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**CYTOKINE AND ANTIBODY RESPONSES TO MALARIA VACCINE
CANDIDATE ANTIGENS, *PLASMODIUM FALCIPARUM* GLUTAMATE RICH
PROTEIN (GLURP) AND MEROZOITE SURFACE PROTEIN (MSP3) IN
GHANAIAI CHILDREN**

BY

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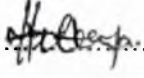


**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN
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DECLARATION

The work described in this thesis was performed by me at the Immunology Department of Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, under the supervision of Dr. Daniel Dodoo of Immunology Department, NMIMR, and Prof. Dominic Edoh of Zoology Department, University of Ghana, Legon. References cited in this thesis have fully been acknowledged.

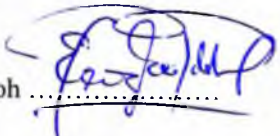
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DEDICATION

I dedicate this work to God Almighty, and to my dear parents Madam Margaret Otoo and Mr. Jacob Benjamin Nartey for their numerous sacrifices.



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Sincere thanks to the Almighty God, for helping me complete this work.

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LIST OF ABBREVIATIONS

ADCI	Antibody-dependent cell mediated inhibition
AMA1	Apical Merozoite Antigen 1
CSP	Circumsporozoite Surface Protein
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment of crystallization
FBS	Foetal bovine serum
GLURP	Glutamate Rich Protein
IFN- γ	interferon- γ
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
LSA	Liver Stage Antigen
MHC	Major Histocompatibility Complex
MOH	Ministry of Health
MSP1	Merozoite Surface Protein 1
MSP3	Merozoite Surface Protein 3
NK cells	Natural Killer Cells
OD	Optical Density
OPD	Ortho-phenylenediamine
<i>P. falciparum</i>	<i>plasmodium falciparum</i>
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PfEMP1	<i>P. falciparum</i> Erythrocyte Membrane Protein
PHA	Phytohaemagglutin
PMA	Phorbol 12-myristate 13-acetate
PPD	Purified protein derivative of tuberculin
RBC	Red blood cell
SCID	Severe combined immunodeficiency
Tc	T-cytotoxic

TGF β	Tumour growth factor β ,
T _H cells	T helper cells
TNF α .	Tumour necrosis factor
WHO	World Health Organization

ABSTRACT

GLURP and MSP3 are targets for antibodies involved in antibody-dependent cellular inhibition that may lead to protective immunity against malaria. Comparative assessment of IgG and subclass responses to GLURP and MSP3 in relation to immunity against malaria will provide relevant information that will be useful in future malaria vaccine development. Several studies have led to the recent recognition of the importance of T-cell in the generation of long-term antibody responses in malaria infection. Therefore, cellular responses to GLURP and MSP3 must be investigated to get a better understanding of the roles the various cytokines play in conferring immunity against malaria.

The antibody responses to GLURP and MSP3 were measured by Enzyme-linked immunosorbent assay (ELISA) in samples obtained from a cohort of 300 children, 3-15 years of age. The plasma samples came from a previous longitudinal morbidity survey carried out over a period of 18 months (1994-1995) covering two malaria transmission seasons, in which children were classified as susceptible or resistant to malaria. In addition, peripheral blood mononuclear cells (PBMC's) were stimulated in culture with GLURP peptides and cytokines were measured.

The pattern of IgG subclass responses to both antigens was similar, indicating higher prevalence for cytophilic antibodies than non-cytophilic antibodies. The association between antibody levels and protection was statistically significant for GLURP IgG ($P < 0.001$) and MSP3 IgG ($P < 0.001$), and for cytophilic IgG1 and IgG3

($0.009 > p > 0.001$) for both GLURP and MSP3. However, when the effect of age was adjusted for in a logistic regression model, GLURP IgG and IgG1 responses were associated with protection in Ghanaian children. For cellular responses to GLURP peptides, higher cytokine responses were raised in antigen-stimulated cultures of exposed individuals than in non-exposed individuals. The study found no significant association between cytokine responses in protected Ghanaian children and those susceptible to malaria ($p > 0.05$). Furthermore, there was no correlation between antibody and cytokine responses in Ghanaian children.

These results confirm the association between cytophilic antibodies against GLURP and MSP3 and protection from clinical malaria. The complementarity of antibodies responses against both antigens supports their use as a hybrid in a future malaria vaccine. The study showed cytokine responses against some GLURP peptides, suggesting that there are T-cell epitopes within the antigen. The knowledge of the level of cellular and antibody responses to GLURP and MSP3 of *P. falciparum*, and possibly the combined effect of these two antigens will be very useful in future malaria vaccine development.

CHAPTER 1

1.0 INTRODUCTION

Malaria is a protozoan disease, which is transmitted through the bite of a female anopheles mosquito. There are four species of the genus *plasmodium* that infect humans. These are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*. These plasmodia species cause four kinds of malaria with different disease presentations. *P. vivax* causes the benign or tertian form of malaria, responsible for about 43% of cases worldwide. It is prevalent throughout the tropics and many temperate regions. *P. malariae* causes quartan malaria, which is responsible for about 7% of malaria cases in the world. Likewise *P. ovale* causes tertian malaria, a rare and milder form of infection, and confined to only tropical Africa and subtropics. *P. falciparum* is the most common malaria parasite in Africa, responsible for about 50% of all malarial cases worldwide, and causes the most lethal form of malaria, especially in children, pregnant women and travelers from non-malaria endemic countries. *Plasmodium falciparum* infection is the major cause of morbidity and mortality in malaria endemic regions (Osuntokun, 1983). The clinical symptoms of human *P. falciparum* infections include chills, fever, headache, nausea, vomiting, and diarrhoea. Severe malaria complication manifests as cerebral malaria, severe anaemia, convulsions and respiratory distress, occurring mainly in children under 5 years of age.

In Africa, studies suggest an annual mortality of 1.0–2.5 million, majority of which are children below five years (Snow *et al.*, 1999; WHO, 2002). However, there is an increasing trend in malaria morbidity and mortality that can mainly be attributed to drug resistant parasite strains and insecticide resistant vectors, thus leading to an increase in

the social and economic burden of the disease. Malaria is said to be a disease of the poor, as well as a cause of poverty in Africa. The annual economic growth in malaria endemic countries has been shown to be lower than in countries without malaria (Roll back malaria, 2001). Malaria also has a direct impact on Africa's human resources, due to mortality and loss of productivity associated with the illness. In addition, it affects children's education and social development as the sickness causes absenteeism from school. The greatest challenges facing malaria eradication in Africa is drug resistance, and the development of insecticide resistance by the mosquito vector (Greenwood, 2002). With the advent of drug resistant parasites and insecticide resistant mosquitoes, much emphasis has been placed on developing a malaria vaccine.

Development of a malaria vaccine or other new immunotherapies may lead to a decrease in malaria morbidity and mortality especially in children. This can be possible by understanding the complex life cycle, and the interactions of the parasite with the host's immune system. The complete life cycle of *P. falciparum* involves an invertebrate host, which is a female anopheles mosquito and an intermediate vertebrate human host. There are three stages of the life cycle in the human host namely the pre-erythrocytic, erythrocytic and gametocytic stages. The parasite is mainly intracellular in all the stages with only brief periods of extracellular existence prior to sporozoite invasion of liver cells, or merozoite invasion of erythrocytes. However, both antibody and T-Cell responses are elicited against all the 3 stages of the parasite in the human host. During the pre-erythrocytic stage, antibodies are raised against the sporozoites that may prevent their entrance into the liver cells. Nevertheless, some sporozoites manage to enter into the liver cells, which leads to induction of cytokine responses by the host immune

system, thus inhibiting the development of the parasites in the liver cells. Furthermore, antibodies raised against merozoites may prevent the invasion of parasites into the erythrocytes, and lead to splenic clearance of infected red blood cells. At the gametocytic stage, antibodies and cytokines are raised against the gametocytes and may thus prevent their development into sporozoites. The multiplication of parasites of the erythrocytic stage is responsible for the clinical symptoms of malaria, and there is therefore a lot of focus on this stage, with the view of identifying antigens important for protective immune responses.

Generally, the human immune system is divided into innate and acquired systems. The innate immunity is inherent and always present to protect the individual from foreign materials and pathogens. It consists of body surfaces and internal organs such as the skin and mucous membrane, other components include interferons, phagocytic cells, macrophages and microglial cells of the central nervous system. Cells of the innate immune system detect the presence of pathogens first and send signals to the adaptive immune system during infection. The acquired immune system on the other hand, consists of antigen specific cells that supplements the protection provided by the innate immune system. They express cell surface molecules that are capable of recognizing a wider range of antigens. The two arms of acquired immunity are humoral immune response, which is mediated by B cells, and cellular immune response, mediated by T-cells. The B cells produce soluble antibodies, which are heterogeneous mixture of serum globulins (immunoglobulins) that circulates in the body, recognize and bind specific microbial antigens for destruction. The antigen-antibody reaction activates the complement system, which consists of serum enzymes resulting in the lysis of microbes

(Benjamini *et al.*, 2000). The cell-mediated immune response consists of T lymphocytes, which bears antigen receptors called T cell receptors. T lymphocytes circulate to antigen directly to destroy it in the context of MHC class I or II reactivity. When an antigen is presented by macrophages or other antigen presenting cells, it results in the activation and proliferation of T helper cells (T_H cells) cells as well as T-cytotoxic (T_c) cells. T_H cells function by releasing cytokines, which activates signals for B cells leading to the production of antibodies to eliminate extra cellular pathogens.

T_H cells can be divided into two functional subsets (T_{H1} and T_{H2}), and these secrete different cytokines. These cytokines are soluble low molecular weight proteins produced by various cells of the innate and acquired immune system. They are chemical messengers that convey information between cells, regulate the development, differentiation of the effector cells and modulate immune responses. These cytokines include interferons, interleukins, tumour necrosis factor and other proteins. T cells also release cytokines that lead to delayed-type hypersensitivity reaction. The cytotoxicity mechanism of cell-mediated immunity may be either through antigen specific T lymphocytes ($CD8^+$ cells), bearing T cell receptors or may involve nonspecific cells like Natural Killer cells (NK cells) and macrophages. These cytotoxic T cells play important role in directly recognizing and eliminating infected cells and antigens. T_H -cell differentiation in the presence of antigens leads to the synthesis and secretion of a range of cytokines like IL-2, which would eventually lead to pathogen destruction. Cytokines produced by activated T_H cells can influence the activity of B cells, NK cells, macrophages, granulocytes and the entire network of cells in the immune system (Benjamini *et al.*, 2000).

Malaria infection results in both activation of antibody and cellular immune responses from the host. These immune responses are regulated by the innate and adaptive immune systems (Perlmann *et al.*, 2002). T-cells and cytokines are also known to be involved in the immune regulation and effector phases of malaria immunity through T helper cells (Weidanz *et al.*, 1988; Riley *et al.*, 1988; Luty *et al.*, 1999). Epidemiological survey performed in malaria endemic communities has shown that gradual acquisition of partial clinical immunity to malaria in individuals is due to repeated exposure to malaria parasites. Infants born to immune mothers are protected from the disease in their early months as a result of in utero acquisition of maternal antibodies (Marsh, 1992). Antibodies have been shown to play a major role in immunity against the blood stages, the stage responsible for clinical malaria (Dodoo *et al.*, 2000; Okech *et al.*, 2004). Several studies have associated naturally acquired protection against malaria in endemic populations, with immune responses to particular malaria parasite antigens (Bouharoun-Tayoun *et al.*, 1990; Egan *et al.*, 1996; John *et al.*, 2004; Okech *et al.*, 2004; Oeuvray *et al.*, 1994; Theisen *et al.*, 1998 and 2001). It is also known that there is participation of cell-mediated effector mechanisms in the establishment and maintenance of protective immunity (Good *et al.*, 1998).

Cell mediated immune responses against malaria is thought to be protective against both pre-erythrocytic and erythrocytic stages of the parasite (Troye-Blomberg *et al.*, 1994). The T-helper cells, which secrete cytokines, are believed to be important for the outcome of infection in human and animal models, either leading to pathology or protection (Omer *et al.*, 2000; Shear *et al.*, 1990; Stevenson *et al.*, 1990). The balance

between pro-inflammatory cytokines and anti-inflammatory cytokines may be important in malaria infection and immunity (Troye-Blomberg *et al.*, 2002). Cell-mediated immune responses are effected through cytokines. It is important to elucidate the role played by cytokines in protective immunity, and whether certain cytokines influence the production of cytophilic antibodies. A number of malaria antigens have been identified and characterized (Figure 2.1), and may be targets for protective immunity to malaria (Warrell, 1993). These antigens include, the Merozoite Surface Protein 1-19 (MSP1₁₉), the Merozoite Surface Protein 3 (MSP3), the Circumsporozoite Surface Protein (CSP), *P. falciparum* Erythrocyte Membrane Protein (PfEMP1), Apical Merozoite Antigen 1 (AMA1), Liver Stage Antigen 1 (LSA1) and the Glutamate Rich Protein (GLURP).

This study focused on GLURP and MSP3, which have been identified as targets for antibodies involved in antibody-dependent cellular inhibition (ADCI). Several studies on antibody responses to GLURP and MSP3 have shown the importance of GLURP and MSP3-specific antibodies, more especially, cytophilic antibodies in mediating immunity against malaria (Theisen *et al.*, 1998; Dodoo *et al.*, 2000; Oeuvray *et al.*, 1994 and 2000; Theisen *et al.*, 2001). Since GLURP and MSP3 are effective in ADCI, there is the need for baseline immunological data in malaria endemic regions that will provide rational for including both antigens in a future multivalent malaria vaccine. Furthermore, to be an effective malaria vaccine, it may be required that GLURP induces T-cell responses to regulate efficient and long lasting antibody responses under natural infection. However, little has been done with regards to cell-mediated immune responses to GLURP, in relation to the pattern of cytokine responses (either pro-inflammatory or anti-inflammatory). Since regulation of antibody production by B-cells is T-cell mediated

and involves cytokines, it is important to elucidate cytokine(s) that may influence production of cytophilic antibody responses (IgG1 and IgG3) that has been consistently found to be associated with protection from malaria (Dodoo *et al.*, 2000; Oeuvray *et al.*, 2000; Theisen *et al.*, 2001).

This study therefore aims to characterize T-cell cytokine responses to selected peptides of GLURP, and to assess which specific cytokine profiles are associated with cytophilic antibodies in Ghanaians. This would give a better understanding of the role of cytokine responses to GLURP and immunity against malaria. In addition, this study also aims to compare antibody levels raised individually against GLURP or MSP3 and a hybrid GLURP-MSP3 antigen in a cohort of Ghanaian children. These antigens are on phase I clinical trials, yet not much has been done to compare the two antigens in their level of antibody response in malaria infections. The knowledge of the level of antibody responses to GLURP and MSP3 of *Plasmodium falciparum*, and possibly the combined effect of these two antigens will be very useful in future malaria vaccine development. Furthermore, characterizing cellular responses to these antigens will provide much needed information regarding which epitopes to include in the GLURP vaccine that will ensure long lasting and protective antibody production. The study therefore hypothesises that GLURP induces cellular responses and antibody responses that are protective, and specific cytokine(s) induced by components of GLURP will enhance cytophilic antibody production.

Objectives

The objectives are

1. To measure and compare immunoglobulin G (IgG), and subclass (IgG1-IgG4) responses to GLURP, MSP3 and Hybrid (GLURP-MSP3) in Ghanaian children.
2. To assess and compare the association between individual antibody responses to GLURP, MSP3, and that of the hybrid GLURP-MSP3.
3. To assess *in vitro* cellular responses to synthetic peptides of GLURP (LR129, LR130, MR186, MR187), as measured by stimulation and production of cytokines (interferon gamma, interleukin-2, interleukin-4 and interleukin-10) in Ghanaian children.
4. To compare cytokine and antibody responses to GLURP in relation to protection, and assess which cytokine(s) may be correlated with cytophilic antibodies measured in Ghanaian children.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Epidemiology of malaria

Malaria mostly occurs in Africa, South East Asia, The Caribbean, Eastern Mediterranean, Western Pacific, Latin America and parts of Europe (WHO, 2002), and it presents major socio economic problems. The global prevalence of malaria is 515 million episodes in 2002, resulting in 1 million deaths annually and most of these are in children under five year old (Snow *et al.*, 2005). About 90% of malaria mortality occurs in Sub-Saharan Africa, this is because the majority of infections are caused by *Plasmodium falciparum*, the most dangerous of the four human malaria parasites. Moreover, *Anopheles gambiae*, which is the most effective vector is widespread in Africa and it is very difficult to control. Children and pregnant women living in malaria transmission areas are at the highest risk of malaria mortality and morbidity. Previous and recent reviews have shown that malaria causes about 10-20% of all deaths in children under five years of age in Africa and kills a child every 30 seconds (Binka *et al.*, 1994; Jaffar *et al.*, 1997; Greenwood, 1999; WHO, 2002; Africa Malaria Report, 2003).

2.1.1 The burden of malaria

The burden of malaria on health in all malaria endemic countries in Africa is enormous. Up to 25-40% of all outpatients clinic visits are due to malaria, and 20-50% of all hospital admissions are as a result of malaria infection (Africa Malaria Report, 2003). Malaria infection poses a big burden on the socioeconomic status of people in endemic areas (Gallup and Sachs, 2001; Shephard *et al.*, 1991). Child malaria mortality rates

have been shown to be higher in poorer households, since poor people are at risk of becoming infected frequently. This has been shown in a demographic surveillance carried out in Tanzania, in which under five years mortality due to malaria was found to be 39% higher in the poorest socioeconomic group (Mwageni *et al.*, 2002). A survey done in Zambia also found a significantly higher prevalence of malaria infection among the poor population (Zambia Roll Back Malaria Report, 2001) and similar observation was made in Northern part of Ghana (Akazili, 2002). These could be due to settlement conditions of poor families, un-affordability of mosquito treated nets, and inability to pay for effective malaria treatment.

2.1.2 Malaria distribution in Africa

There is a wide distribution of malaria in Sub Saharan Africa, approximately 63% of people in Sub Saharan Africa live in malarious areas. Malaria is endemic throughout Zambia, an estimate of 4.8 million (32%) cases of malaria each year among a population of about 10 million. 35.6% hospital admissions is due to malaria and 14.8% mortality rate. Chloroquine resistance to malaria has been estimated between 20-50% in Zambia (RBM/WHO, 2000; Zambia Fact Sheet, 2001). 90% of the Ugandan country is highly endemic, with an estimated 5.3 million cases of malaria per year for a total population of 21 million. Outpatient malaria cases are between 25-40%, and 20% hospital admissions and 9-14% death cases is attributable to malaria infection (RBM/WHO, 2000). In Rwanda 1.2 million cases of malaria is reported every year. In Kenya, an estimated 8.2 million cases of malaria are reported annually for a total population of 30 million (Kindermans, 2002), resulting in the death of about 26000 children under five years everyday. In Tanzania, malaria is a leading cause of mortality and morbidity with 16

million cases every year. It is estimated that 93.7% of Tanzanian's 32.8 million people are at risk of the disease. In Ghana, malaria is hyperendemic, *P. falciparum* accounts for 90% of infection, *P. malariae* (9.9%) and *P. ovale* (0.1%). Clinical malaria results in 42-45% of outpatient hospital visits and 22% of under-5 year old mortality (Binka *et al.*, 1994; RBM, 2005).

The morbidity of malaria has increased yearly due to deteriorating health systems, insecticide and drug resistance, climatic changes and civil war. The greatest challenges facing malaria eradication in Africa is drug and insecticide resistance, since malaria control in Africa is based mainly on chemotherapy (WHO, 1999; White, 1999; Trape, 2001; Greenwood, 2002). Resistance to chloroquine is now widespread in 80% of the 92 countries where malaria continues to be a major killer, while resistance to other drugs of choice for treatment is increasing (RBM/WHO, 2000). Development of a malaria vaccine or other new immunotherapy's may lead to a decrease in malaria morbidity and mortality especially in children. This can be possible by understanding the complex life cycle, and the interactions of the parasite with the immune system of the host.

2.2 Life cycle

The complete life cycle of *P. falciparum* involves both the mosquito and the human host (Figure 2.1). Briefly, malaria transmission begins with the bite of an infected female anopheles mosquito, which injects sporozoites into a human host during feeding. These sporozoites enter the blood circulation, and invade the hepatocytes, where they undergo asexual stage of reproduction for 9-16 days. The liver cells then rupture to release merozoites into the bloodstream. These merozoites invade the red blood cells, reside in the parasitophorous vacuole, undergo development to the early ring stage

trophozoite, late trophozoite, and by mitotic division mature into the schizont stage that contains about 32 merozoites. The schizonts are released into the blood when the red blood cells rupture and invade new red blood cells for repetitive intra-erythrocytic cycle. After a number of intra-erythrocytic cycles, the merozoites develop and, differentiate into infective female and male gametocytes, which are ingested by the mosquito during a blood meal. Sexual reproduction of gametocytes takes place in the midgut of the mosquito, resulting in the production of zygote, which develops into oocysts through the process of sporogony to release sporozoites. The sporozoites invade the salivary glands, and enter into man when the infected female mosquito feeds on man and the cycle continues.

2.3 Case definition of clinical malaria

Clinical disease caused by the parasite is due to asexual multiplication of blood stage parasites, therefore a lot of focus has been put on the erythrocytic stage with the view of identifying antigens targeted for protective immune responses. The clinical manifestation of *P. falciparum* infection includes chills, fever, headache, nausea, vomiting, and diarrhoea. Major complications of *P. falciparum* malaria in children are cerebral malaria, severe anaemia, convulsions and respiratory distress. The definition of clinical malaria is usually based on the microscopic detection of malaria parasitemia, fever, and febrile temperature ≥ 37.5 °C. It has been established that fever due to malaria is induced by the release of parasite toxins when schizont infected red blood cells rupture to release merozoites (Kwiatkowski *et al.*, 1989). Hence, malaria disease is said to be directly caused by parasite multiplication that leads to febrile temperatures. The use of febrile temperature ≥ 37.5 °C and parasitaemia at various levels results in highly sensitive and specific malaria case definition (Armstrong *et al.*, 1994). It should however

be noted that individuals in highly endemic areas may have parasites without fever or other symptoms associated with malaria.

2.4 Malaria immunity

2.4.1 Innate immune response

The innate immune system mediates a nonspecific protection through monocytes, macrophages, dendritic cells, natural killer (NK cells) cells, eosinophils, neutrophils, mast cells, complement and acute phase proteins. It also includes physical barriers like epithelial layers, and anti microbial substances on these surfaces. In innate immunity, neutrophils, mononuclear phagocytes and NK cells are known to play a role in malaria infections. NK cells have been shown to be involved in the lyses of *P. falciparum* infected erythrocytes *in vitro*. They elicit the production of cytokines such as interferon- γ (IFN- γ), which activates macrophages leading to phagocytosis of invading foreign particles (Orago *et al.*, 1991; Artavanis-Tsakonas; Riley, 2002). In addition, innate immune mechanisms by NK cells leads to the stimulation of IFN γ , which limits the initial phase of parasite replication, this has also been demonstrated in studies done on murine malaria (Doolan and Hoffmann 1999; Fell and Smith, 1998; Mohan *et al.*, 1997).

2.4.2 Acquired immunity

2.4.2.1 Humoral immune response

Malaria infection induces strong humoral immune responses through the production of high concentrations of immunoglobulins (Ig), especially IgG and IgM as well as IgE. The extent of protective immunity acquired against malaria infection in humans and mice has been shown to be associated with the level of antibody against the asexual blood stage antigens (Piper *et al.*, 1999; Hirunpetcharat *et al.*, 1998; Astagneau *et al.*,

1995). The importance of antibody in malaria immunity is evident from the protection conferred to neonates and infants by malaria specific antibodies acquired by mothers (McGregor *et al.*, 1963; Sabchareon, *et al.*, 1991). Passive transfer of monoclonal antibody against plasmodium parasite antigens conferred protection in naïve mice (Spencer *et al.*, 1998; Narum *et al.*, 2000). Although various immunoglobulins may confer protection to individuals with malaria, IgG is the most important of all. The use of purified immunoglobulin from sera of African adults in clinical trials to treat some sick children drastically reduced clinical symptoms and parasitaemia (Bouharoun-Tayoun *et al.*, 1990). This has established that immunoglobulin G (IgG) is a main component of protection against the asexual blood stage of *P. falciparum* (Druilhe *et al.*, 1994).

Cytophilic antibodies have been shown to play critical role in anti-malaria immunity. They may act in collaboration with monocytes and macrophages by attaching to certain parasite antigens by their Fc receptors and lead to phagocytosis or release of toxic factors that kill infected cells. Studies done on the role of subclass responses in naturally acquired immunity are important. Increased levels of cytophilic antibodies (IgG1 and IgG3 subclasses) have been found in individuals protected from malaria (Bouharoun-Tayoun *et al.*, 1992; Sarthou *et al.*, 1997; Oeuvray *et al.*, 2000). Further studies done by Aribot *et al.*, 1996 revealed the association between high levels of parasite specific IgG3 and malaria. In addition, higher levels of IgG3 antibodies have been found in certain populations and its association with malaria infection has been reported (Aribot *et al.*, 1996 and Rzepezyk *et al.*, 1997; Ndungu *et al.*, 2002). It has also been revealed that

repeated malaria infections are associated with elevations in total IgE and its regulation in T-cell activities (Perlmann *et al.*, 1994).

Mechanism of antibody response: Antibodies are known to mediate protection against malaria, through various mechanisms. Results from studies performed *in vivo* suggest that one mechanism may confer clinical immunity to malaria by antibody interruption of parasite multiplication (McGregor *et al.*, 1964; Sabchareon *et al.*, 1991). Antibodies against blood-stage merozoite antigens may block parasite invasion of erythrocytes or make them susceptible to phagocytosis, leading to reduction in parasitemia (Blackman *et al.*, 1994; Holder *et al.*, 1992). Other mechanisms are clearance of infected erythrocytes from circulation by antibodies binding to their surface via Fc receptors and its elimination from the body (Udeinya, 1981; Bouharoun-Tayoun *et al.*, 1990 and 1995). Some of the parasites elicit antibodies that form clumps or rosettes which the immune system recognize and clear them from circulation by opsonization or phagocytosis (Treutiger *et al.*, 1992). Antibody dependent cell-mediated cytotoxicity of parasites may be elicited through cytophilic antibodies, as well as parasite inhibition by effector cells like neutrophils and monocytes (Bouharoun-Tayoun *et al.*, 1990; Groux and Gysin, 1990). Parasite agglutination and indirect effects like the antibody dependent cellular inhibition (ADCI) (Oeuvray *et al.*, 1994) are other immune mechanisms that may protect against *P. falciparum* malaria. The ADCI associated killing of parasites is mediated by cytophilic antibodies, which act in collaboration with monocytes to release soluble factors from macrophages and monocytes to destroy parasites (Bouharoun-Tayoun *et al.*, 1995). The malarial parasites also mount up immune evasion mechanisms. These mechanisms involve antigenic variation, since so many different parasite antigens are

presented to the immune system. Others are manipulation of the host immune response that could contribute to pathological changes, polymorphism of parasite protein and competition between protective and non-protective responses (Troye-Blomberg *et al.*, 1999).

2.4.2.2 Cell mediated immunity

Although antibody plays a major role in malaria immunity, T-cells and cytokines are also known to be involved in the immune regulation and effector phases of anti malaria immunity through T helper cells (Weidanz *et al.*, 1988). Cell mediated immune responses against malaria is thought to be protective against both pre-erythrocytic and erythrocytic parasite stages (Troye-Blomberg and Perlmann, 1994). The importance of cytokines in conferring protective immunity to malaria infection in animal models has been documented (Kobayash *et al.*, 1996; Shear *et al.*, 1990; Stevenson *et al.*, 1990). Several studies have revealed cellular mechanisms such as lymphocyte proliferation, IFN γ production, activation and killing of parasites by macrophages when peripheral blood mononuclear cells (PBMC) from immune individuals were stimulated with malaria antigens *in vitro* (Troye-Blomberg *et al.*, 1984 and 1985; Ballet *et al.*, 1985; Brown *et al.*, 1986; Ockenhouse *et al.*, 1984). When similar studies were done on non-immune individuals infected with *P. falciparum*, it revealed a decreased cellular recognition of plasmodial antigens (Ho *et al.*, 1988; Theander *et al.*, 1986). T-cells that regulate antibody production are also involved in both inflammation and its regulation via cytokine production. The differentiation of T_H-cells into their subsets T_H1 and T_H2 may have important biological and immunological implications towards the susceptibility or resistance to particular diseases or infection (Troye-Blomberg and Perlmann, 1994).

Thus different subsets of T_H cells play different roles in terms of inflammation or anti inflammation in malaria infection.

Mechanisms of cellular immune response: Studies have revealed that T_{H1} and T_{H2} cells are responsible for the regulation of antibody mediated immunity and cell-mediated immunity. This is evident from a study in which T-cells regulated antibody production via T_{H1} and T_{H2} induced cytokine production (Weidanz et al., 1988). T_{H1} cells secrete cytokines like interleukin-2 (IL-2), interferon (IFN) γ , Tumour growth factor (TGF) β , and tumour necrosis factor (TNF) α . These cytokines activates macrophages that helps in opsonization through IgG antibodies, promotes inflammatory response leading to tissue injury, and mediates delayed hypersensitivity reactions (Abbas *et al.*, 1996). T_{H2} cells secrete cytokines like IL-4, IL-5, IL-6, IL-10, and IL-13. These cytokines stimulates the proliferation of mast cells and eosinophils, and provides help to B-cells during infection (Abbas *et al.*, 1996). These T_H cells cross-regulate the differentiation and activities of each other through the cytokines that they produce. Studies conducted revealed that T_{H2} secreted cytokines regulate the functional activity, and production of T_{H1} secreted cytokine in *P. falciparum* infection (Ho *et al.*, 1995). The T_{H1} secreted cytokines are known to down regulate the development of T_{H2} cytokines, whereas the T_{H2} cells also secrete cytokines that down regulates T_{H1} cell maturation (Troye-Blomberg and Perlmann, 1994). For instance, IFN γ secreted by T_{H1} cells inhibits the development and proliferation of T_{H2} cells, as well as IL-4 and IL-10 secreted by T_{H2} cells inhibit the development of T_{H1} cells. It has been shown that cytokines like IL-1, IL-6, IFN- γ , and TNF- α may be protective by inducing parasite killing by macrophages

and neutrophils (Kumaratilake and Ferrante, 1994; Taylor-Robinson *et al.*, 1993; Troye-Blomberg *et al.*, 1999).

2.4.2.2.1 Role of cytokines in malaria immunity

The role of T_H1 secreted cytokines: T_H1 cells are responsible for cell-mediated immunity, by activating macrophages and other effector cells to release inflammatory cytokines. A number of cytokines play a significant role in the development of acute or chronic inflammatory response to *P. falciparum* antigens. IFN γ plays an important role in resistance to blood stage malaria infection through enhanced activities of macrophages. Work done by Luty *et al.*, 1999, have shown an association between IFN γ responses to the liver and blood-stage antigens, and resistance to malaria re-infection. When macrophages were activated with IFN γ , it resulted in the activation of phagocytic cells and killing of malaria parasites (Ockenhouse *et al.*, 1984). Studies done with human serum have shown a correlation between IFN γ levels with resistance to *P. falciparum* malaria (Deloron *et al.*, 1991; Riley *et al.*, 1988). In addition, it has been demonstrated that IFN γ concentrations increased in plasma of individuals with symptomatic malaria infection than in asymptomatic individuals (Mshana *et al.*, 1991). Furthermore, high levels of IFN γ production by antigen stimulated PBMC *in vitro* correlates with reduced risk of clinical malaria in Ghanaian children (Dodoo *et al.*, 2002).

An association between IFN γ production from peripheral blood mononuclear cells (PBMC's) in response to soluble antigens of *P. falciparum* and increased risk of malarial

infection has been demonstrated (Riley *et al.*, 1991). PBMC's from children with mild *Plasmodium falciparum* infection produced high levels of IFN γ when stimulated *in vitro* with malaria antigen. These children indicated a lower risk of re-infection when cleared from parasitaemia by drug treatment. Whereas children with severe malaria showed lower levels of IFN γ , when their PBMC's were stimulated *in vitro* with malaria antigens, which makes them more susceptible to re-infection (Luty *et al.*, 1999). In animal models, IFN γ -deficient mice with *P. c. chabaudi* infection resulted in increased malaria morbidity and mortality (Su and Stevenson, 2000). This indicates the important role that IFN γ plays in protection against malaria infection. Other research in malaria have revealed an increased expression of IFN γ during parasite resolution, as well as decreased IL-2 production, suggesting a regulatory mechanism of cytokines in malaria infection (Winkler *et al.*, 1998). Although IFN γ is known to be essential in the resolution of primary infection, its over-production predisposes individuals to severe immunopathology (Waki *et al.*, 1992).

IL-2 cytokine, which is secreted by T_H1 cells, induces proliferation of antigen-primed cells and enhances activity of NK cells. A study done in which PBMC's were stimulated with malaria-specific antigens during acute malaria infection, revealed no detectable levels of IL-2 production. This suggests that there may be defective cell mediated immune response to these malaria specific antigens during acute *P. falciparum* infection, which might have resulted in immune unresponsiveness (Ho *et al.*, 1988). Measurement of plasma IL-2 levels in some West African children showed no significant association of IL-2 response and malaria infection. Furthermore, studies conducted in a malaria-endemic community in West Africa revealed no association between plasma soluble IL-

2 receptor levels and *in vitro* proliferative responses of peripheral blood mononuclear cells (PBMC's) to some malaria antigens. In contrast, a high soluble IL-2 receptor was detected in the plasma of malaria infected individuals (Riley *et al.*, 1993), a finding which did not support the idea of immune suppression in malaria infection from previous studies (Ho *et al.*, 1988).

The role of T_H2 secreted cytokines: T_H2 cells regulate humoral immunity by providing help to B cells in antibody production. Cytokines such as interleukin-4 (IL-4) and interleukin-10 (IL-10), which are Th2 secreted cytokines are known to elicit anti-inflammatory responses to malarial antigens. IL-10 cytokine has been shown to regulate the functional activity and the production of Th1 cytokine (Ho *et al.*, 1995 and 1998). It is also known to induce B-cell proliferation, immunoglobulin production, leading to the development and maturation of anti-malaria antibodies (Moore *et al.*, 1993). It has also been reported that higher IL-10 levels were found in children and adults with clinical malaria than healthy controls (João *et al.*, 1997; Peyron *et al.*, 1994; and Deloron *et al.*, 1994). Other studies done to compare specific cytokine responses of patients with or without malaria in Malawi revealed higher serum IL-10 levels in malaria patients than in other patients (Jason *et al.*, 2001). Experiments done have also shown an increased IL-10 as well as TNF α levels during malaria infection (Wenisch *et al.*, 1995). Othoro *et al.*, 1999 predicted that higher plasma IL-10 over TNF α levels might provide protection against severe malaria anemia by down regulating the immuno-pathologic effects of the later. The same study revealed that children with mild malaria had significantly higher IL-10 to TNF α ratios than children with severe malaria. Furthermore, TNF α to IL-10 ratios were found to be significantly lower in patients with severe malaria anaemia than

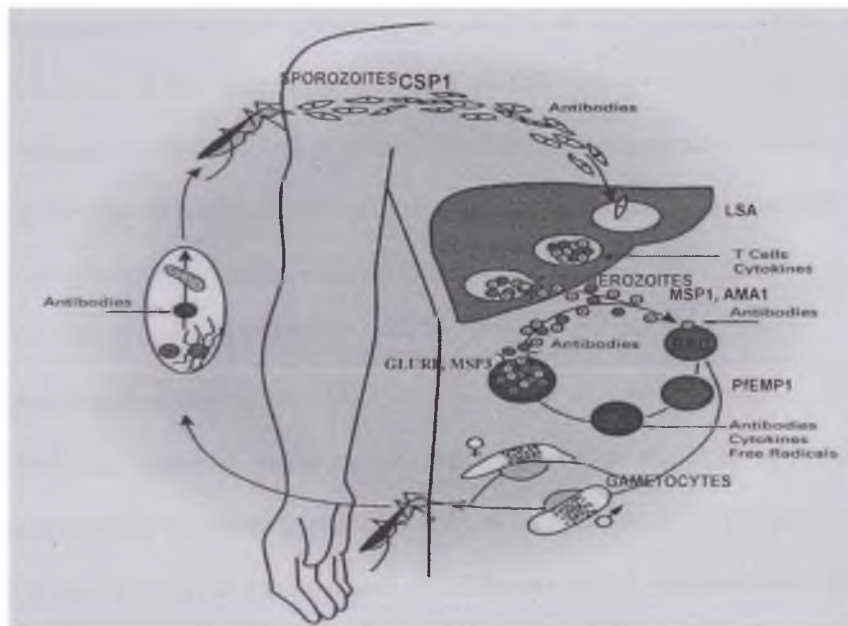
in patients with uncomplicated malaria (Kurtzhals *et al.*, 1998). This means that the lack or insufficient IL-10 response in comparison to TNF α may predispose an individual to severe malaria. Studies done with animal models also suggest the significant role that IL-10 plays in down regulating severe malaria. Although IL-10 plays a role in humoral immunity, it has been shown to play other roles, for instance in an *in-vitro* model, IL-10 was found to decrease the production of IL-6 and TNF α and IFN γ secretion and function (Moore *et al.*, 1993; de Waal *et al.*, 1991). These findings support the idea that IL-10 is a critical factor in down regulating the pathogenesis of severe malaria.

Interleukin-4 (IL-4), a T_H2 secreted cytokine induces the proliferation and differentiation of B cells. Studies have demonstrated that IL-4 production by CD4⁺ T cells is frequently associated with serum antibodies (Troye-Blomberg *et al.*, 1990; Riley *et al.*, 1991; Kulane *et al.*, 1997), suggesting that IL-4 plays a role in certain anti-malarial antibody responses. The finding of high serum IL-4 levels in parasitemic individuals in a holoendemic *P. falciparum* region (Mshana *et al.*, 1991) indicates the significant role that IL-4 plays in clearing parasites. However, IL-4 has been shown to facilitate parasite survival by suppressing anti-parasite activities by macrophages (Kumaratilake and Ferrente, 1992). Research conducted by Winkler *et al.*, 1998 revealed no direct involvement of IL-4 in the clearance of parasitemia, since IL-4 was shown to suppress macrophage anti-*P. falciparum* activity *in vitro* (Kumaratilake and Ferrente, 1992). The percentage of lymphocytes making IL-4 cytokines in adults was found to be significantly lower in malaria patients than those without malaria infection in an endemic area (Jason *et al.*, 2001). In addition, the lack of IL-4 production in malaria patients found in a number of studies suggests that IL-4 may not be a critical factor in

the pathogenesis of malaria (Othoro *et al.*, 1999). There has been a limitation into studies in IL-4 responses in human infection because it has been difficult to detect IL-4 cytokine in supernatants (Kurtzhals *et al.*, 1992).

2.5 Malarial antigens

There have been attempts to identify and characterize antigens on the asexual stage of the parasite (Figure 2.1), that may be of importance in the development of protective immunity to malaria (Warrell, 1993). Some of the antigens found on *P. falciparum* include, the merozoite surface protein 1-19 (MSP1₁₉), the Merozoite surface protein 3 (MSP3), the Circumsporozoite Surface Protein (CSP), *P. falciparum* Erythrocyte Membrane Protein (PfEMP1), Apical Merozoite Antigen 1 (AMA1), Liver stage antigen (LSA) and the Glutamate Rich Protein (GLURP).



Good *et al.*, 1988 (Modified)

Fig 2.1 The life cycle of the *P. falciparum* parasite indicating stage specific antigens.

In a number of recent immuno-epidemiological studies, some antigens have been identified as possible targets of protective antibody-immunity to malaria. These include GLURP, MSP1-19, AMA1, and MSP3, which are now currently in phase I clinical trials (Dodoo *et al.*, 2000; Oeuvray *et al.*, 2000; Theisen *et al.*, 2001). Also, CSP (Hoffman *et al.*, 1986), LSA and PfEMP1 (Bull *et al.*, 1998) have been studied in association with protection.

2.5.1 Pre-erythrocytic stage antigens

Circumsporozoite surface protein (CSP) antigen is found on the surface of matured sporozoites. When host antibodies bind to CSP on sporozoites, the CSP-antibody complexes are shed through a process called the circumsporozoite protein reaction and may serve as defense against host immunity. Antibody directed against CSP can inhibit sporozoite invasion into liver cells (Hoffman *et al.*, 1986).

Liver Stage Antigen (LSA)

Liver stage antigens are processed and presented by the liver cell. This leads to recognition by T-cytotoxic cells and killing of the infected cell. It evokes the stimulation of T cells to produce cytokines such as γ -interferon and IL-10 that ultimately lead to the death of the intracellular parasite. Thus a pre-erythrocytic stage vaccine will prevent the establishment of parasites in the blood stage and will provide protection against clinical malaria.

2.5.2 Asexual blood stage antigens

The Merozoite Surface Protein 1 found on the surface of blood stage merozoites is a possible malaria vaccine candidate. A conserved 19-kDa part of the protein is attached to the merozoite during erythrocyte invasion and expressed by the parasite during the early ring stages. Antibodies raised against the conserved fragment may block merozoite invasion into erythrocytes and inhibit parasite multiplication (Holder *et al.*, 1992). The Merozoite Surface Protein-3 (MSP3) is a 48KDa protein; antibodies raised against MSP3 antigen protect subjects clinically from malaria through ADCI mechanism. Cytophilic antibodies raised against this antigen can clear parasites by opsonization or phagocytosis (Bouharoun-Tayoun *et al.*, 1995).

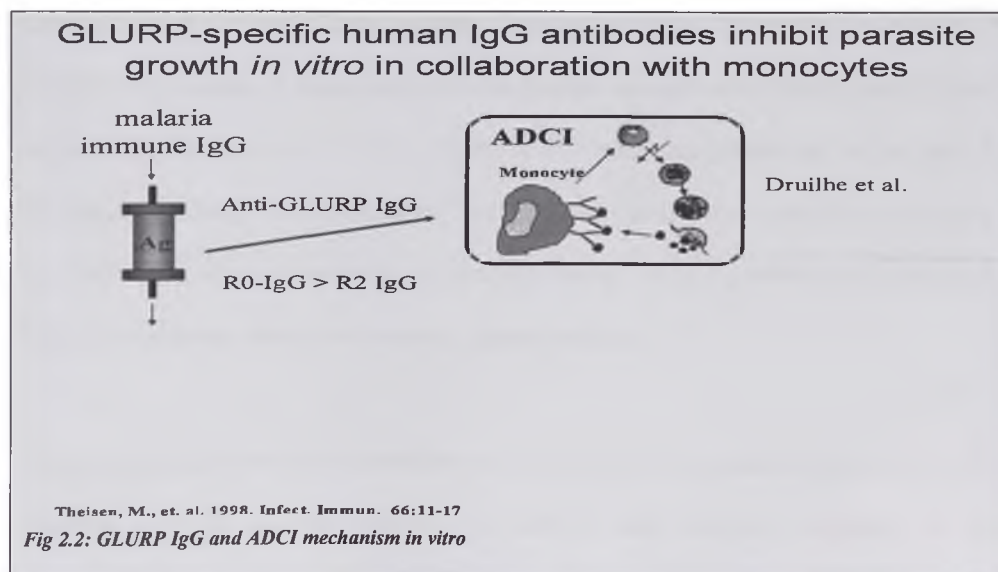
Plasmodium falciparum Erythrocyte Membrane Protein-1 (PfEMP-1) is a highly variant antigen of approximately 300 kDa expressed on surface of erythrocytes infected with late schizont stage of the parasite. It is coded for by about 50 genes of the 'var' multigene family (Smith *et al.*, 1995). PfEMP-1 has been identified as the molecule responsible for intra-vascular sequestration of *P. falciparum* infected blood cells. The role of PfEMP-1 in cytoadherence of infected erythrocyte antibodies to variants of this protein have been reported to occur in people living on malaria endemic regions. (Baruch *et al.*, 1997; Biggs *et al.*, 1990).

The Glutamate Rich Protein (GLURP) is a 200-kDa parasite protein found on the surface of merozoites, located in the parasitophorous vacuole of exo-erythrocytic, erythrocytic and mature schizont-infected erythrocytes (Borre *et al.*, 1991). GLURP is synthesized during all stages of the parasite in the host and is found on the surface of

newly released merozoites (Borre *et al.*, 1991). GLURP has been characterized molecularly (Borre *et al.*, 1991), and several studies done on antibody responses to GLURP indicate the importance of anti-Glurp antibodies, more especially, cytophilic antibodies in mediating immunity against malaria (Dodoo *et al.*, 2000; Oeuvray *et al.*, 2000; Theisen *et al.*, 1998 and 2001).

2.5.3 Immune responses against GLURP and MSP3

A large number of parasite antigens play important role in conferring protection to malaria infection. GLURP and MSP3 have been identified as target antigens for antibodies involved in antibody dependent cellular inhibition mechanism (Figure 2.2) (Oeuvray *et al.*, 1994; Theisen *et al.*, 1998). Human monocytes can cooperate with antibodies to exert this ADCI mechanism *in vitro* (Bouharoun-Tayoun *et al.*, 1990; Khusmith *et al.*, 1983; Oeuvray *et al.*, 1994; Theisen *et al.*, 1995) and also, in humanized SCID mouse model (Badell *et al.*, 2000). High levels of GLURP and MSP3-specific antibodies were associated with acquired protective immunity to malaria (Bouharoun-Tayoun *et al.*, 1995; Dodoo *et al.*, 2000; Oeuvray *et al.*, 1994). Furthermore, high affinity-purified human IgG antibodies to GLURP have been found to promote a strong monocyte-dependent inhibition of *P. falciparum in vitro* (Theisen *et al.*, 1998).



In Ghana, GLURP IgG1 and IgG3 responses were significantly associated with protection from *P. falciparum* malaria infection in children (Dodoo *et al.*, 2000).

These data on GLURP and MSP3 suggests that cytophilic antibodies against both antigens contributed to the development of clinical immunity in West African children (Oeuvray *et al.*, 1994 and 2000). Previous studies have shown that purified human IgG antibodies against non-repeat epitopes of GLURP inhibit parasite growth *in vitro* in a monocyte-dependent manner (Theisen *et al.*, 1998 and 2001). Further studies conducted in Asia have shown an association between cytophilic antibodies against MSP3 and protection against clinical malaria (Soe *et al.*, 2004). The levels of human IgG antibodies found against GLURP peptides were significantly associated with the absence of disease in Ghanaian children (Theisen *et al.*, 2001). In addition, these peptides were demonstrated to be targets of cytophilic antibody responses in individuals living in endemic areas. T-cell responses to these peptides also revealed that T-cell epitopes were detected in LR 67 and LR 68 peptides. This suggests that the non-repeat region of GLURP may contain T-helper-cell epitopes that are recognized by individuals exposed to malaria (Theisen *et al.*, 2001). There is evidence that production of an anti *P. falciparum* antibody *in vitro* requires T-cell-derived signals from cytokines (Garraud *et al.*, 1999). It is still unknown which cytokines induce cytophilic antibody production in humans to enhance protective immunity against malaria.

This present study aims to characterize cell-mediated and responses to selected GLURP peptides (LR129, LR130, MR186 and MR187) and antibody responses to the recombinant Non-repeat N-terminal antigen, R0. In addition, to identify cytokines

associated with GLURP, and to assess which specific cytokine profiles are associated with cytophilic antibodies in a cohort of Ghanaians. Most studies done on GLURP were on antibody responses, which do not provide much information on cytokine responses to synthetic peptides and recombinant antigens of GLURP. Much information is needed regarding which epitopes to include in the GLURP and MSP3 vaccine being developed, which will ensure long lasting and protective antibody production.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Study area and study population

Plasma and cell samples obtained in a previous immuno-epidemiological project conducted in Dodowa were used for the study. Dodowa is a semi rural town approximately 50km from Accra, mainly a subsistence farming community and has a population of about 6,500. It has two rainy seasons; the major rainy reason is from May-August, and the minor one from October-November, followed by a dry season from December to April. Although malaria transmission is perennial, it is highest during or immediately after the rainy season (high-transmission season) and lowest during the dry season (low-transmission season). It has been estimated that individuals in Dodowa are exposed to about 20 infective bites per year, and 98% of the infections are due to *P. falciparum* (Afari *et al.*, 1995). Dodowa can thus be described as an area of hyperendemic and seasonal malaria transmission. The transmission is stable because it does not vary considerably from year to year.

Plasma and peripheral blood mononuclear cells (PBMC's) were obtained from a cohort of 300 children, 3-15 years of age prior to and at the end of the malaria transmission season for immunological studies. A longitudinal morbidity survey was done over a period of 18 months covering two malaria transmission seasons. The children were enrolled into the study after informed consent has been obtained from the parent of each child. Those included in the study were children who do not carry the sickle cell trait or genotype. Each child was visited weekly, during which physical examination was done, and the body temperature measured. In the case of fever above 37.5°C or other

symptoms of malaria, a malaria blood slide was made and treatment offered if the slide was positive for malaria parasites. Children were said to have malaria if they had febrile temperature above 37.5°C and parasitemia > 5000 μ l in blood. In the case of severe symptoms like convulsions, respiratory distress and severe anaemia, the child was referred to the hospital. Children with asymptomatic malaria *i.e.* parasitemia without clinical symptoms, were not treated. During the clinical and parasitological follow up, once a month, a blood smear was prepared by finger pricking from all children irrespective of symptoms to identify asymptomatic malaria infections. The parents or guardian and children were encouraged not to start self-treatment or seek treatment elsewhere unless the field team was not available. They were also encouraged to inform the research team if they did otherwise.

3.2 Study procedures

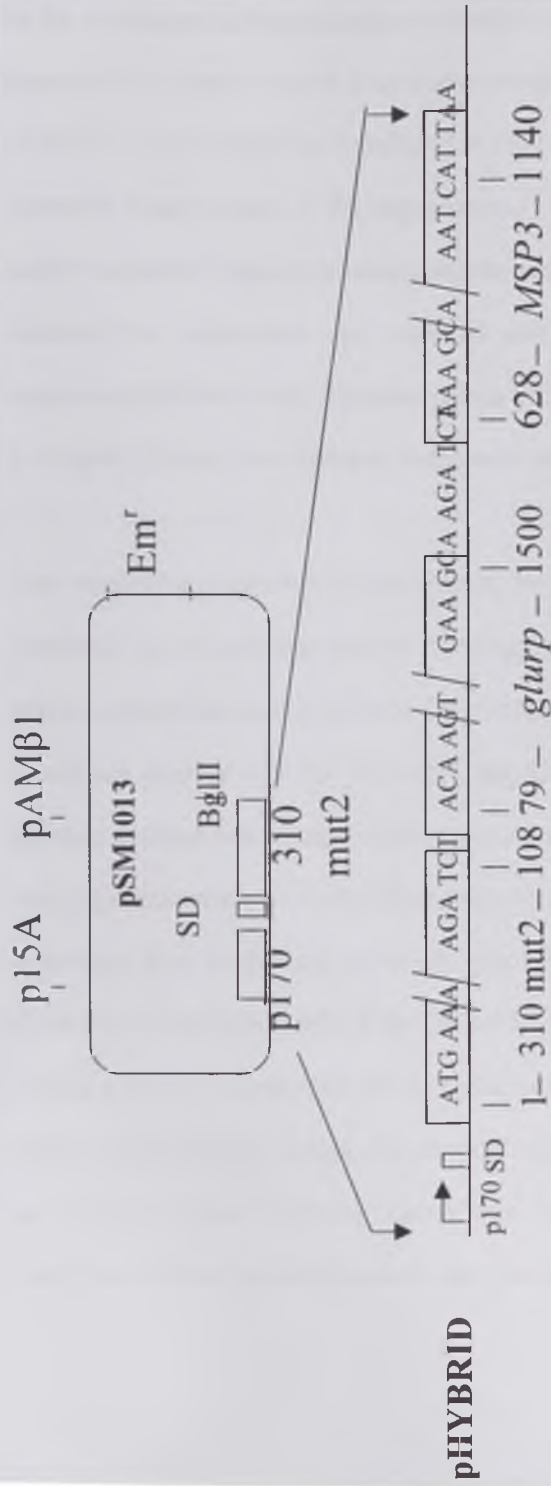
3.2.1 Antigens

The *P. falciparum* GLURP recombinant protein R0 and peptides from the N-terminal non-repetitive region of GLURP, LR129, LR130, MR186 and MR187, and LR55 from MSP3 antigen were kindly supplied by Dr. Michael Theisen from the State serum Institute, Denmark. Amino acid sequences of GLURP peptides, MSP3, and the GLURP-MSP3 hybrid are shown in Table 3.1 and Figure 3.1. Crude malaria schizont antigens were prepared and used in stimulation assay to assess responses to total blood stage soluble antigens.

3.2.1.1 Sequences of antigens

aa position	aa sequence
GLURP ₂₇₋₉₈ (MR186)	TSENRNKRIGGPKLRGNVTSNIKLPNNKGKIRGSND ELNKNSDEVLEQSEKSLVSENVPSGLDIDDIPKE
GLURP ₂₇₄₋₃₅₂ (LR129)	NLSIPNDPIEQILNQPEQETNIQEQLYNEKQNVEEKQN SQIPSLDLKEPTNEDILPNHNPLENIKQSESEINHVDHAL
GLURP ₃₄₀₋₄₁₈ (LR130)	SESEINHVDHALPKENIIDKLDNQKEHIDQSQHNINVL QENNINNHQLEPQEKPNIESFEPKNIDSEIILPENVETEE
GLURP ₄₀₆₋₅₀₀ (MR187)	SEIILPENVETEEIIDDVPSPKHSNHETFEETSESEHEEA VSEKNAHETVEHEETVSQESNPEKADNDGNVSQNSNNELNENEFVESEKSEHEA
MSP3 ₁₆₁₋₂₇₆ (LR55)	RKTKEYAEKA KNAYEKAKNA YQKANQAVLK AKEASS YDYI LGWEFGGGVP EHKKEENMLS HLYVSSKDKE NISKENDDVL DEKEEEAEET EEEEE

Table 3.1: Amino acid sequences of GLURP peptides and MSP3 antigen



Deduced amino acid sequence of the GLURP-MSP3 Hybrid:

AERSTSENRRNKRI GGP KLRGNVT SNIKLP SNNGKGIIRGSND ELNKNSEDVLEQSEKSLVSENVP SGLDIDDIPKESIFIQ
 EDQEGQTHSELNPETSEHSKDLNNDSKNESDII SENNKS NKVQNHFESLSDL ELL ENSSQDNLDKDTI STEPFPNQKHK
 DLQDDLND EPLEPFPPTQIHKDYKEKNLINEEDSEPFPRQEHKKVVDNHNEEKNVFHENG SANGNQGS LKLSFDEHLKDEKI
 EN EPLVHENLSIPNDPIEQILNQPEQETNIQEQLYNEKQNV EEKQNSQIPSLDLKEPTNEDILPNHNPLENIKQSESEINH
 VQDHALPKENIIDKLDNQKEHIDQSQHNINVLQENNINNHQLEPQEKPNIESFEPKNIDSEIILPENVETEEIIDDVSPK
 HSNHET FEEETSESEHEEA VSEKNAHETVEHEETVSQESNPEKADNDGNVSQNSNNELNENEFVESEKSEHEARSKAKIAS
SYDYILGW EFGGVPEHKKKEENMLSHLYVSSKDKENISKINDDLDEKLEEAETEEEEELEEKNEETEESEISEDEEEEEE
EEKEEENEKKKKEQEQSNENNDQKDMEAQNLI SKQNNNNEKNVKEAAESIMKTLAGLIKGNNDIDTLKDLVEELSKEYF
 KNH

3.2.2 Continuous cultivation of malaria parasites

In vitro cultivation of *P. falciparum* was done using cryopreserved infected erythrocytes, by the continuous culture technique with slight modifications. Frozen laboratory strain parasites (3D7) were removed from liquid nitrogen, thawed quickly in a water bath pre-set at 37°C. The mixture was centrifuged at 1500 rpm for 10 minutes and the supernatant discarded. Equal volume of thawing mixture (3.5% sodium chloride in sterile distilled water) was added, thoroughly mixed, and the suspension spun again at 1500 rpm for 10 minutes. The supernatant was removed and 1ml of complete parasite medium (consisting of RPMI-1640, Albumax (Gibco BRL, USA), Gentamycin and L-glutamine at 10µg/ml, (Sigma) were added to the parasite culture, and resuspended for washing.

After washing and removal of supernatants, the parasites were added to culture flasks containing 5ml of complete parasite culturing medium and 200µl O+ packed red blood cells to provide optimum conditions for growth. The culture medium was gassed with a special gas mixture (2% O₂, 5.5% CO₂ and 92.5% N₂) and incubated at 37 °C. The culturing medium was changed routinely to prevent changes in pH and accumulation of toxic substances that may inhibit parasite growth. This was done by gently removing the supernatant from the parasite culture and then mixed thoroughly. After thorough mixing of the infected red blood cells in the culture flask, a thin slide preparation was made by placing a drop of parasite culture on a slide and smeared to a thin smooth layer. The slide was then fixed in methanol for about 10 minutes and stained in 10% Giemsa buffer for 15 minutes, after which the Giemsa stain was washed off with water and the slide dried. The erythrocytes were counted under the microscope (Olympus BH2 Microscope)

at 100X magnification. The parasitemia was estimated, and the various parasite stages identified and recorded.

The rate of parasite growth is estimated by: % parasitaemia = $\frac{\text{Number of parasite infected RBC}}{\text{Total number of red blood cells}} \times 100$

When parasitemia level was about 2.5% with a high number of the late trophozoites and schizonts, a sub-culture was made by transferring 1.5ml of parasite culture into a bigger culture flask for optimal growth. Then 1ml of packed RBC and 20ml of complete parasite medium added and gassed. The cultures were maintained until the parasites matured to the schizont stage with parasitemia of approximately 5% or higher for use in the preparation of crude schizont antigen.

3.2.3 Preparation of the crude malaria schizont antigen

Crude malaria schizont antigen was prepared as soluble antigens of *P. falciparum*. Erythrocytes infected with *P. falciparum* in the late trophozoite and mature schizont stages (5% parasitaemia or higher) were separated from uninfected and ring-stage infected erythrocytes by magnet-activated cell sorting (MACS; Miltenyi-BioTec, Bergisch Gladbach, Germany), (Miltenyi *et al.*, 1993). The MACS column was flushed initially with 2% foetal bovine serum (FBS) in phosphate buffered saline (PBS). Infected erythrocytes from *in vitro* cultures were mixed and passed gradually through a size C MACS column mounted with a 0.9mm x 40mm needle (Becton, Dickinson, Fraga, Spain). The column was further washed with 2% foetal bovine serum (FBS) in PBS until no erythrocytes were seen in the eluate. The erythrocytes infected with parasites in the late developmental stages were retained specifically due to their high content of paramagnetic haemozoin (Paul *et al.*, 1981). The column was removed from the magnetic field and the late stage infected erythrocytes eluted by washing the column with buffer (2.0% FCS in PBS). The eluate collected was centrifuged at 2000 rpm for 10 minutes and the supernatant discarded. The cells were resuspended in 3ml wash medium, and 50µl of cell suspension was then added to 950µl of wash medium (20-fold dilution) and pipetted into the slide chamber (Neubauer improved 0.100mm, Germany) for counting under a microscope. The schizont enriched RBC preparation is estimated

$$\text{by: Number of RBC/ml} = \frac{\text{Number of cells counted}}{\text{Number of fields}} \times \text{dilution factor} \times 10^4$$

After counting, the cell suspension was adjusted to a concentration of 5.0×10^6 cells/ml by adding complete parasite medium. Parasite lysate antigen was prepared from the

eluate enriched for the schizont stage by 3 cycles of freezing and thawing. The parasite antigens from the lysed RBC released into solution was centrifuged at high speed of 16000 rpm for 30 minutes to remove the cellular debris. The supernatant containing the parasite soluble antigens was removed and stored at -80 °C until required. The prepared crude antigen was used as controls in stimulation assays.

3.2.4 Antibody measurements

Antibody levels in plasma against GLURP and MSP3 antigens were measured by an indirect ELISA method (Sousa *et al.*, 1998). Ninety-six well microtitre plates (Maxisorp; Nunc, Denmark) were coated (0.1M NaHCO₃ pH 9.6) overnight at 4°C with 100µl/well of goat anti-human F(ab)₂ antibody (Sigma, Aldrich) in PBS and 0.1M NaCl at 2µg/ml in columns 1-2 of each plate. Then 100µl/well of 0.2µg/ml recombinant GLURP-R0, and 1µg/ml LR55 (State Serum Institute, Denmark) were coated in columns 3 to 12 of each Elisa. The plates were washed four times in washing buffer containing 0.5M NaCl in Phosphate-buffered saline (PBS) and 0.1% Tween 20. This was done by dispensing washing buffer into all the wells and allowing it to stand for 1 minute before discarding and padding the plates to dry. The plates were blocked for 1 hour with 150µl/well of blocking buffer containing 3% milk powder in PBS-Tween 20. Two-fold serial dilutions of the reference standard were prepared in dilution buffer (1% milk powder in PBS and 0.1% Tween-20) for each isotype and subclass at various starting concentrations; purified polyclonal IgG (BP055) at 50ng; purified human IgM kappa (BP090) at 2000ng; purified human IgG1 kappa (BP078) at 500ng; purified human IgG2 kappa (BP080) at 50ng; purified human IgG3 kappa (BP082) at 1000ng; and purified human

IgG4 kappa (BP084) 1000ng. All standard antibodies were from The Binding Site, Birmingham UK. The standards were added at 100µl/well in columns 1-2 to be captured by the anti-Fab. Afterwards, 100µl/well of diluted test plasma samples 1/200 (IgG and IgM) and 1/50 (IgG subclasses) in serum dilution buffer (1% milk powder in PBS and 0.1% Tween-20 and 0.02% Na azide) were added to antigen-coated portions of the plate, and incubated for two hours. The secondary antibodies added were peroxidase-conjugated goat anti-human IgG (H17007, Catlag, 1mg/ml) and Peroxidase-conjugated rabbit anti-human IgM (H15007, Catlag, 1mg/ml) at 1µg/ml, (1:3000 in dilution buffer at 100µl/well). For IgG subclasses, polyclonal sheep anti-human peroxidase conjugated subclass-specific antibodies (The Binding Site, Birmingham, UK) were added at 100µl/well, for IgG1 (AP006, 1mg/ml) at 1:1000, IgG2 (AP007, 1mg/ml) at 1:1000, IgG3 (AP008, 1mg/ml) at 1:2000 and IgG4 (AP009, 1mg/ml) at 1:800 dilutions. After one hour of incubation, the level of bound secondary antibody in the test sample was quantified by colouring with 0.4 mg/ml of o-phenylenediamine (OPD) substrate with Hydrogen peroxide (H₂O₂), (Sigma-Aldrich, St. Louis, USA) in citrate buffer (100µl/well) for 30 minutes in the dark. The reaction was stopped by adding 50µl of 3M H₂SO₄. The plates were washed four times with PBS-Tween between each incubation step. The optical density (OD) at 492nm was determined by ELISA plate reader (Multiskan Ascent, Labsystems, Helsinki-Finland).

3.2.5 Cellular assays

3.2.5.1 Lymphocyte cultivation and proliferation assays

Frozen Peripheral Blood Mononuclear Cells (PBMC) from 56 children, and 21 Ghanaian adults were thawed quickly at 37 °C, washed by adding 30 ml of washing medium (RPMI 1640, 5% Fetal Bovine Serum (FBS), L-glutamine at a concentration of 10µg/ml and streptomycin-penicillin (5µg/ml) and centrifuged at 1500rpm for 10 minutes. The supernatant was discarded and the cell pellets resuspended in washing medium and centrifuged at 1500rpm for 10 minutes. The washing and centrifugation steps were repeated and after discarding the supernatant, the cell pellets were then resuspended in 3ml of complete parasite medium (RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 4ml of 10µg/ml L-glutamine and 5ml of 5µg/ml streptomycin-penicillin) and enumerated under the microscope (Olympus BH2 Microscope) at 40X magnification. The cell concentration was estimated as follows,

$$\text{Number of cells/ml} = \frac{\text{Number of cells counted}}{\text{Number of fields}} \times \text{dilution factor} \times 10^4/\text{ml}$$

After counting, the cell suspension was adjusted to 1.0×10^6 cells /ml by adding complete parasite medium. The 96-well round-bottom plates (Nunc, Denmark) were dosed initially with 20µl of GLURP peptide antigens (LR129, LR130, MR186, and MR187) for a final concentration of 10µg/ml. For the positive control antigens, purified protein derivative of tuberculin (PPD, Sigma), and phytohaemagglutinin (PHA, Sigma), at a final concentration of 5µg/ml and 2.5µg/ml were used respectively. The crude schizont antigen was tested at a concentration of 4.0×10^5 cells/well, 150,000 cells in a total

volume of 150µl of complete culture medium were added to each antigen dosed well. Cultures were done in triplicates and the plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Parallel cultures were done for the various cytokines, and IL-2 was harvested on day 2. On day 5, some cultures were stimulated with Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, P8139) and Calcium Ionophore Hemicalcium (Sigma-Aldrich, C9275) mixture at 1µg/ml and 15µg/ml respectively to enhance the detection of IL-4 in the culture supernatants. After 24 hours, supernatants from triplicate cultures were then pooled for IL-4 measurement. The other parallel cultures were harvested on day 5 for analysis of *in vitro* measurement of IL-10, IFNγ by ELISA. The harvested supernatants were stored in -80°C until needed for cytokine measurements.

3.2.5.2 Cytokine measurements

Measurement of *in vitro* cytokine production was done by ELISA using culture supernatants of antigen stimulated, and unstimulated cells. IFN γ , IL-2, IL-4 and IL-10 were detected in culture supernatants by double sandwich ELISA according to manufacturer's protocol. Briefly, microtitre plates were coated (1 x PBS and 0.001% phenol red) at 50 μ l/well of anti-cytokine capture antibody at 4 μ g/ml (IL-10, IL-4), and 2 μ g/ml for IL-2. IFN γ (MABTACH AB) capture antibody was coated with 0.1M NaHCO₃ buffer, pH 8.2 at 2 μ g/ml and incubated overnight at 4°C. Plates were washed 4 times in washing medium (PBS plus 0.05% Tween 20, pH 7.4), followed by blocking with 150 μ l/well of blocking medium (5% heat inactivated Foetal Bovine Serum (FBS) in PBS) for 1 hour at room temperature to block non-specific binding. After this step, the plates were washed 2 times. Cytokine standards (BD PharMingen, San Deigo) at starting concentrations between 2000 pg/ml–4000 pg/ml were prepared in two-fold serial dilutions in standard diluent (RPMI 1640 plus 1% NHS) and 50 μ l/well added. Then, 50 μ l/well of unstimulated and antigen stimulated culture supernatants were added in duplicates and incubated for 2 hours at room temperature on a rocker.

After washing four times, 50 μ l/well of biotinylated anti-human monoclonal cytokine antibodies were added at 1 μ g/ml, but 6 μ g/ml for IFN γ , and incubated for 45 minutes (room temperature). Plates were washed five times and avidin peroxidase (1:1000) was added at 50 μ l/well for 30 minutes at room temperature. For IFN γ plates, 50 μ l/well of streptavidin alkaline phosphatase in 5% FCS in PBS was added at 2.5 μ g/ml and incubated for 45 minutes

on a rocker. Microtitre plates were washed eight times afterwards to remove excess unbound enzyme conjugates. The plates were developed with 0.4 mg/ml of o-phenylenediamine (OPD) substrate with Hydrogen peroxide (H_2O_2), (Sigma-Aldrich, St. Louis, USA) in citrate buffer for 30 minutes to obtain colour, the reaction was stopped with 2.5 M H_2SO_4 and absorbance read at 492nm for IL-4, IL-2 and IL-10 ELISA's. Whereas, for $IFN\gamma$, 100 μ l/well of phosphate substrate (Blue Phos TM, Kirkegaard & Perry Laboratories, USA) (mixture of equal volumes of solutions A and B) were added and incubated for 20 minutes in the dark. The plates were stopped with stop solution (Blue Phos TM) and absorbance read at 630nm.

3.3 Statistical analysis

All data were entered into micro-soft excel program, and the Sigma stat statistical software (Jandel Scientific, San Rafael, Calif.) used for data analysis.

Spearman rank correlation was used to assess the strength of association between age of Ghanaian children and antibody responses to GLURP, MSP3 and the hybrid, and also the correlation between individual GLURP or MSP3 antibody responses and antibody responses to the GLURP-MSP3 hybrid. In addition, spearman rank correlation was used to estimate the correlation between antibody responses and cytokine levels. Mann Whitney's test was used to compare antibody responses in children categorized as susceptible to or protected from clinical malaria. R-sept and STATA 8.0 program (Stata Corporation, Texas, USA) were used for Logistic regression analysis to correct for the confounding effect of age. Differences were considered statistically significant if the *P value* < 0.05.

CHAPTER 4

4.0 RESULTS

4.1 The pattern of malaria in the cohort

Amongst the cohort of 300 children ages between 3 to 15 years, parasite infection seem to be stable, with about 50 percent of the children being asymptotically infected at any particular time (Fig 4.1). However, the incidence of clinical malaria was lowest prior to the rainy season (April), and was highest during the peak transmission season (July-September). Using the case definition of malaria as fever above 37.5 and parasitaemia above 5000 parasites/ μ l of blood, 63 children were categorized as having at least one episode of malaria (susceptible), and 202 children did not have any episode of malaria during the transmission season (protected). Ten children had fever above 37.5 and parasitaemia below the cut-off of 5000 and were categorized as indeterminate. The protected and susceptible groups of children were used in subsequent statistical analysis. The median age of the susceptible children was significantly lower than that of the protected children (median age-susceptible = 5years, median age-protected = 8years, $p < 0.001$)

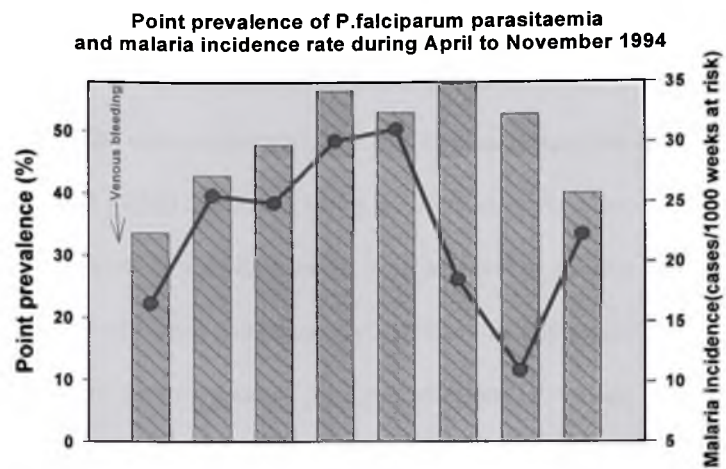


Fig. 4.1: Prevalence of *P. falciparum* parasitaemia in one transmission season in Dodowa (Dodoo *et al.*, 1999).

4.2 Antibody responses to GLURP, MSP3 and GLURP-MSP3 hybrid antigens

4.2.1 IgG / IgM responses to GLURP, MSP3 and GLURP-MSP3 hybrid antigens

Isotype and IgG subclasses responses to recombinant GLURP, MSP3 and GLURP-MSP3 hybrid antigens were measured by ELISA in plasma samples of 275 children out of an original cohort of 300 children residing in Dodowa. Fifty plasma samples obtained from healthy Danish adults who have never been exposed to malaria were also tested as controls. Prevalence of positive responses was defined as any response above the mean antibody level of the control plasmas plus two standard deviations ($\text{Mean}+2\text{SD}$). The prevalence of IgG and IgM responses to GLURP antigens were 48% and 58% respectively. The isotype responses to MSP3 were 38% for IgG and 20% for IgM. The prevalence of IgG and IgM responses to the hybrid GLURP-MSP3 were 81% and 60% respectively (Fig 4.2).

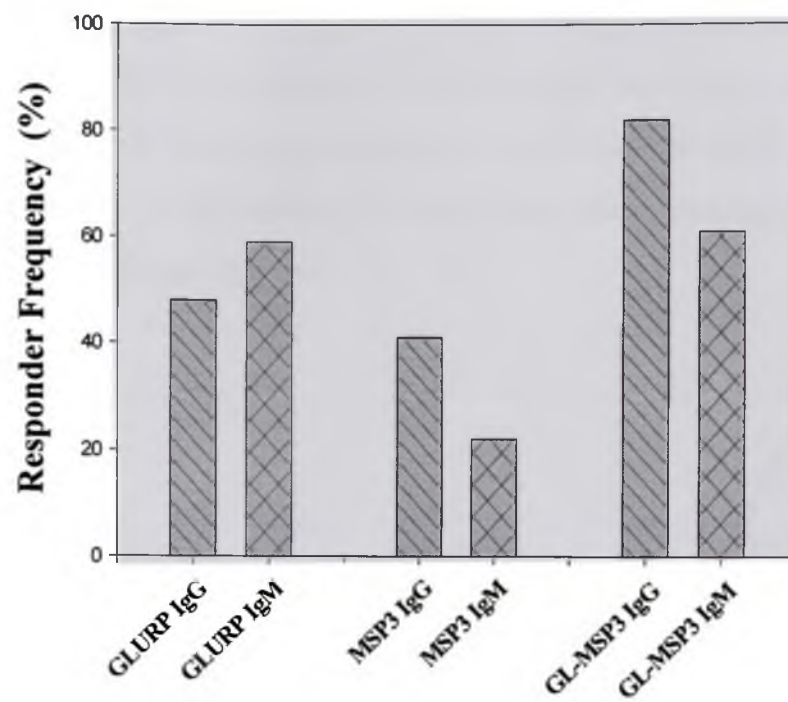
Prevalence of IgG and IgM Responses to GLURP, MSP3 and GLURP-MSP3 Hybrid**Antibodies**

Fig 4.2: IgG and IgM responses in all three antigens in Ghanaian children.

4.2.2 IgG subclass responses to GLURP, MSP3 and GLURP-MSP3 antigens

The IgG1-IgG4 subclass responses raised against GLURP were 29%, 1%, 9% and 4% respectively, while subclass responses to MSP3 were 27%, 0%, 13%, and 4% for IgG1-4, respectively. The prevalence of cytophilic antibody levels (IgG1 and IgG3) were higher in all the three antigens than the non-cytophilic antibodies (IgG2 and IgG4), (Fig 4.3). Among the three antigens, GL-MSP3 Hybrid had significantly higher antibody responses to the IgG subclasses.

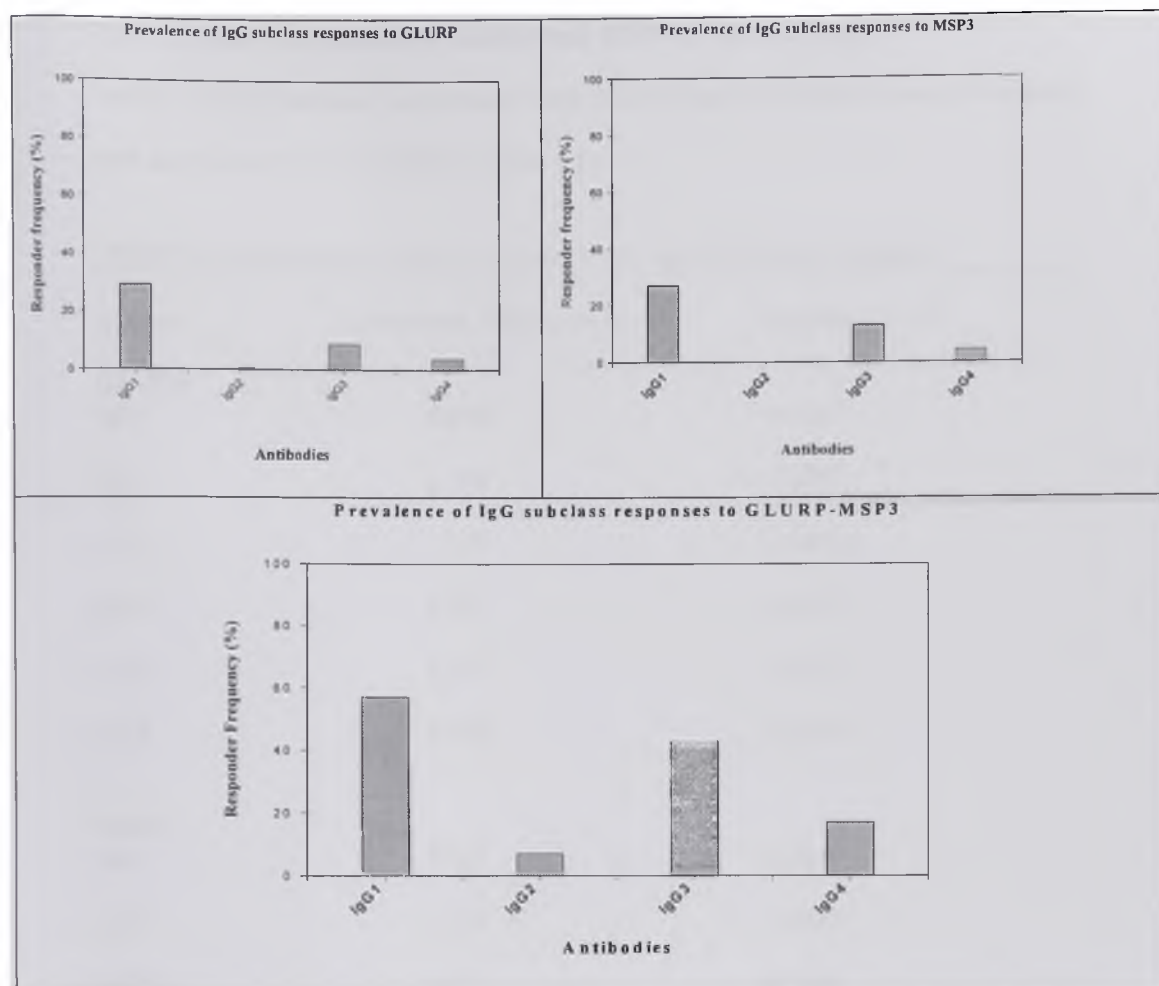


Fig 4.3: Graph of IgG subclass responses to GLURP, MSP3 and GLURP-MSP3 antigens in Ghanaian children.

4.2.3 Antibody responses to GLURP and MSP3 in relation to age

Isotype and IgG subclass responses to both GLURP and MSP3 significantly correlated with age ($0.54 > r > 0.31$, $p < 0.0001$) (Table 4.1).

Table 4.1: Association of antibody responses with age of Ghanaian children.

Antigen	Correlation Coefficient (r)	Significant Level
GLURP		
IgG	0.414	<0.0001
IgM	0.486	<0.0001
IgG1	0.512	<0.0001
IgG2	0.473	<0.0001
IgG3	0.347	<0.0001
IgG4	0.470	<0.0001
MSP3		
IgG	0.541	<0.0001
IgM	0.310	<0.0001
IgG1	0.393	<0.0001
IgG2	0.367	<0.0001
IgG3	0.520	<0.0001
IgG4	0.453	<0.0001

Table 4.1: done with spearman's rank-order correlation.

4.2.4 Antibody responses to GLURP and MSP3, and protection from malaria

IgG and IgM antibody levels of GLURP and MSP3 were compared between individuals categorized as susceptible or protected from malaria, and the data showed that antibody levels were significantly higher in protected individuals than those susceptible to malaria ($p < 0.05$) (Table 4.2). IgG subclass responses to both GLURP and MSP3 in the protected individuals were significantly higher than in the susceptible individuals ($0.03 > p > 0.001$, Table 4.3).

Table 4.2: IgG and IgM antibody responses in Ghanaian children protected or susceptible to malaria.

Antigens		Antibody levels		
		(Median and Interquartile range)		
		<u>Protected (n=202)</u>	<u>Susceptible (n=63)</u>	P-Value ^a P-Value ^b
GLURP				
IgG	545.0 (153.3 – 2224.4)	152.0 (51.3 – 541.0)	<0.001	0.046
IgM	1422.8 (857.5 – 3450.2)	874.7 (541.3 – 1589.4)	<0.001	0.089
MSP3				
IgG	437.9 (229.8 – 1385.8)	240.2 (97.7 – 521.9)	<0.001	0.118
IgM	380.2 (233.3 – 636.3)	287.6 (140.0 - 530.0)	0.021	0.258

P-value^a done with Mann-Whitney rank-sum test. P-value^b Multiple Logistic Regression Analysis (r-Sept); Data was transformed to normality using various equations. Data in ng/ml. P>0.05 NOT Significant.

Table 4.3: IgG subclass responses to GLURP and MSP3 in Ghanaian children protected or susceptible to malaria.

Antigen	Antibody levels			
	(Median and Interquartile range)			
	Protected (n=202)	Susceptible (n=63)	P-Value ^a	P-Value ^b
GLURP				
IgG1	123.9 (30.0 – 1177.1)	30.0 (30.0 – 133.3)	<0.001	0.050
IgG2	1.8 (1.0 – 9.9)	1.0 (1.0 – 2.3)	<0.001	0.167
IgG3	16.0 (10.0 – 47.4)	15.0 (8.0 – 20.6)	0.009	0.201
IgG4	14.5 (8.0 – 56.4)	8.0 (5.0 – 16.3)	<0.001	0.013*
MSP3				
IgG1	120.0 (36.1 – 808.6)	37.2 (20.0 – 167.0)	<0.001	0.105
IgG2	1.79 (1.15 – 2.90)	1.47 (1.0 – 2.43)	0.032	0.576
IgG3	14.5 (10.0 – 165.3)	10.0 (10.0 – 15.7)	<0.001	0.126
IgG4	13.7 (10.0 – 35.7)	10.0 (7.1 – 15.7)	0.001	0.541

From Mann-Whitney rank-sum test. P-value ^b Multiple Logistic Regression Analysis (r-Sept); Data was transformed to normality using various equations. Data in ng/ml. P>0.05 NOT Significant. * data had extremely low OD's (92% of OD's below 0.2).

Antibody ratios in relation to protection from clinical malaria: The cytophilic versus non-cytophilic antibody ratios (IgG1 + IgG3) : (IgG2 + IgG4) of the two antigens were compared between protected and susceptible individuals in relation to protection. The data indicated significantly higher ratios in protected compared to susceptible children with regards to responses against MSP3 (P=0.006), but not for GLURP. However, no significant difference was found when the ratios were compared after correcting for age (Table 4.4).

Table 4.4: Cytophilic to non-cytophilic antibody ratios in children protected or susceptible to malaria from Dodowa.

Antigens	Antibody Ratios (Median and interquartile range)		P-Value ^a	P-Value ^b
	Protected (n=202)	Susceptible (n=63)		
GLURP	7.68 (4.2 – 21.6)	6.3 (4.9 – 10.0)	0.254	0.596
MSP3	7.85 (3.62 – 46.4)	4.9 (2.78 – 12.7)	0.006	0.075

P-value ^a By Mann-Whitney rank-sum test. P-value ^b Multiple Logistic Regression Analysis (r-Sept); Data was transformed to normality using various equations. Data in ng/ml P>0.05 NOT Significant.

4.2.4.1 Age related exposure and protection from clinical malaria

Anti-GLURP or anti-MSP3 antibody levels and age were significantly associated with protection, and therefore require correction to establish a true relationship between the specific antibody responses and immunity to malaria. The data was therefore re-analyzed using a logistic regression model in which both age and antibody levels were included as explanatory variables for the outcome of infection, whether susceptible or protected. This analysis showed a significant association with protection for GLURP IgG ($P=0.04$) and IgG1 and IgG4 subclasses ($P=0.05$; $P=0.01$, respectively) (Table 4.3). However, IgG4 responses though statistically significant, the OD's were extremely low (92% below 0.2) comparable to the background levels. Although the majority of individuals with OD above 0.2 belong to the protected group.

After correcting for the confounding effect of age-related exposure, isotype and IgG subclass responses to MSP3 were not significantly associated with protection ($P>0.05$, Table 4.2 and 4.3).

4.3 Antibody responses to GLURP-MSP3, GLURP and MSP3 antigens

Antibody responses to either GLURP or MSP3 were compared to that of a recombinant hybrid antigen, GLURP-MSP3 in view of the fact that a future hybrid vaccine antigen is anticipated. The results showed a significant relationship between IgG antibody levels of GLURP-MSP3 versus GLURP and MSP3 antibody levels ($r=0.756$, 0.695 ; $P= 0.0001$) respectively. There was also a significant correlation between IgG1 and IgG3 responses to the hybrid antigen and that of GLURP ($r=0.696$, $P= 0.0001$; $r=0.363$, $p=0.0001$, respectively) or to that of MSP3 ($r= 0.713$, $p=0.0001$; 0.676 ; $P= 0.0001$, respectively), (Fig 4.4).

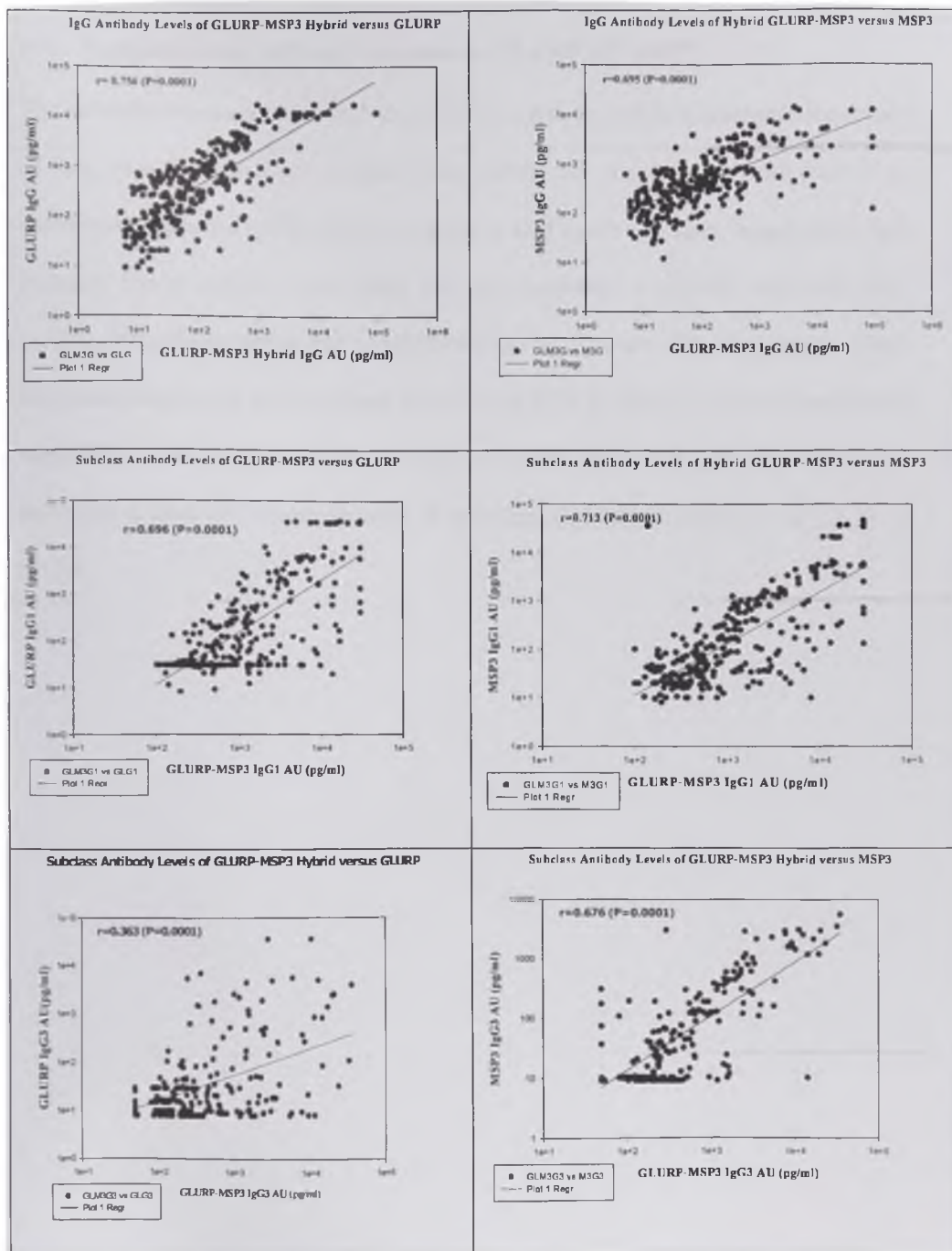


Fig. 4.4: Correlation of GLURP-MSP3 hybrid and GLURP or MSP3 antigens.

4.4 Complementary antibody responses to GLURP and MSP3

The antibody responses to GLURP and MSP3 were compared to characterize the pattern of responses in individual samples. The pattern of responses showed that some individuals responded to GLURP only, others to MSP3 only and some responded to both antigens. The prevalence of IgG, IgG1 and IgG3 responses to GLURP were 48%, 29% and 9%, respectively whiles that of MSP3 were 41%, 27% and 13%, respectively. When responder frequencies were analyzed for either GLURP or MSP3, a larger proportion of responders were indicated; 60% for IgG, 52% for IgG1 and 20% for IgG3, thus indicating a level of complementarity in inducing antibody responses to GLURP and MSP3.

4.5 T-cell responses to GLURP peptides

4.5.1 Cytokine responses in Ghanaian children and adults

Cryo-preserved peripheral blood mononuclear cells (PBMC) from a selected number of children were tested for cellular responses against GLURP specific peptides (LR129, LR130, MR186 and MR187). Control antigens included *P. falciparum* crude antigens, PHA and PPD. Fourteen PBMC's obtained from healthy adults from Denmark who have never been exposed to malaria were used as control cells. IFN γ , IL-10, IL-2 and IL-4 responses to GLURP peptide stimulated cultures of Ghanaian children were higher compared to the Danish controls (Fig 4.5). However, cultures of both Ghanaian and Danish volunteers stimulated with control antigen (PPD) produced elevated levels of cytokines (Fig 4.6).

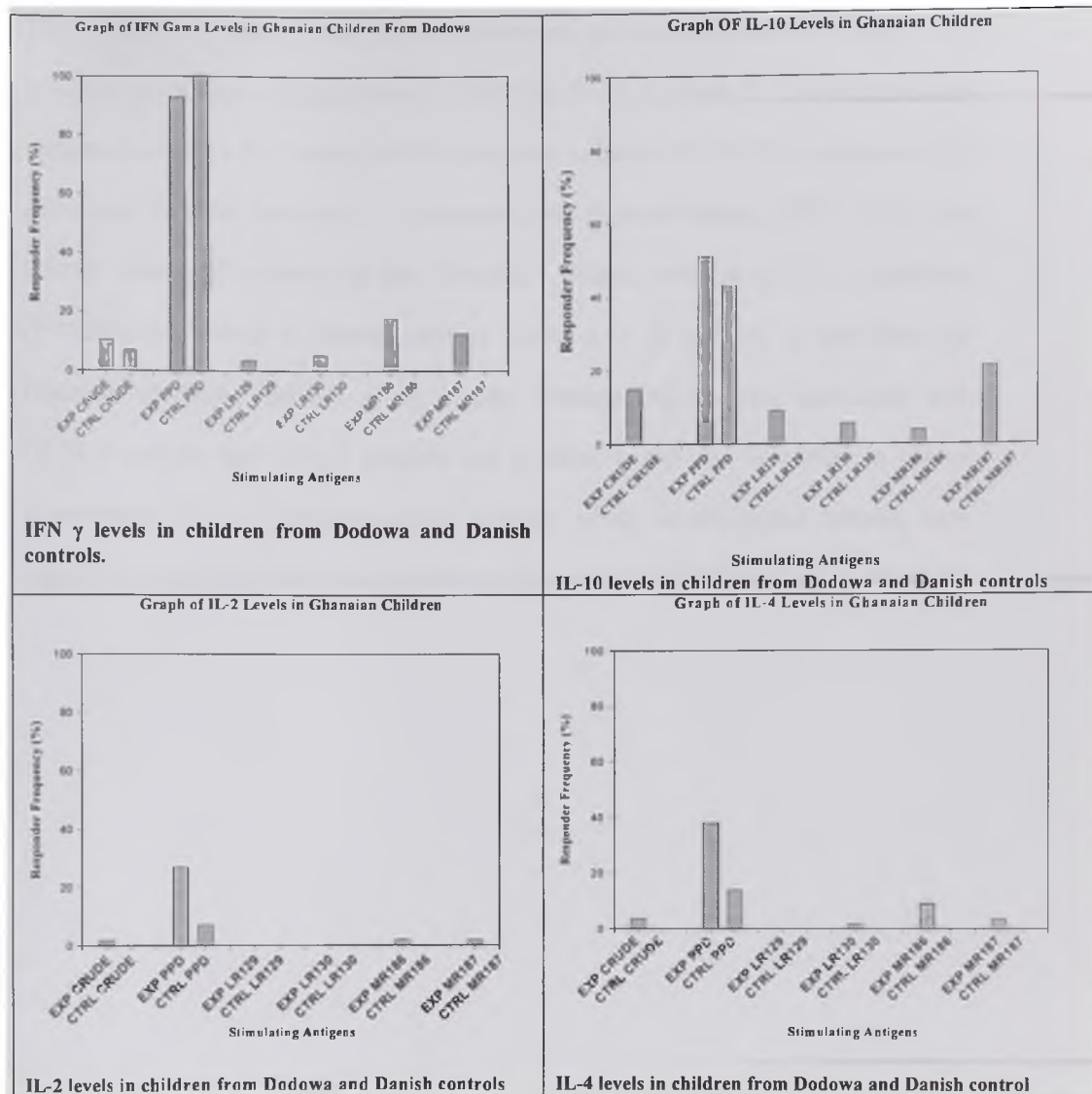


Fig 4.5: Cytokine levels in exposed (EXP) and non-exposed groups (CTRL).

The cytokine responses in the exposed individuals and non-exposed individuals were, however, not statistically significant ($P > 0.05$) for IL-10, IL-4 and IL-2 in the stimulated cultures, except for IL-2 levels in PPD stimulated cultures ($P = 0.002$). Furthermore, the differences in IFN γ levels to *P. falciparum* crude schizont antigen, PPD, LR129 and LR130 stimulated cultures in the Ghanaian children were statistically significant ($P < 0.050$) than levels in Danish controls (Table 4.5). In addition to data from the Ghanaian children, PBMC's of 21 healthy Ghanaian adults were stimulated with GLURP peptides and control antigens and cytokine levels were measured in culture supernatants. In the Ghanaian adults, cytokine levels in stimulated cultures were significantly higher ($p > 0.05$) than levels in cultures from adult Danish controls (Fig 4.6).

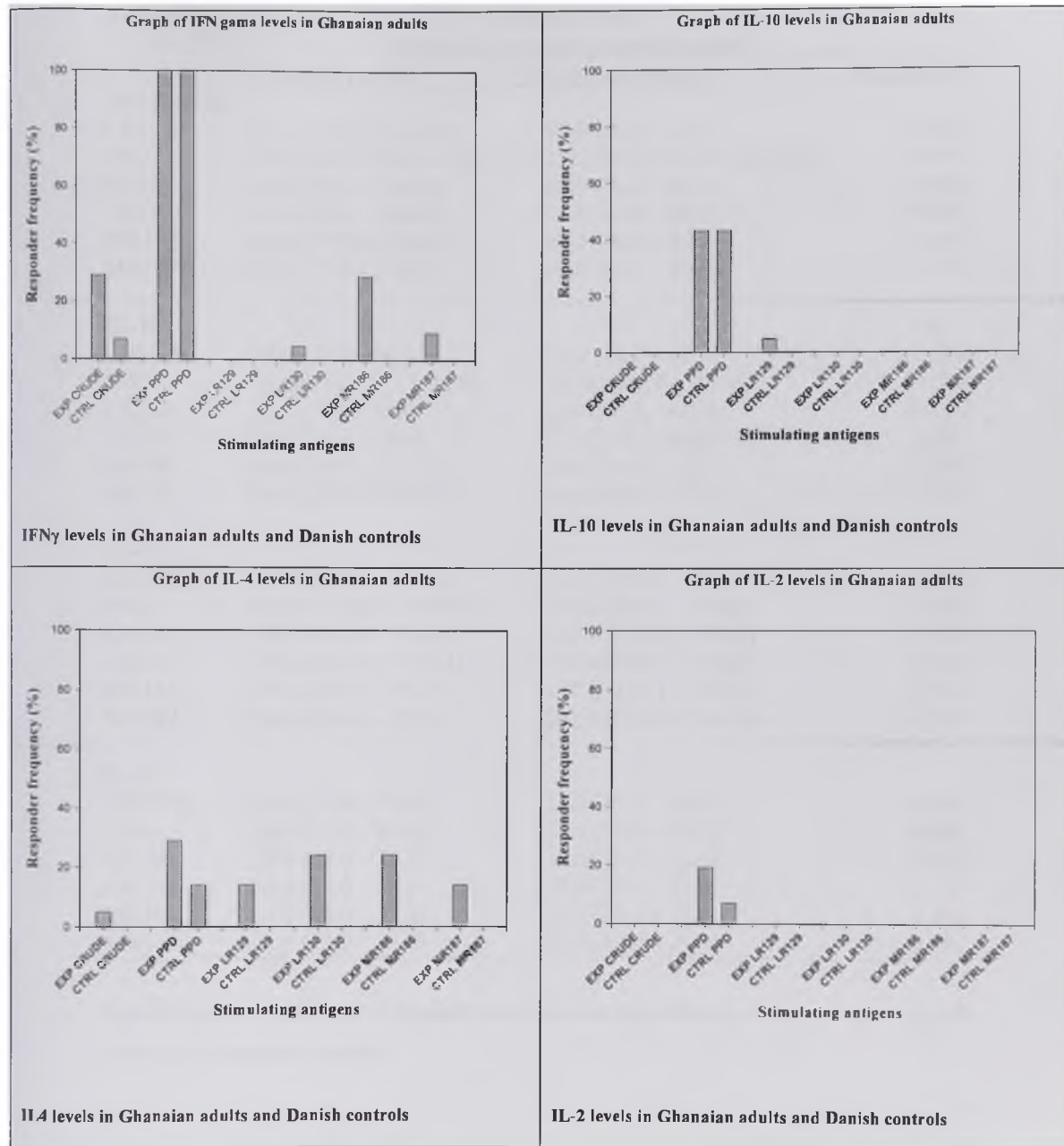


Fig 4.6: Cytokine levels in Ghanaian adults (EXP) and Danish controls (CTRL)

Table 4.5: Cytokine levels in exposed and unexposed individuals

Antigens	Cytokine Levels (Median and Interquartile range)		Sig. Level
	Exposed (n=56)	Unexposed (n=14)	
IFN GAMA			
CRUDE	60.0 (56.93 – 100.0)	80.0 (80.0 – 80.0)	0.019
PPD	1333.55 (612.20 – 3141.16)	3711.97 (1421.88 – 4000.0)	0.011
LR129	60.0 (55.0 – 100.0)	80.0 (80.0 – 80.0)	0.036
LR130	60.0 (60.0 – 100.0)	80.0 (80.0 – 80.0)	0.022
MR186	60.0 (57.50 – 100.0)	80.0 (80.0 – 80.0)	0.237
MR187	60.0 (55.0 - 100.0)	80.0 (80.0 – 80.0)	0.077
IL-10			
CRUDE	30.0 (25.0 - 56.80)	35.0 (25.0 – 40.0)	0.336
PPD	71.64 (41.74 - 131.19)	80.78 (38.76 - 96.52)	0.660
LR129	30.0 (25.0 - 50.0)	35.0 (25.0 – 40.0)	0.628
LR130	30.0 (25.0 – 49.4)	35.0 (25.0 – 40.0)	0.953
MR186	30.0 (25.0 – 43.17)	35.0 (25.0 – 35.0)	0.782
MR187	30.0 (27.31 – 68.89)	35.0 (25.0 – 35.0)	0.252
IL-4			
CRUDE	269.6 (85.4 – 733.4)	182.9 (107.7 – 391.9)	0.308
PPD	655.9 (123.0 – 1529.9)	236.2 (135.1 – 436.8)	0.065
LR129	300.9 (72.6 – 741.5)	152.7 (122.9 – 334.7)	0.274
LR130	294.4 (100.3 – 752.4)	159.4 (110.7 – 350.3)	0.261
MR186	345.6 (87.3 - 752.6)	187.5 (137.1 – 390.3)	0.315
MR187	266.6 (86.2 – 755.4)	202.4 (152.8 – 334.9)	0.390
IL-2			
CRUDE	25.0 (25.0 – 30.0)	25.0 (25.0 – 25.0)	0.081
PPD	30.0 (25.0 – 55.1)	25.0 (25.0 – 25.0)	0.002
LR129	25.0 (25.0 – 30.0)	25.0 (25.0 – 25.0)	0.124
LR130	25.0 (25.0 – 30.0)	25.0 (25.0 – 25.0)	0.100
MR186	25.0 (25.0 – 27.2)	25.0 (25.0 – 25.0)	0.124
MR187	25.0 (25.0 – 25.0)	25.0 (25.0 – 25.0)	0.182

Table 4.5: Median values (pg/ml) of stimulated culture supernatants in Ghanaian children and Danish controls.

P>0.05 is not statistically significant.

4.5.2 Cytokine responses in relation to age

PPD and PHA-induced IFN γ and PPD-induced IL-2 levels significantly correlated with age of Ghanaian children ($r = 0.372$, $P = 0.004$ and $r = 0.430$, $P = 0.001$, $r = -0.279$, $P = 0.037$, respectively). However, for the GLURP peptide and crude antigen stimulated cultures, there was no significant association with age for the other cytokines measured ($r = -0.06$ to -0.30 , $P > 0.05$).

4.5.3 Cytokine responses in relation to protection from malaria

Of the 4 GLURP specific peptides tested (LR129, LR130, MR186, MR187), MR186-induced IFN γ was significantly higher in the protected Ghanaian children compared to that in the susceptible children ($p = 0.003$). None of the other cytokine levels including IL-10, IL-2 and IL-4 measured in the peptide and crude antigen stimulated cultures correlated with protection ($p > 0.05$, Table 4.6).

Table 4.6: Cytokine levels in Ghanaian children susceptible to, or protected from clinical malaria

Antigens	Cytokine levels (Median and interquartile range)		
	Susceptible (n=17)	Protected (n=39)	Sig. Level
IFN GAMA			
CRUDE	60.0 (55.0 – 100.0)	60.0 (60.0 – 100.0)	0.527
PPD	912.4 (334.1 – 2913.3)	1343.8 (767.7 – 3211.7)	0.318
PHA	3472.7 (3143.6 – 4019.3)	4000.0 (3000.0 – 5000.0)	0.470
LR129	60.0 (55.0 – 100.0)	60.0 (55.0 – 100)	0.755
LR130	60.0 (58.8 – 100.0)	60.0 (60.0 – 100.0)	0.708
MR186	60.0 (55.0 – 63.1)	100.0 (60.0 – 154.7)	0.003
MR187	60.0 (55.0 – 100.0)	60.0 (60.0 – 100)	0.305
IL-10			
CRUDE	30.0 (25.0 – 71.5)	30.0 (25.0 – 54.8)	0.979
PPD	57.3 (33.9 – 196.5)	73.1 (44.2 – 120.8)	0.972
PHA	394.8 (297.2 – 608.7)	605.7 (336.3 – 939.9)	0.187
LR129	30.0 (25.0 – 42.5)	30.0 (26.0 – 50.0)	0.611
LR130	30.0 (25.0 – 40.0)	30.0 (25.0 – 50.0)	0.327
MR186	30.0 (25.0 – 40.0)	30.0 (26.0 – 50.0)	0.438
MR187	40.0 (28.8 – 42.5)	30.0 (26.2 – 81.9)	0.845
IL-4			
CRUDE	243.0 (102.2 – 620.0)	366.6 (46.5 – 808.40)	0.397
PPD	551.5 (152.7 – 1193.6)	752.5 (96.2 – 1622.8)	0.539
PHA	136.6 (89.6 – 208.4)	182.6 (114.0 – 334.5)	0.134
LR129	187.5 (69.0 – 479.9)	361.6 (85.8 – 745.9)	0.289
LR130	227.9 (104.3 – 468.0)	351.9 (88.6 – 817.5)	0.226
MR186	191.4 (93.0 – 844.5)	420.4 (78.2 – 731.3)	0.630
MR187	219.1 (88.4 – 502.2)	325.4 (74.3 – 860.9)	0.363
IL-2			
CRUDE	25.0 (25.0 – 30.0)	25.0 (25.0 – 30.0)	0.986
PPD	30.0 (25.0 – 49.4)	30.0 (25.0 – 59.9)	1.00
PHA	894.9 (638.6 – 1527.3)	1049.8 (746.1 – 1765.4)	0.407
LR129	25.0 (25.0 – 26.3)	25.0 (25.0 – 30.0)	0.788
LR130	25.0 (25.0 – 27.8)	25.0 (25.0 – 30.0)	0.957
MR186	25.0 (25.0 – 26.3)	25.0 (25.0 – 27.9)	0.789
MR187	25.0 (25.0 – 26.3)	25.0 (25.0 – 25.0)	1.00

Table 4.6: Median values (pg/ml) of cytokine responses of Ghanaian children susceptible or protected from malaria. P>0.05 is not statistically significant.

4.5.4 Cytokine ratios in relation to malaria infection

The capacity to regulate inflammation has been previously associated with protection from clinical malaria, however, ratios of pro-inflammatory cytokines (IFN γ and IL-2) to anti-inflammatory cytokines (IL-10 and IL-4) assessed in this study were not significantly associated with protection from malaria ($p > 0.05$, Table 4.7).

Table 4.7: Cytokine ratios in Ghanaian children susceptible to or protected from clinical malaria

Antigens	Cytokine Levels		
	(Median and interquartile range)		
	Susceptible (n=17)	Protected (n=39)	Sig. Level
IFNγ/IL-10			
CRUDE	2.0 (1.50 – 2.20)	2.0 (1.28 – 2.65)	0.229
PPD	7.9 (3.18 – 33.63)	13.8 (5.2 – 43.20)	0.465
PHA	8.89 (3.5 – 11.43)	5.54 (3.20 – 8.73)	0.176
LR129	2.0 (1.43 – 2.25)	2.0 (1.0 – 2.72)	0.533
LR130	2.2 (1.89 – 2.45)	2.0 (1.2 – 2.95)	0.796
MR186	2.0 (1.67 – 2.20)	2.2 (1.74 – 4.0)	0.157
MR187	2.0 (1.50 – 2.20)	2.0 (1.58 – 2.40)	0.796
IL-2/IL-4			
CRUDE	0.16 (0.06 – 0.49)	0.07 (0.03 – 0.30)	0.173
PPD	0.10 (0.04 – 0.19)	0.07 (0.03 – 0.60)	0.605
PHA	9.49 (5.27 – 12.60)	6.32 (3.56 – 9.18)	0.181
LR129	0.16 (0.06 – 0.38)	0.08 (0.03 – 0.36)	0.363
LR130	0.11 (0.06 – 0.23)	0.08 (0.03 – 0.41)	0.293
MR186	0.11 (0.03 – 0.23)	0.06 (0.03 – 0.38)	0.782
MR187	0.11 (0.05 – 0.28)	0.08 (0.03 – 0.73)	0.527
IFNγ/IL-4			
CRUDE	0.39 (0.13 – 0.67)	0.21 (0.08 – 1.52)	0.340
PPD	1.16 (0.62 – 4.49)	1.95 (0.86 – 9.47)	0.593
PHA	28.73 (15.30 – 42.50)	22.96 (10.80 – 27.86)	0.187
LR129	0.30 (0.17 – 0.84)	0.23 (0.09 – 1.38)	0.533
LR130	0.43 (0.13 – 0.68)	0.28 (0.09 – 1.39)	0.612
MR186	0.38 (0.08 – 0.70)	0.40 (0.09 – 1.50)	0.433
MR187	0.27 (0.12 – 1.67)	0.24 (0.08 – 1.67)	0.593
IL-2/IL-10			
CRUDE	0.83 (0.36 – 1.0)	0.83 (0.54 – 1.0)	0.873
PPD	0.52 (0.24 – 0.83)	0.51 (0.32 – 0.73)	0.735
PHA	2.34 (1.17 – 4.05)	1.52 (1.07 – 2.68)	0.345
LR129	0.83 (0.62 – 1.0)	0.83 (0.58 – 0.96)	0.702
LR130	0.83 (0.63 – 1.0)	0.83 (0.52 – 1.0)	0.363
MR186	0.83 (0.63 – 1.0)	0.83 (0.60 – 1.0)	0.412
MR187	0.63 (0.62 – 0.88)	0.83 (0.31 – 1.0)	0.776

Table 4.7: Cytokine ratios of Ghanaian children susceptible to or protected from malaria. P>0.05 is not statistically significant.

4.5.5 Relationship between humoral and cellular responses

It is known that cell mediated immune responses generally regulate antibody responses, thus GLURP induced cytokine responses in the selected Ghanaian children were related to antibody responses to GLURP recombinant antigens. The data however, could not demonstrate any relationship between cytokine and antibody responses to GLURP ($-0.09 < r < 0.03$, $p > 0.05$) as a whole, or in relationship with immunity from malaria ($0.01 < r < 0.07$, $p > 0.05$).

CHAPTER 5

5.0 DISCUSSION

Several malaria immuno-epidemiological studies have shown that antibodies play important role in protective immunity against malaria (Aribot *et al.*, 1996; Baruch *et al.*, 1997; Bouharoun-Tayoun *et al.*, 1990; Bull *et al.*, 1998; Dodoo *et al.*, 1999 and 2000; Hoffman *et al.*, 1986; Holder *et al.*, 1992; Oeuvray *et al.*, 1994 and 2000; Okech *et al.*, 2004; Smith *et al.*, 1995; Soe *et al.*, 2004; Theisen *et al.*, 1998 and 2001). Although both cellular and antibody responses have been shown to be important for immunity to malaria, antibody seem to promote effector cell mechanism against the malaria parasite, and thus protection from disease (McGregor *et al.*, 1963; Sabchareon, *et al.*, 1991). Multiplication of blood stage parasites are responsible for clinical malaria, and of the several identified blood stage antigens, *Plasmodium falciparum* glutamate rich protein (GLURP) and merozoite surface protein (MSP3) have been unequivocally demonstrated to be important in immunity against malaria. They have been associated with acquired protective immunity to malaria in many field studies (Bouharoun-Tayoun *et al.*, 1995; Dodoo *et al.*, 2000; Oeuvray *et al.*, 1994 and 2000; Soe *et al.*, 2004; Theisen *et al.*, 1998 and 2001). Affinity purified antibodies from both GLURP and MSP3 have been shown to inhibit parasite growth *in vitro* in the antibody dependent cellular inhibition (ADCI) assay, which involves monocytes and macrophages that release factors to indirectly kill parasites (Bouharoun-Tayoun *et al.*, 1995; Oeuvray *et al.*, 1994).

Previous immuno-epidemiological study in Ghana was done with a subset of a larger cohort, and focused on only serological responses to GLURP. In the present study, the larger cohort was assessed for antibody responses to both GLURP and MSP3 and cell

mediated responses to overlapping peptides of GLURP. It is important to assess immune responses to both GLURP and MSP3, since both antigens are being considered in a future vaccine, either as single antigens or as a hybrid construct.

The prevalence of Isotype and IgG subclass levels to GLURP and MSP3 antigens in plasma was significantly high in Ghanaian children. For both GLURP and MSP3 antigens, the prevalence of cytophilic antibody responses (IgG1 and IgG3) were higher than non-cytophilic antibodies (IgG2 and IgG4). This agrees with several studies done to show the critical role cytophilic antibodies play in individuals protected from malaria (Aribot *et al.*, 1996; Luty *et al.*, 1994; Ndungu *et al.*, 2002; Oeuvray *et al.*, 1994 and 2000; Soe *et al.*, 2004). In a previous study done in Ghana, the prevalence of GLURP (R0)-specific cytophilic antibodies (52%-89%) were higher than non-cytophilic antibodies (7%-51%), (Dodoo *et al.*, 2000). In another study conducted in Senegal, antibody responses to GLURP (R0) showed a similar trend in which cytophilic antibodies were higher in protected children than susceptible children (Oeuvray *et al.*, 2000).

The antibody prevalence against GLURP and MSP3 antigens were found to be significantly associated with age of Ghanaian children ($P < 0.0001$, $r = 0.54 > r > 0.31$). This occurrence has been frequently reported of antibody responses to various *P. falciparum* blood stage antigens (al-Yaman *et al.*, 1995; Aribot *et al.*, 1996; Dodoo *et al.*, 2000; Dziegiel *et al.*, 1993; Oeuvray *et al.*, 2000). This shows how partial protective immunity is acquired as individuals mature in age. Various studies conducted in endemic areas have shown an age-dependent increase in malaria immunity and a decrease in clinical

malaria episodes (al-Yaman *et al.*, 1995; Deloran *et al.*, 1987; Marsh and Greenwood 1992).

Several studies have confirmed the important role GLURP and MSP3-specific antibodies play in conferring protection against malaria. The children were therefore categorized into susceptible and protected groups to assess the role of antibodies in these groups. The data generated revealed a significant higher antibody levels in the protected children than susceptible children ($P < 0.05$), indicating a significant association between the antibody levels and protection. A study done by Theisen *et al.*, (2001), showed higher GLURP-specific antibodies in protected individuals than susceptible individuals. In addition, a study conducted in Asia, also showed elevated levels of cytophilic antibodies against MSP3 antigen in protected individuals than in susceptible children (Soe *et al.*, 2004). Furthermore, cytophilic to non-cytophilic antibody ratios have also been related to clinical malaria in immuno-epidemiological studies (Dodoo *et al.*, 2000) with the view that the interactions between the types of antibodies may be more important than relating individual cytophilic antibodies to clinical outcome. From this study, when the cytophilic to non-cytophilic ratios were compared, the cytophilic antibody ratio was higher in protected group than in susceptible individuals. However, the difference was statistically significant between protected and susceptible groups for MSP3 ($P = 0.006$), but not significant for GLURP-specific antibody responses ($P > 0.05$), probably due to higher non-cytophilic antibody levels to GLURP. This was not in agreement with previous work done in which cytophilic to non-cytophilic antibody ratios against GLURP (R0) were significantly higher ($p = 0.002$) in the protected group than in susceptible individuals (Dodoo *et al.*, 2000). Oeuvray *et al.*, (2000) have also

demonstrated the association of high cytophilic antibody ratios and ADCI activity *in vitro*, indicating that incorporation of cytophilic antibodies and monocytes is important for parasite clearance.

In this present study, however, after correcting for the effect of age in a logistic regression model, GLURP-specific IgG and IgG1 were associated with protection ($P < 0.05$). None of the MSP3 IgG and subclass levels were significantly associated with protection against malaria when the confounding effect of age was corrected ($P > 0.05$). This is in contrast with work done in which levels of IgG and cytophilic antibody subclass responses against GLURP and MSP3 were associated with protection after the effect of age was adjusted (Aribot *et al.*, 1996; Dodoo *et al.*, 2000; Oeuvray *et al.*, 2000). Interestingly, GLURP-specific IgG4 was found to be associated with protection in this study, this is not consistent with what has been documented by others in which non-cytophilic antibodies were not associated with protection (Aribot *et al.*, 1996; Dodoo *et al.*, 2000; Oeuvray *et al.*, 2000; Soe *et al.*, 2004). It may also be possible that the measured GLURP-specific IgG4 levels in this study associated with protection may not be of biological significance, since 92% of the measurements were comparable to the background levels.

From this study, some individuals, who did not respond to GLURP, responded strongly to MSP3 antigens. This suggests that in each individual, antibodies could be raised against either GLURP or MSP3 or both antigens at the same time to enhance protection against malaria. This complementarity of responses to GLURP and MSP3, which could lead to more effective immunity against malaria, was in agreement with a study done in

Asia in which there was complementary antibody responses to GLURP and MSP3 antigens (Soe *et al.*, 2004). Hence, it would be important to consider combining both antigens in a future vaccine against malaria.

Antibodies raised against GLURP and MSP3 have been shown to be protective, it is therefore important to look at their combined responses. Hence, epitopes of these two antigens were genetically coupled into a recombinant hybrid protein, GLURP-MSP3 in view of the fact that a future hybrid vaccine antigen is anticipated. Higher antibody levels were raised against GLURP-MSP3 hybrid in Ghanaian children compared to the individual GLURP and MSP3 antigens. Furthermore, there was a correlation between antibody levels of GLURP-MSP3 hybrid with MSP3 or GLURP antigens in Ghanaian children ($P < 0.05$). This is consistent with a study done in which plasma of Liberian adults showed higher antibody levels to the hybrid antigen compared to individual GLURP and MSP3-specific antibody levels *in vitro* (Theisen *et al.*, 2003). This suggests that the hybrid protein might have produced adequate presentation of the GLURP and MSP3 epitopes or antigenic determinants. Furthermore, in the same study, upon immunizing mice with GLURP, MSP3 and the hybrid antigens, antibody responses to hybrid protein exceeded that of individual GLURP or MSP3-specific antibody responses (Theisen *et al.*, 2003). Hence these two antigens when combined into a hybrid vaccine, may elicit higher antibody responses, which could lead to protective immunity against malaria.

Several studies have led to the recognition of the importance of T-cell in the generation of long-term antibody responses in malaria infection (Ballou *et al.*, 1987; Good *et al.*, 1988). Further studies have shown that cell-mediated immune responses play important role in malaria infection (Troye-Blomberg *et al.*, 1994). T-cell responses to GLURP peptides also revealed that T-cell epitopes were detected in LR67 and LR68 peptides. This suggests that the non-repeat region of GLURP may contain T cell epitopes that are recognized by individuals exposed to malaria (Theisen *et al.*, 2001). Previous study has been shown that production of an anti *P. falciparum* antibody *in vitro* requires T-cell-derived signals from cytokines (Garraud *et al.*, 1999).

This study further assessed the *in vitro* cellular responses in PBMC culture supernatant of children stimulated with some overlapping peptides of GLURP by the production of cytokines. The data showed lower cytokine responses to the antigen-stimulated cultures of the Danish controls who have never been exposed to malaria, compared to the antigen-stimulated cultures of exposed children. The cytokine responses observed in the Ghanaian children to the peptide antigens could be due to the role of memory T-cells acquired through repeated exposure to *P. falciparum* infection. Studies done by Theander *et al.*, (1997), have shown similar results in which cytokine responses were higher in PBMC's of exposed individuals than controls from non-endemic area after stimulating with *P. falciparum* synthetic MSP2 peptide.

From this study however, although cytokine responses in exposed group were higher than in unexposed group, the difference between stimulated cultures of exposed and non-exposed groups for IL-10, IL-4 and IL-2 levels were not statistically significant

($P > 0.05$). Only PPD induced IL-2 level ($P = 0.002$), crude, PPD and LR129 induced-IFN γ levels were significantly different in the two groups ($P < 0.05$). The level of cytokines produced in the peptide-stimulated cultures of the exposed group could be due to exposure to *P. falciparum* infection. Studies have shown that *P. falciparum* parasites are able to induce the production of several cytokines (Allan *et al.*, 1995; Jakobsen *et al.*, 1993). Surprisingly, from this study, a single Danish control sample produced IFN γ in crude antigen stimulated cultures. This is in agreement with studies done in which unexposed individual responded positively to malarial antigens (Jakobsen *et al.*, 1993; Rzepczyk *et al.*, 1989). Previous study done by Gabrielsen *et al.*, (1982) has demonstrated that responses to crude malaria antigens may be due to other mitogenic reactions. The cytokine responses to malarial antigens in unexposed individuals may be due to previous exposure to cross-reacting antigens from other infections (Hviid *et al.*, 1992 and Jakobsen *et al.*, 1993).

Although these children are in a malaria endemic area and their cells were expected to have generated cytokine production after stimulating with malaria antigens, only a small proportion of individuals responded to all the peptide antigens. Generally, the low cytokine responses observed in Ghanaian children could be due to lack of T-cell epitopes and MHC restriction. High crude antigen-induced cytokine responses (IL-2, IL-4, IL-10 and IFN γ) were observed in most of the cultures of Ghanaian children. The reason could be that the crude antigen has a repertoire of other *P. falciparum* antigens, which elicit a wide range of cytokine responses. This is in agreement with studies conducted in which significant levels of IL-2 and IFN γ were seen when stimulated with crude antigens compared with other purified malarial antigens or peptides (Butcher *et*

al., 1990 and Riley *et al.*, 1988). Cytokine levels between Ghanaian adults and children revealed a higher IFN γ levels from antigen stimulated cultures of adults than in children. There were more positive responders to IL-10 induced cytokines in children than in adults; however the levels of IL-4 and IL-2 were not significantly different in adults and children. Studies conducted have shown that T-cell responses to malaria antigens were higher in children than in adults (Jakobsen *et al.*, 1993), this was in agreement with what was observed in IL-10 induced cytokines produced. However, it contrasts with elevated IFN γ levels found in adults than in children in this study. IL-10 induction may be higher in children as anti-inflammatory responses, whilst higher IFN γ levels in adults may reflect immune status.

There was no significant relationship between cytokine levels of peptide stimulated cultures and age of Ghanaian children ($p>0.05$). Previous studies conducted in Ghana, found no correlation with induced cytokine responses with age in children (Dodoo *et al.*, 2002). A study conducted in Kenya found an association between IFN γ responses to pre-erythrocytic antigens (LSA1 and MSP1) and age of children (Chelimo *et al.*, 2003), however, IL-10 levels to LSA1 were not age dependent in another study (John *et al.*, 2000). This suggests that some cytokine responses may be age-dependent and thus relate to age specific acquisition of immunity, while others may be related to the pathogenesis of malaria infection.

Cytokine responses were compared between Ghanaian children categorized into susceptible or protected from malaria. Of the four peptides tested, MR186-induced IFN γ showed a significant association with protection. This agrees with a previous study that

associated high malaria specific IFN γ levels with reduced risk of clinical malaria (Dodoo *et al.*, 2002; Chelimo *et al.*, 2003). Previous studies have demonstrated the important role IFN γ plays in resistance to blood and liver- stage malaria infection and its association with protective immunity (Riley *et al.*, 1988; Luty *et al.*, 1999). The levels of IL-4 response were higher in protective group than in susceptible children, however, the difference was not statistically significant. However, some previous studies have shown that the induction of IL-4 against malaria could lead to protective immunity against malaria (Smythe *et al.*, 1988; Clark *et al.*, 1989). In contrast, IL-4 has been shown in other studies to facilitate parasite survival by suppressing anti-parasite activities of macrophages (Kumaratilake and Ferrente, 1992).

Various studies have shown significantly higher levels of IL-10 in children with malaria infection than healthy controls (Joao *et al.*, 1997). Other studies done to compare specific cytokine responses of patients with or without malaria in Malawi revealed higher serum IL-10 levels in malaria patients (Jason *et al.*, 2001). However, this study could not show any significant difference ($P>0.05$) in IL-10 levels between sick and protected individuals. IL-2 levels between the sick and protected children were not significantly different. This is in agreement with studies done involving IL-2, in which PBMC's stimulated in response to malaria-specific antigens during acute malaria infection, revealed no detectable levels of IL-2 production. This suggests that there may be defective cell mediated immune response to these malaria specific antigens during *P. falciparum* infection, which might have resulted in immune unresponsiveness (Ho *et al.*, 1988).

It has been shown that a balance between pro-inflammatory and anti-inflammatory cytokines plays a critical role in determining the outcome of clinical malaria (Troye-Blomberg and Perlmann, 1994). From this study, the ratio of pro-inflammatory cytokines (IFN γ and IL-2) and anti-inflammatory cytokines (IL-10 and IL-4) between sick and protected children showed no significant association with protection ($P>0.05$), which agrees with previous studies conducted by Dodoo *et al.*, (2002), that failed to establish a significant association between cytokine ratios and clinical malaria. Furthermore, when the cellular and antibody responses to GLURP were compared, to assess which cytokine(s) correlate with cytophilic antibodies, the data did not show any significant association between cytokine levels or ratios and cytophilic antibodies ($P>0.05$). The low proportion of individuals responding to the GLURP peptide antigens by the production of cytokines could be due to MHC restriction (Theander *et al.*, 1992).

5.1 CONCLUSION

In conclusion, cytophilic antibodies raised against both GLURP and MSP3 were associated with protection in Ghanaian children. There was an association between antibodies and age; however it was not significant for all antigens when the effect of age was corrected in a logistic regression model. In addition, antibody responses to GLURP and MSP3 antigens were complementary, suggesting that individuals could raise antibodies against either GLURP or MSP3 or both antigens and thus afford protection against malaria for a larger population. This finding provides evidence for the inclusion of these two antigens in a future multivalent antigen development. IFN γ responses to the GLURP peptide MR186 correlated with protection, and this suggests that there are T-cell epitopes in GLURP. The study did not however find an association between cytokine responses and antibody responses. The study has confirmed the importance of cytophilic antibody responses to GLURP and MSP3 in malaria immunity and that the combination of both antigens in a future vaccine is promising. GLURP specific T-cell cytokine responses found in the study further indicates its potential as a future malaria vaccine.

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