for lymphoproliferation in adults residing in this holoendemic area suggests immune activation induced by frequent exposure to malaria.