

**Licentiate thesis from the Department of Immunology,
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**Malarial anaemia: the potential involvement of
Plasmodium falciparum rhoptry proteins**

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**Curiosity unsatisfied dies
a death of boredom**

Adventures of Don Quixote

SUMMARY

Malaria remains a challenging health problem in malaria endemic regions. Infection with malaria invariably leads to anaemia. The groups at risk of developing malarial anaemia include children below the age of five years and pregnant women, especially primigravidae. Several factors have been suggested to be responsible for its aetiology, including increased destruction of infected and normal red blood cells together with bone marrow suppression. However, until recently, the molecular mechanisms involved have remained elusive. The aim of the work presented herein was to investigate the mechanisms responsible for the destruction of normal red blood cells in anaemia, and more specifically to define the role of the ring surface protein (RSP/RAP) -2 and other members of the low molecular weight rhoptry associated protein (RAP) complex, RAP-1 and -3.

In the first study we showed that antibodies to the RAP complex could mediate the destruction of RSP-2 tagged erythroid cells by phagocytosis or by complement activation and then lysis. In addition, antibodies to RAP-1 and RAP-2 could induce the death of RSP-2/RAP-2 tagged erythroblasts. We further investigated the frequency and functionality of naturally occurring RSP-2/RAP-2 antibodies in the sera of anaemic and non-anaemic Cameroonian children. We found that all sera investigated contained RSP-2/RAP-2 reactive antibodies by both immunofluorescence and flow cytometry. The anaemic group of children had significantly higher levels of antibodies of the IgG isotype than the non-anaemic individuals, while the levels of IgM were similar in both groups. With respect to IgG subclasses, low levels of IgG1 and -3 antibodies were detected. Higher levels of IgG3 were seen in the non-anaemic individuals as compared to anaemic subjects. With regards to antibody functionality, the non-anaemic individuals recognised a greater proportion of RSP-2/RAP-2 tagged erythrocytes and activated complement to a greater extent than the anaemic individuals.

From our findings, we can conclude that antibodies to the RAP complex are potentially involved in erythroid cell destruction during malaria which may result in anaemia, and that high levels of such antibodies may be detrimental to the host.

LIST OF PAPERS

This thesis is based on the following original papers, which will be referred to by their Roman numeral.

- I. **Nancy W. Awah**, Marita Troye-Blomberg, Klavs Berzins, Jürg Gysin. Mechanisms of anaemia: involvement of the rophyry associated protein complex. *Manuscript*.

- II. **Nancy W. Awah**, Marita Troye-Blomberg, Klavs Berzins, Jürg Gysin. Antibodies to the *Plasmodium falciparum* rophyry protein RSP-2 in relation to anaemia in Cameroonian children. *Manuscript*.

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ABBREVIATIONS

CR1	Complement receptor 1
Fe	Iron
G6PD	Glucose-6-phosphate dehydrogenase
GPI	Glycophosphatidylinositol
Hb	Haemoglobin
Hz	Hemozoin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LP	Lipid peroxidation
MHC	Major Histocompatibility complex
MIF	Migratory inhibitory factor
MSP	merozoite surface protein
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PAMPS	Pathogen associated molecular pattern
PRRS	Pathogen recognition receptor
RAP	Rhoptry associated protein
RAP	Rhoptry associated protein
RBCs	Red blood cells
RIG	Retinoic acid inducible gene I
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RSP-2	Ring surface protein-2
TLR	Toll-like receptor
TNF	Tumour necrosis factor

INTRODUCTION: AN OVERVIEW

Prelude

Generally speaking, anaemia is a public health problem that affects populations in both rich and poor countries. Although the primary cause is iron (Fe) deficiency, it is a multifactorial condition, particularly in malaria endemic areas. As such, establishing the relative contribution of malaria to anaemia is pretty difficult, as malarial anaemia is seldom present in isolation. Apart from Fe deficiency, it more frequently coexists with a number of other causes, such as, other parasitic infections (helminthes), nutritional deficiencies, and haemoglobinopathies, and several aetiologies might operate in the same individual. Malarial anaemia on its own affects predominantly people in the developing world, with children below 5 years of age and pregnant women (mostly primigravid) representing the most vulnerable groups. Until recently, malaria-related anaemia has received very little attention compared to other complications of severe malaria, notably cerebral malaria, perhaps due to its less dramatic presentation and the ease of treatment by blood transfusion. With the advent and spread of HIV, blood transfusion is no longer deemed a safe and attractive option, especially in the developing world where malaria is most prevalent, thus warranting alternative strategies for the treatment of malarial anaemia. Developing an effective vaccine against malaria has been the ultimate goal (priority) of the research community for the last decade. However, concerted efforts by several investigators/groups to develop a vaccine have so far been relatively unfruitful. This is probably due to the complex nature of the *Plasmodium* parasites amongst several factors. The observations from vaccine studies which suggest that following immunisation with blood stage antigens, monkeys developed anaemia even though their parasitaemia had been successfully controlled (Egan *et al.*, 2002, Jones *et al.*, 2002), might explain the recent renewed interest in severe malarial anaemia and the mechanisms responsible for its aetiology.

The immune system

The immune system in humans is categorized as 'innate' or 'adaptive'. The innate immune system detects the presence and the nature of infection, and provides the first line of host defence before the adaptive immune response sets in. During the onset of an infection, the host must launch a rapid innate response to control pathogen proliferation and spread until the adaptive response of specific T and B cells has fully developed. The detection of pathogens is first carried out by sentinel cells of the innate immune system, such as macrophages and dendritic cells, located in tissues that are in contact with the host's natural environment, and then by circulating granulocytes and monocytes that are rapidly recruited to the site of infection. These cells possess a set of germ line-encoded

receptors, known as pathogen recognition receptors (PRRs), which mediate the recognition of pathogen associated molecular patterns (PAMPs) on pathogens. The germ line encoded Toll-like receptors (TLRs) allow the direct recognition of distinct microbial structures that are shared by microorganisms, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), flagellin and bacterial DNA. Toll-like receptors (TLRs) are central components of the innate immune system. On ligand binding, they activate signaling cascades leading to the synthesis of proinflammatory molecules and chemokines. Chemokines recruit innate immune effector cells such as granulocytes, monocytes, macrophages, and NK cells (Coban *et al.*, 2005, Parroche *et al.*, 2007). The mannose, scavenger and dectin receptors mediate binding and phagocytosis of microorganisms and foreign particles. Fc, complement and scavenger receptors further facilitate the clearance of microorganisms that are coated with opsonic proteins, such as immunoglobulins, complement fragment C3b and mannose-binding lectin. More recently, two additional families of innate receptors have been described that join the TLRs as key pathogen sensors. These are the nucleotide-binding oligomerization domain (NOD-like; NLR) and the retinoic acid inducible gene I (RIG-I-like; RLR) receptors. To date, NLRs have only been shown to detect bacteria, whereas RLRs only recognize viruses (reviewed by Ulevitch 2004, reviewed by Creagh & O'Neill, 2006, reviewed by Meylan *et al.*, 2006, reviewed by Coban *et al.*, 2007). The activation of the innate immune response can, in addition, serve as a prerequisite for the triggering of the adaptive immunity. Here, the antigen-specific lymphocytes of the adaptive immune response are activated by co-stimulatory molecules that are induced on cells of the innate immune system during their interaction with microorganisms. The cytokines produced during these early phases also have an important role in stimulating the subsequent adaptive immune response and shaping its development (reviewed by Aderem and Underhill, 1999, Akira *et al.*, 2001, Colonna 2003).

Adaptive immunity is usually triggered when an infection eludes or overwhelms the innate defence mechanisms. Adaptive immunity is mediated by B and T lymphocytes.

T lymphocytes are primarily responsible for cell-mediated immunity, and B lymphocytes are responsible for humoral immunity. T cells can further be divided into $\alpha\beta$ T cells and $\gamma\delta$ T cells based on their antigen receptors (reviewed by Chien and Bonneville, 2006). The T cells are further divided into CD4+ and CD8+ T cells. CD4+ cells can be differentiated into Th1 and Th2 helper T cells and they are characterized by their different cytokine profiles. Th1 cells produce interleukin (IL)-2, interferon (IFN)- γ and tumour necrosis factor (TNF)- α whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (reviewed by Wipasa *et al.*, 2002). While $\alpha\beta$ T cells recognize processed protein antigens in the form of peptides associated with major histocompatibility genes (peptide/MHC), there is no known antigen processing and presentation requirement for ligand recognition by $\gamma\delta$ T cells (reviewed by Chien and Bonneville, 2006).

B lymphocytes are essential in the generation of humoral immune responses through the production of immunoglobulins (Igs). In humans, there are five classes of immunoglobulins divided into nine subclasses (IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgE and IgD) (reviewed by Pleass and Holder, 2005). Serum levels vary considerably for Igs of the different classes. The levels of IgG are the most abundant, making up about 85% of total serum Ig levels in humans. Roughly 5% of serum Ig is IgM, mainly pentameric. Monomeric IgA accounts for about 7-15% of serum Ig. Owing to its low rate of synthesis and its high susceptibility to degradation, IgD is rare making up about 0.3%. IgE is the lowest of all the serum Ig classes, accounting for 0.02% of total serum antibody (reviewed by Manz *et al.*, 2005). The primary function of Igs, is to bind antigen resulting in a direct effect e.g. neutralization. However, binding to antigen can also be ineffective unless secondary effector functions are recruited, including Fc-receptors (FcRs), and complement components amongst others (reviewed by Daëron, 1997, and Pleass and Holder, 2005). Different FcRs are expressed on all cell types of the immune system and they can serve as key immune modulators bridging the innate and adaptive immunity. Interaction between antibodies of a given class and the corresponding FcR results in a variety of cellular responses such as phagocytosis, antioxidant production, antibody-dependent cellular cytotoxicity (ADCC) and the release of inflammatory mediators.

Malaria

Malaria is an ancient disease that continues to take a toll on human existence. It remains one of the world's greatest public health problems. Today, an estimated 40% of the world's population remains at risk of malaria, with 500 million cases annually, resulting in 1–2 million deaths, mostly of young children, each year with a death from malaria occurring every 30 seconds (Greenwood and Mutabingwa, 2000). Ninety percent of the deaths occur in sub-Saharan Africa, but all tropical poor countries are affected. Four species of *Plasmodium* have been known to infect man namely: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* (Prudencio *et al.*, 2006). In addition to these, there are over a 100 *Plasmodium* species that infect a variety of other hosts, including reptiles, rodents, birds, primates and other mammals. The dogma of only four species of Plasmodia infecting humans has recently been challenged by the discovery of a large focus of simian malaria parasite *P.knowlesi* in the Malaysian population (Singh *et al.*, 2004). Therefore, a fifth species *P.knowlesi* (monkey parasite) is now known to infect humans (Cox-Singh and Singh, 2008, Cox-Singh *et al.*, 2008, Nishimoto *et al.*, 2008). *Plasmodium falciparum* is so far the deadliest of the five species, and is responsible for most of the morbidity and mortality associated with malaria (Prudencio *et al.*, 2006). *P. knowlesi* could become potentially dangerous if control measures are not taken.

The parasite and its life cycle

Malaria parasites are obligate intracellular parasites with a complex life cycle involving both a vertebrate (e.g. man) and an invertebrate host (mosquito vector), where they undergo different developmental stages. The life cycle of *P. falciparum* (and other plasmodia) commences when *Plasmodium* sporozoites enter the mammalian host through the bite of an infected female *Anopheles* mosquito. Sporozoites enter the blood vessel at the site of the bite and travel through the blood stream to the liver where they develop into merozoites.

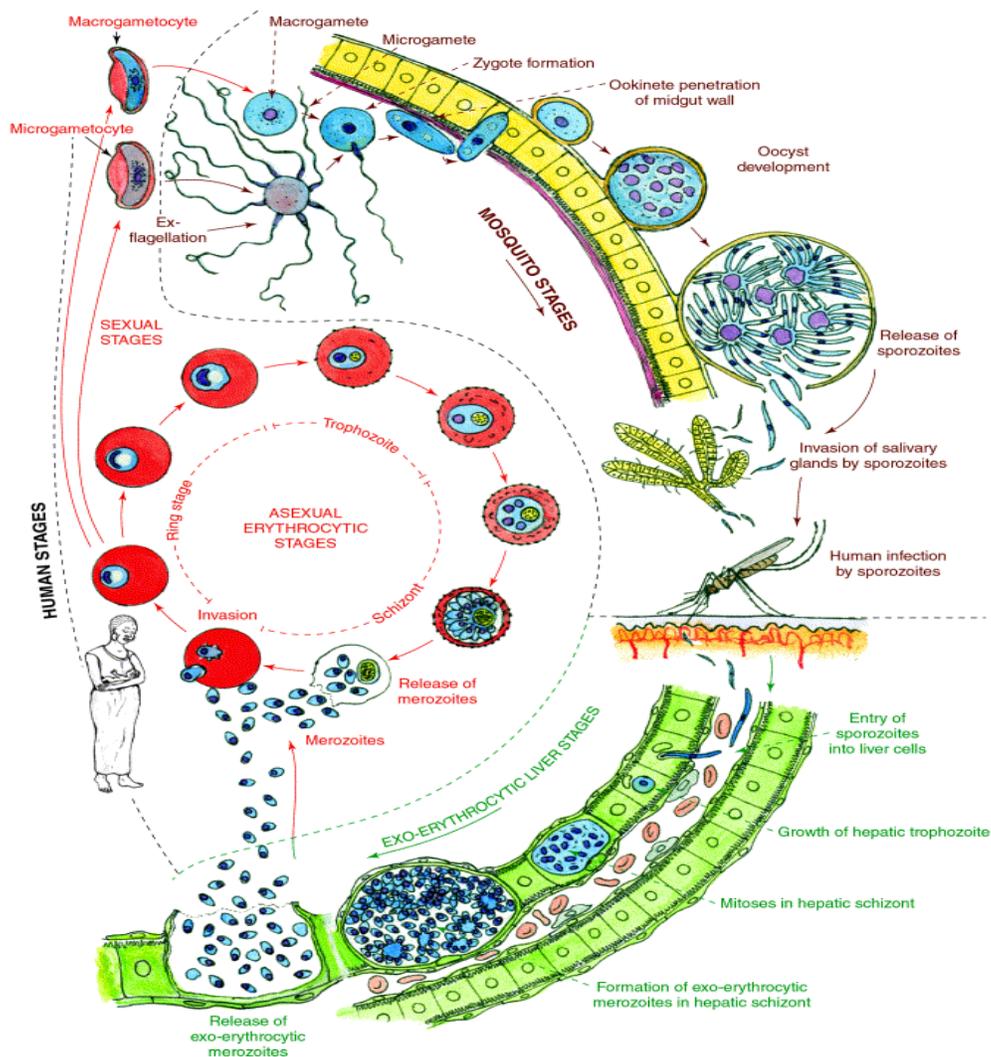


Figure 1: The life cycle of *Plasmodium falciparum* (Adapted from Bannister, L. and Graham Mitchell. *Trends in Parasitology* 2003; 19: 209-213)

For two of the five human species (*P. vivax*, *P. ovale*), dormant hypnozoite forms can develop in the liver leading to delayed clinical attacks months or years later, but for *P. falciparum*, there are no dormant forms. In the liver, merozoites replicate within the hepatocytes and several thousands of merozoites are released into the circulation.

Merozoite will invade erythrocytes, initiating a replication cycle of 48 hours, during which a single invaded merozoite develops into “ring” trophozoite, mature trophozoite, and finally a schizont. Each erythrocytic schizont, depending on the species, releases approximately 6 to 32 merozoites, which then in turn initiate a new asexual cycle by infecting other RBCs (Kumaratilake *et al.*, 1992). This phase of the life cycle constitutes the erythrocytic cycle, and it is the phase of the parasite’s life cycle that gives rise to all of the clinical symptoms and mortality due to malaria. At this stage, the parasite is known to cause a number of changes on the red cell membrane including the insertion or binding of parasite-encoded molecules to the red cell surface, which are important in host parasite interactions (Kirk 2001). At about this time, parasitized RBCs are detectable microscopically, and symptoms of disease occur, which are characterized by fever, chills, headache, lassitude, and gastro-intestinal symptoms.

The *Plasmodium* life cycle continues when some merozoites develop into the sexual parasite stages, the male and female gametocytes, which can be taken up by mosquitoes during subsequent blood meals. The gametocytes undergo fertilization and maturation in the mosquito midgut, forming an infective ookinete form, which migrates through the mosquito midgut into the hemocele, developing into an oocyst in which sporozoites are formed. When fully matured, the oocysts burst and release sporozoites, which migrate into the mosquito's salivary glands, ready for the next transmission step (Kirk 2001, Prudêncio *et al.*, 2006, Good *et al.*, 2005, Sargeant *et al.*, 2006).

The disease: clinical manifestations of malaria

The signs and symptoms of malaria and their gravity in humans are caused by the asexual blood stage of the malaria parasite and are quite diverse with a wide range of outcomes and pathologies (Weatherall *et al.*, 2002).

In fact, the spectrum of the disease may range from mild asymptomatic infections (reflecting the ability of adaptive immune mechanisms to prevent disease) through symptomatic (fever), to severe disease and this is greatly influenced by the infecting parasite, host genetic, geographic, nutritional and social factors (Fig. 2) (Miller *et al.*, 2002).

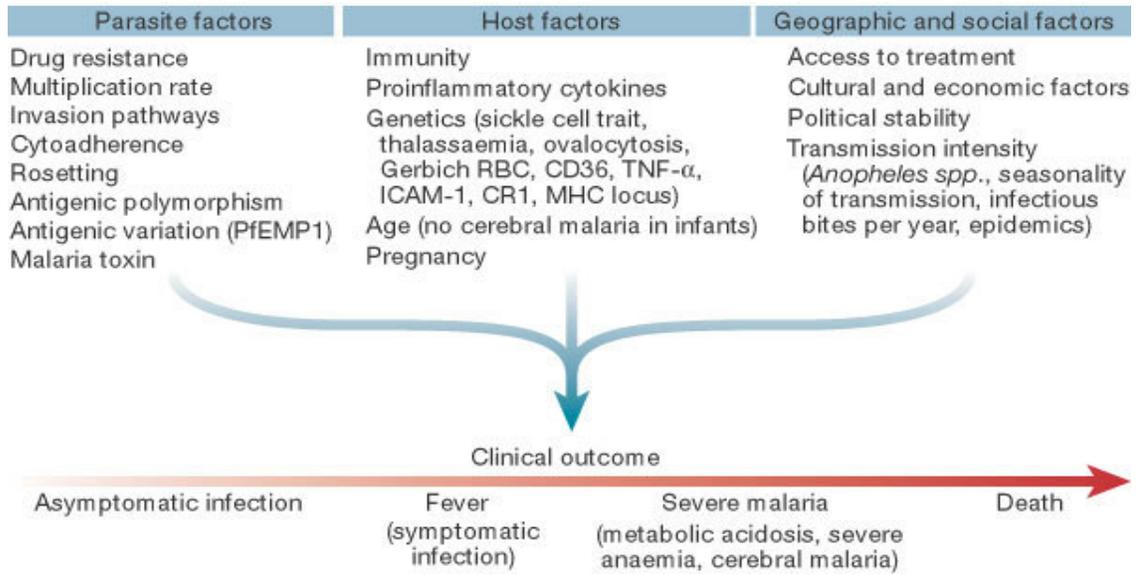


Figure 2: The spectrum of malarial infection depends on the infecting parasite, host, geographic and social factors. *Adapted from Miller et al (2002).*

The initial symptoms of the disease are also quite variable, particularly in children, and may include irregular fever, malaise, headaches, muscular pains, sweats, chills, nausea and vomiting (Najera and Hemple 2001). However, the principal symptom is fever. The classical fever paroxysms last 8-12 hours and occur in three stages:-

- The 'cold stage' which is characterised by marked vasoconstriction and lasts for 30 minutes to 1 hour. The patient feels intensely cold and uncomfortable and there is marked shivering though the temperature rises rapidly, often as high as 41°C.
- The 'hot stage' follows abruptly and lasts for 2-6 hours. The patient feels intensely hot and uncomfortable with intense headache and delirium maybe present.
- The sweating stage then occurs with profuse sweating and a drop in temperature. The patient feels fatigued and exhausted, but otherwise well and often sleeps off. Headache and other pains are relieved until the next rigour. However, in some instances, there might be an overlap in symptoms and signs between malaria and other acute febrile illnesses such as influenza or gastro-intestinal infections (Rooth and Björkman 1992, Font *et al.*, 2001). Redd *et al.* (1992) observed that 95% of children with a clinical definition for pneumonia also met the malaria case definition. In a small fraction of children, the disease may present with more severe disorders (severe malaria).

Severe falciparum malaria

Annually, severe malaria (SM) causes at least one million deaths, most of which occur in African children less than five years of age (WHO, 2000). It encompasses the major life-threatening clinical syndromes in malaria patients and includes cerebral malaria (CM), malarial anaemia (MA), respiratory distress syndrome (RDS) and placental associated malaria (PAM) in pregnant women (Miller *et al.*, 2002, Weatherall *et al.*, 2002, Schofield and Grau, 2005). Only *P. falciparum* causes the above mentioned complications and, in some cases, more than one syndrome may be present in a single individual. Hence, the disease might be accompanied by single organ, multi-organ and or systemic involvement.

Cerebral malaria

Cerebral malaria is defined by an unarousable coma not attributable to other causes, with any level of parasitaemia. It is a major cause of death in *falciparum* infection. Approximately 1% of the infection progresses to CM. The greater majority of cases occur in sub-Saharan Africa, and here, approximately 10-20% of individuals who develop CM die. Meanwhile, despite adequate treatment, survivors develop neurological sequelae. These sequelae are more common in children. A key feature of this manifestation is the sequestration of parasitized red cells in the microvasculature of various organs, most notably in the brain (reviewed in Good *et al.*, 2005).

Respiratory distress syndrome

The pathogenesis of respiratory distress syndrome (RDS) in malaria is poorly understood. It is defined as the presence of tachypnea and one or more of nasal flaring, in drawing, grunting or deep breathing in life-threatening malaria (Menendez *et al.*, 2000). It is most commonly associated with metabolic acidosis, usually involving lactic acidosis which has been shown to be a poor prognostic sign in children with severe malaria (Menendez *et al.*, 2000, Marsh *et al.*, 1995). The main clinical sign of RDS is deep breathing caused by attempts to release CO₂ to compensate for acidosis. There are several causes of lactic acidosis in children with severe malaria ranging from: - (1) Reduced oxygen delivery to tissues as a result of anaemia, causing increased tissue production of lactate; (2) Decreased clearance of lactate as a result of reduced liver function in malaria; (3) Effects of pro-inflammatory cytokines, e.g. TNF contributes to increased lactate production; (4) Lactate production by *P. falciparum* (through direct stimulation by cytokines) represents a minor contribution in this context (Miller *et al.*, 2002, reviewed by Good *et al.*, 2005).

Pregnancy associated malaria and anaemia

Pregnant women are particularly vulnerable to the effects of malaria, especially primigravid women, despite previously acquired protective immunity (reviewed by Hviid and Salanti, 2007). They are more likely than their non-pregnant counterparts to be infected with *P. falciparum* and once infected; there is a tendency towards increased disease severity. This was once speculated to be caused, in part, by the transient immune-suppression or –modulation, characteristic of pregnancy in order to sustain the fetal allograft (Menendez, 1995, reviewed by Hviid and Salanti, 2007). However, pregnancy-induce immune-modulation has been shown to mainly affect the cellular arm of immunity, whereas the humoral immunity is largely unaffected (reviewed by Hviid & Salanti, 2007). The effects of malaria on pregnant women may differ with regard to the level of acquired immunity, gravidity, trimester of pregnancy and the presence or absence of co-morbidity (reviewed by Duffy, 2007, reviewed by Lagerberg, 2008). An adverse consequence of an infection with *P. falciparum* during pregnancy (pregnancy associated malaria-PAM) is the sequestration of malaria parasites in the placenta resulting in placental malaria. Placental malaria is thus defined as the accumulation of *Plasmodium* infected erythrocytes in the intervillous spaces in the placenta, causing histologic changes, including leukocyte induced damage to the trophoblastic basement membrane (Uneke 2007). One major outcome of infection is maternal, which may lead to maternal death. The effects on the developing fetus and new born include intrauterine growth retardation, infant anaemia and low birth weight (due to preterm delivery), which in turn is an important determinant of infant mortality (Rogerson *et al.*, 2003, Duffy, 2007). An estimated 75000-200 000 infants are believed to die as a direct result of PAM each year (Steketee *et al.*, 2001). The accumulation of infected erythrocytes in the maternal circulation or intervillous spaces of the placenta occurs through adhesion of infected erythrocytes to placental receptors, such as chondroitin sulfate A (CSA) on the syncytiotrophoblasts surface or within the intervillous space. Infected erythrocyte adhesion is mediated by the clonally variant parasite protein PfEMP1 expressed on the surface of infected erythrocytes (reviewed by Rogerson and Boeuf, 2007).

Malarial anaemia

The blood stages of *P. falciparum* inevitably cause life-threatening anaemia, (Miller *et al.*, 2002). Compared to CM, not much is known about the host and parasite factors that contribute to malarial anaemia. A hemoglobin value of less than 5g/dL is considered to represent severe disease (Weatherall *et al.*, 2002, reviewed in Good *et al.*, 2005). Several factors may contribute to malaria anaemia. Since anaemia is the running theme for this piece of work, it will be discussed in greater detail in the following sections (see related background).

Immunity to malaria

The immune response in humans against natural infections due to malaria parasites is complex and varies with the level of endemicity, genetic makeup, age of the host and parasite stage and species. In addition, repeated infections and continued exposure are required to achieve clinical immunity (Marsh 1992). In highly endemic areas, children born to immune mothers rarely get infected during the first 6 months of life, and they are presumed to be protected by transplacentally transferred maternal antibodies. However this protection is lost during early childhood, rendering infants susceptible to severe forms of the disease with high mortality until the age of about 3-5 years. Once this stage of high parasitaemia infection is passed, acquired immunity (premunition) gradually develops. The developed immunity is not sterile, but individuals usually show low-grade parasitaemia without apparent clinical symptoms (Marsh 1992). Nevertheless, this acquired “anti-disease” immunity is not stable and requires exposure to repeated infections. The mechanisms of acquired immunity are poorly understood, but are thought to be cell mediated as well as humoral. An early response by monocytes or macrophages to parasite toxins is known to induce fever and other non-specific immune mechanisms, which lead to reduction of blood stage parasite densities. Subsequent steps leading to the protective state of premunition include the T cell mediated activation of macrophages and neutrophils as well as the induction of a B cell response. Extensive antigenic variation, immunodominance of non-protective antigenic structures in combination with low immunogenicity of epitopes critical for protection, represents major obstacles that account for the inability of the host to develop a sterilising immunity.

Innate immunity to malaria

Innate immune responses are the first line of defence against pathogens, and have an important role in malaria. Due to a complex life cycle of the parasite, polymorphism in many parasite antigens, host–parasite interactions, the resulting innate immune responses to malaria parasites are still poorly understood.

Recent evidence suggests that Toll like receptors (TLRs) are involved in the innate immune responses to *Plasmodium* (Coban *et al.*, 2005). *P. falciparum* blood-stage parasites have been shown to activate human plasmacytoid dendritic cells (DCs) as well as murine DCs through MyD88- and TLR9-dependent pathways (Coban *et al.*, 2005, Parroche *et al.*, 2007). Furthermore, glycosylphosphatidylinositol (GPI) from *Plasmodium falciparum* has been reported to interact with immune cells through the activation of TLR2 and TLR4 (Parroche *et al.*, 2007).

Variation in the host response to infection is known to have a genetic basis. Studies have shown that, individuals with certain red blood cell disorders are to some extent protected from malaria.

Genetic disorders such as sickle cell trait, thalasaemia, and glucose-6-phosphate dehydrogenase (G6PD) affect parasite survival and provide resistance to the host, probably by impeding the intraerythrocytic development of the parasite. West African children with the African form of G6PD had a 46-68% reduction in the risk of severe malaria, and HbS carriers had more than 90% protection from severe malaria (Yuthavong and Wilairat, 1997, Weatherall *et al.*, 2002). Persons with ovalocytosis have lower parasite densities following infection with *P. vivax* and *P. falciparum*, due to the elliptical red blood cells resisting parasite invasion. There is also evidence that HbC protects against severe *falciparum* malaria. Studies performed in Burkina Faso showed HbC to be associated with a 29% reduction in the risk of clinical malaria in HbAC heterozygotes, and with 93% in HbCC homozygotes (Modiano *et al.*, 2001). Also HbF in new borns inhibits *P. falciparum* development (Miller *et al.*, 1994).

Associations between the severity of *P. falciparum* malaria and the antigens of the ABO blood group have also been reported. In a Gambian study, a weak but significant association of blood group O with resistance was found (Hill, 1992). Indeed, rosetting has been shown to be reduced with blood group O erythrocytes compared with the non-O blood groups (A, B, and AB) in *P. falciparum* laboratory strains and field isolates. Rosetting is a known parasite virulence factor and is thought to contribute to the pathogenesis of severe malaria by obstructing the microvascular blood flow (Rowe *et al.*, 2007).

Non-specific phagocytosis and clearance of parasitized red cells from the circulation by the action of the reticulo-endothelial system in the liver and the spleen also contributes to innate resistance to malaria infection (Looareesuwan *et al.*, 1991).

Also, there is evidence that both *P. malariae* and *P. vivax* can provide some protection against subsequent *P. falciparum* episodes, or at least, prevent severe symptoms, suggesting that cross-species immunity between *falciparum* and *vivax* might be capable of controlling *P. falciparum* parasite densities (May *et al.*, 1999, Smith *et al.*, 2001). In contrast, the *P. malariae* effect is thought to be a consequence of down regulation of the cytokine production by this parasite (Black *et al.*, 1994).

Acquired Immunity to Malaria

Acquired immunity to malaria involves both the humoral and cell mediated arm of the adaptive immune system. The specific immune response to malaria may limit the rising parasitaemia, and with exposure to a sufficient number of parasite strains, it may eventually confer resistance to disease but not infection. Consequently, asymptomatic parasitaemias are commonly found in older children and adults living in hyper- or holoendemic areas.

Endemicity is traditionally defined in terms of the spleen rate and parasite rate and has been classified by the WHO as hypo-, hyper- and holoendemic areas (Wickramasinghe and Abdalla, 2000). In holoendemic areas, the spleen rate in children is > 75% and that in adults is low. Transmission is intense and continuous and there is a considerable morbidity and mortality from malaria in the first 2-3 years of life, followed by the acquisition of immunity. In hyperendemic areas, spleen rates are >50% in children and >25% in adults. Transmission is intense but seasonal and immunity within the population is low and symptomatic disease can be observed in all age groups. In hypoendemic areas, the spleen rate in children is less than <10% and transmission is weak (unstable). Epidemics can occasionally occur in such areas.

Humoral immunity to malaria

Antibody responses in malaria are crucial for the clearance of infection. Evidence for the importance of antibody responses in malaria was long demonstrated by passive transfer experiments using sera or purified immunoglobulins (IgG) from adult residents of hyperendemic regions of malaria (Cohen *et al.*, 1961, McGregor *et al.*, 1963 Sabchareon *et al.*, 1991). In malaria, acute infections are usually associated with a non-specific polyclonal B cell activation. It has been estimated that only 6% of the IgG and 11% the IgM are specific against malaria antigens, and a high proportion those antibodies are unrelated to protection (White and Plorde, 1991).

Protection has been mainly associated with IgG, and this seems to be largely dependent on the balance between cytophilic (IgG1 and IgG3) and non-cytophilic (IgG2 and IgG4) antibodies (Bouharon-Tayoun and Druilhe, 1992, Aribot *et al.*, 1996). The predominance of cytophilic IgG1 and IgG3 in individuals living in endemic areas has been associated with either lower parasitemia or a lower risk of malarial attack (Bouharon-Tayoun and Druilhe, 1992, Sarthou *et al.*, 1997, Tangteerawatana *et al.*, 2001, reviewed by Wipasa *et al.*, 2002). However, IgG2 antibodies to certain antigens have been associated with protection in some studies (Aucan *et al.*, 2000). IgG4 antibodies, on the other hand, are non-protective and might instead compete with cytophilic antibodies, thus inhibiting certain effector mechanisms (Warmerdam *et al.*, 1991, Bouharon-Tayoun and Druilhe, 1992, reviewed by Wipasa *et al.*, 2002, Garraud *et al.*, 2003).

Anti-malaria specific IgE seems to mediate both pathogenic and protective responses during malaria (Duarte *et al.*, 2007). Both IgE levels and IgE anti-plasmodial antibody are elevated in human and experimental malaria infections, but their role in protection and/or pathogenesis is not yet well-established in malaria. Total and anti-malarial IgE have been shown to be higher in patients with cerebral malaria than in patients with uncomplicated malaria (Perlmann *et al.*, 1994). However, high levels of anti-*falciparum* IgE have also been associated with a reduced risk of acute malaria in Tanzania (Berezcky *et al.*, 2004). IgE-containing immune complexes are thought to be

pathogenic via cross-linking of IgE receptors on monocytes/macrophages, with subsequent overproduction of local TNF- α , a major pathogenic factor in malaria (Perlmann and Troye-Blomberg 2000).

The mechanisms by which *Plasmodium*-specific antibodies mediate their effects is by either specifically binding to antigens on the surface of red blood cells or on the merozoites thereby blocking invasion, or by inhibiting schizont development, either alone or in cooperation with ancillary cells (e.g monocytes in ADCI) (Bouhroun-Tayoun *et al.*, 1995). Parasite neutralization may also be mediated by antibody-dependent phagocytosis involving monocytes or polymorphonuclear leucocytes or initiating complement-mediated damage. Antibodies may also prevent the adherence of infected cells to endothelial surfaces or rosetting of parasitized red blood cells to uninfected red cells, thereby preventing severe disease (reviewed by Wipasa *et al.*, 2002, Jafarshad *et al.*, 2007). Since protective immunity to malaria seems to be associated with different antibody classes and subclasses, the antibody quality and effector function will therefore be important in defining the antibody immune response in infected individuals.

Cell mediated immunity to malaria

Cell mediated immunity (CMI) plays a very important role in malarial immunity. The CD4+ T-cell subset is of major importance for the induction of blood-stage immunity, while the CD8+ subset has been shown to be cytolytic against liver stages of the parasite (Oliveira-Ferreira and Daniel-Ribeiro, 2001). Both Th1 and Th2 cells are involved in protective immunity against blood stage malaria and the balance of cytokines produced by these two subsets appears to be crucial in determining the disease outcome. During the initial phases of infection, Th1 cells are the main players, they appear to be important in the initial control of primary parasitaemia while a shift to Th2 later during the course of infection is crucial for the eventual resolution of infection via B cell/T cell cooperation and subsequent antibody production (reviewed by Troye-Blomberg *et al.*, 1994, Wipasa *et al.*, 2002). A role for $\gamma\delta$ T cells in protection has also been described, involving a granulysin mediated mechanism (Farouk *et al.*, 2004). Although the CD4+ T cells are critical for protection, they are also implicated in the pathogenesis of lethal complications (reviewed by Wipasa *et al.*, 2002).

RELATED BACKGROUND

General introduction to anaemia

Compared with available knowledge on the pathogenesis of CM, relatively little is known about the host/parasite factors that contribute to the etiology of anaemia during malaria infection and the mechanisms involved. Severe anaemia can be observed in patients despite low levels of parasitaemia during infection or as a result of chronic subclinical infection and can persist for weeks following complete therapeutic clearance of parasites (Abdalla *et al.*, 1980; Weatherall and Abdalla 1982, Phillips and Pasvol 1992, Camacho *et al.*, 1998, Weatherall *et al.*, 2002).

The *P. falciparum* life cycle includes a non-pathogenic, asymptomatic hepatic stage (extra-erythrocytic), which is followed by the invasion of mature erythrocytes by infective forms (merozoites) and the initiation of the pathogenic intra-erythrocytic stage. As the parasite develops within the host's erythrocytes, a number of changes take place, including the modification of the red cell membrane to form knob-like structures and increasing the rigidity of the cell, alterations in metabolic transport and the insertion of a number of parasite-derived proteins on the surface of the infected erythrocyte. The importance of these changes has not been fully dissected, but the development of immunity to malaria is thought to involve responses to malaria antigens expressed at the surface of the infected erythrocytes (reviewed by Craig and Scherf, 2001). A number of such proteins have been described to date, some of which have been implicated in the pathogenesis of severe disease, such as PfEMP-1, which is involved in antigenic variation, cytoadherence and sequestration, leading to different outcomes depending on sequestration site (reviewed by Craig and Scherf, 2001).

More recently, the parasite protein RSP-2/RAP-2, on which these studies are based, has been implicated in the development of severe malarial anaemia. Studies by Layez *et al.*, 2005 demonstrated that the immune response against RSP-2/RAP-2 could lead to the destruction of normal red blood cells and bone marrow erythroid precursor cells through mechanisms such as opsono-phagocytosis and complement activation. The immune mechanisms underlying severe malaria anaemia are poorly understood, but apparently involve both humoral (e.g. antibody and/or complement) as well as cellular (e.g. cytokine) immune responses. Hence, the immunologic basis of severe malarial anaemia is of particular interest because of its potential relevance to malaria vaccine development. This is especially so following observations from vaccine studies which suggest that immunized monkeys with acquired protection from acute infection may succumb to anaemia during a subacute or chronic phase of infection (Egan *et al.*, 2002, Jones *et al.*, 2002) and data suggesting the possible involvement of the RSP-2/RAP-2 protein in the development of severe malarial

anaemia as shown by Layez *et al.*, (2005) and confirmed by us in this thesis. Hence, an improved understanding of the pathogenic basis of severe malarial anaemia and the mechanisms involved will therefore have important implications in the design, development and deployment of safe and effective interventions against malaria, including vaccines.

The epidemiology/prevalence of malaria- related anaemia

As earlier mentioned, malarial anaemia is a common complication during malaria infections in endemic areas. Its prevalence and or severity is determined by a number of interacting factors including: the species of the infecting parasite; the intensity of transmission (endemicity); age and pregnancy status of the host; associated host genetic factors; and causes of anaemia other than malaria, e.g. hookworm infections (Geoffrey and Abdalla 2004). Severe malarial anaemia varies geographically (Nagel 2002), and is most frequently seen in areas with very high malaria transmission, and most commonly in young children and pregnant women (Greenwood 1997). The prevalence of anaemia, defined as a haematocrit <0.33 as measured in community surveys in malaria endemic areas of Africa, varies between 31 and 91% in children and between 60 and 80% in pregnant women (Menendez *et al.*, 2000, Schellenberg *et al.*, 2003, Casals-Pascaul and Roberts, 2006). The severity, incidence and predominant age for the onset of severe malarial anaemia (SMA) are dependent on the intensity of malaria transmission. In areas of high and stable malaria transmission, children between the ages 1 and 3 years present with SMA as a result of early and frequent exposure and quicker development of immunity. Meanwhile, in less endemic areas, it is common in older children due to less frequent infections and a delay in the acquisition of immunity (Menendez *et al.*, 2000, Schellenberg *et al.*, 2003). The mortality rate of malaria-related anaemia is between 5.6% and 16% for children 4-6 years of age and 6% for pregnant women, especially in primigravidae (Chang *et al.*, 2004). Children born in endemic areas are largely protected from severe malaria in the first 6 months of life by the passive transfer of maternal immunoglobulins and by fetal hemoglobin. Also, the rate at which anaemia develops and its severity is dependent on the level of *P. falciparum* parasitaemia (Achidi *et al.*, 1996). However, asymptomatic low density parasitaemia may also result in anaemia, particularly if the parasitaemia persists for prolonged periods upon lack of, or ineffective treatment (McElroy *et al.*, 2000). Price *et al.*, (2001) observed that even a single episode of uncomplicated malaria, diagnosed and treated properly, could still cause anaemia, which resolves slowly. Therefore, establishing the relative contribution of malaria infection to anaemia is essential for both clinical management and development of prevention strategies.

Features of malarial anaemia

Severe malarial anaemia

Severe malarial anaemia is most frequently seen in areas of very high transmission most commonly in young children and pregnant women. It is usually observed at low parasitaemia levels during chronic infections, and even after complete chemotherapeutic clearance of infection (Abdalla *et al.*, 1980, Philips *et al.*, 1986). According to the WHO, severe malarial anaemia is defined as a haemoglobin (Hb) concentration $<5\text{g/dL}$ or a haematocrit (Hct) <0.15 in the presence of a *P. falciparum* parasitaemia $>10,000$ parasites per μl , with a normocytic blood film. This definition is however considered specific but of low sensitivity, and of limited use both for the clinical management of patients and for epidemiological studies of anaemia. Over a third of the children admitted with severe anaemia had blood smears negative for malaria parasites, but responded to anti-malarial treatment (reviewed by Pascual-Casals and Roberts, 2006). Also, SMA may occur in acute or chronic infection, accompanied by a background of normal or low haemoglobin. Children with SMA may present with malaise, fatigue and dyspnoea or respiratory distress, defined by tachypoea, deep gasping breathing (Menendez *et al.*, 2000).

Hematologic features

The anaemia of *P. falciparum* is typically normocytic, with a notable absence of reticulocytes. It may be accompanied by changes in white cell and platelet counts. The leukocyte counts are usually low to normal in most cases of malaria. Increased leukocyte counts indicate either a severe infection or secondary bacterial infection. Reduction in the leukocyte counts is attributed to hypersplenism or sequestration in the spleen. Relative lymphocytosis, and/or monocytosis are also seen in acute infections. The presence of neutrophils containing pigment is associated with severe disease and unfavourable outcome (reviewed by Casals-Pascaul and Roberts, 2006).

Blackwater fever

Black water fever (BWF) received attention in early studies of expatriates living in endemic areas with low or no immunity against malaria. The syndrome is characterized by severe intravascular hemolysis, hemoglobinaemia and haemoglobinuria (Rogier *et al.*, 2003). Its symptoms include a rapid pulse, high fever and chills, extreme prostration, a rapidly developing anaemia, and the passage of urine that is black or dark red in colour (hence the disease's name). Abdominal pain, jaundice, hepatosplenomegaly, vomiting, and renal failure, especially in adults, have also been reported. The pathogenesis of BWF remains unclear, but has been largely associated with the irregular use of quinine for chemoprophylaxis in non-immune individuals and patients with severe *falciparum* malaria, as well as with other related drugs. BWF is also occasionally observed in

malaria patients with G6PD deficiencies that are treated with quinine, artemisinin or primaquine (Rogier *et al.*, 2003, Gobbi *et al.*, 2005). It is most prevalent in Africa and Southeast Asia. Individuals with increased susceptibility, such as non-immune immigrants or individuals who are chronically exposed to malaria, are classic sufferers from the complication. BWF seldom appears until a person has had at least four attacks of malaria and has been in an endemic area for six months (Weatherall *et al.*, 2002 Rogier *et al.*, 2003, Gobbi *et al.*, 2005).

The pathogenesis of malarial anaemia

Pathogenesis relates to the various host and parasite factors that are responsible for causing pathology. Several mechanisms have been implicated in the pathogenesis of severe anaemia including erythrocyte lysis and splenic phagocytosis, increased sequestration of parasitized red blood cells, and immune-mediated erythrocyte destruction, bone marrow dyserythropoiesis and ineffective erythropoiesis, and lower erythroblast proliferative rates and numbers (Wickramasinghe and Abdalla, 2000). The relative contributions to anaemia by the various mechanisms differ according to the age, pregnancy state, anti-malarial immune status and genetic constitution of infected individuals, and the local endemicity of malaria. In general, haemolysis is considered to be of greater importance in non-immune children experiencing acute malaria, whereas dyserythropoiesis is seen in persons experiencing recurrent or frequent *falciparum* malaria, although it is thought that several mechanisms are likely to operate in any one individual. In summary, SMA is thought to arise from mechanisms involve increased destruction of non-parasitized and parasitized RBCs as well as a decreased production of RBCs (Menendez *et al.*, 2000).

Clearance of infected RBC

Plasmodium parasites reside within host erythrocytes, schizogony and the subsequent release of merozoites invariably leads to intravascular haemolysis. This may suggest that hemolysis of the infected erythrocytes by the parasite is the most likely cause of anaemia. However, the destruction of infected red blood cells alone cannot explain the dramatic drop in hemoglobin levels frequently observed in anaemic malarious children. In humans, SMA is typically associated with parasite burdens that are considerably lower than those required for marked, direct destruction of RBCs (Jakeman *et al.*, 1999).

Red blood cell surface changes are commonly observed in malaria following parasitization. Normal red blood cells have an average diameter of approximately 7.5 μm and possess an amazing ability

to elongate, allowing them to squeeze through capillaries with a patent lumen much smaller than their own diameter (Mokken *et al.*, 1992, Dondorp, 2004). However, maturation of the parasite inside the red blood cell progressively abolishes this deformability, whereby the normally flexible biconcave disc becomes progressively more spherical and rigid and the surface becomes irregular with the presence of electron dense knobs. RBCs that have become rigid and inflexible as a result of malaria infection, are held up in the splenic microvasculature (and might contribute to tissue hypoxia) and subsequently cleared up by the spleen (Burchard *et al.*, 1995, Dondorp 2004).

The pitting of malaria parasites from red cells has been shown to have a role in controlling malaria infection. Pitting is a phenomenon wherein parasites are removed from erythrocytes and the once infected erythrocyte returned into circulation. On the down side, pitting alters the membrane of the red cells left behind, cells becoming more spherical and less deformable, hence susceptible to removal by stromal cells. Pitting of parasitized red cells has been proven to be responsible for the presence of ring infected erythrocyte surface antigen (RESA/Pf155) on non-parasitized red blood cells (Angus *et al.*, 1997, reviewed in Casals-Pascaul and Roberts 2006).

Band 3, a RBC transmembrane protein, known as the anion channel, marks senescent RBC for death by triggering the binding of specific auto-anti-band 3 antibodies, with subsequent complement activation. The antigenicity of band 3 is thought to result from its cleavage, clustering or and from exposure of cryptic epitopes (Santos-Silva *et al.*, 2001). The growth of *P. falciparum* induces profound modifications in the host erythrocyte membrane that lead to increased binding of autologous IgG, and complement. Further, the parasite development induces in sequence, deposition of haemichromes and oxidative aggregation of band 3, which become sites for deposition of autologous IgG and complement and hence subsequent recognition by phagocytes, a phenomenon typical of what may be seen with normally senescent RBCs (Giribaldi *et al.*, 2001).

In addition, *P. falciparum* development within infected red cells alters the distribution of phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (reviewed by Nagel 2002). Cells with exposed phosphatidylserine at their surface, probably due to oxidative stress, are hence readily recognized by macrophages, engulfed and degraded (Koka *et al.*, 2007).

Clearance of normal RBC

During malaria, many uninfected RBCs are destroyed in the spleen and liver, and their destruction has been identified as a major contributing factor to the onset of malarial anaemia. Both mathematical modeling and clinical studies have shown that approximately 12 uninfected RBCs are lost per every parasitized RBC, thus implicating the destruction of normal RBCs as a significant cause of the observed anaemia in malaria (Jakeman *et al.*, 1999, Price *et al.*, 2001). Different

mechanisms that target uninfected RBCs for destruction by intravascular hemolysis or for clearance mediated by the reticulo-endothelial system (extravascular hemolysis) remain unclear but are thought to include: increased oxidative damage (Greve *et al.*, 1999, Kremsner *et al.*, 2000), phosphatidylserine externalisation (Haldar *et al.*, 2007) and reduced deformability of red cells (Dondorp *et al.*, 1999). The reduction in RBC deformability has been shown to parallel disease severity and to correlate with anaemia, possibly due to splenic clearance. At hospital admission, a severe reduction in cell deformability is seen as a strong predictor of mortality, both in adults and children with severe malaria (Dondorp, 1997, Dondorp *et al.*, 1999, Dondorp 2002).

However, other mechanisms leading to destruction of normal RBCs may also involve IgG antibodies targeting parasite antigens such as RSP-2 (Layez *et al.*, 2005) or the *P. falciparum* glycosylphosphatidylinositol (GPI) (Brattig *et al.*, 2008) bound at the RBC surface and which may cause cell destruction by phagocytosis or complement mediated lysis.

Furthermore, during malaria infection, macrophages release reactive oxygen intermediates and nitric oxide which can cause damage to the parasites as well as to ‘innocent bystanders’ such as uninfected erythrocyte membranes and contribute towards erythrocyte destruction and subsequent anaemia in the host (Kremsner *et al.*, 2000, Griffiths *et al.*, 2001). Increased oxidation of normal red cell membranes has been reported in children with severe *P. falciparum* malaria (Griffiths 2001).

Stoute and *et al.* (2003), observed an association of reduced complement receptor 1 (CR1: CD35) and an increase in immune complexes on the surface of red blood cells and malarial anaemia. Complement regulatory protein CRI is important in protecting cells from complement-mediated damage by controlling the complement activation cascade (reviewed by Chang and Stevenson, 2004).

Decreased production of RBCs

Although red cell destruction plays a major role in anaemia of acute malaria, reduced production of erythrocytes in the bone marrow seems to be an important factor in maintaining anaemia, which usually takes 3-4 weeks to resolve. Decreased erythrocyte production is thought to involve bone marrow hypoplasia/suppression, dyserythropoiesis and ineffective erythropoiesis, and inappropriately low erythropoietin levels (Nagel 2002).

RBCs normally stay in circulation for about 120 days and during normal homeostasis, RBC numbers are balanced through the destruction of old RBC by the reticulo-endothelial system (RES) and the production of new RBCs through erythropoiesis. The ability of the bone marrow to compensate for a sudden increase in RBC loss is termed “effective erythropoiesis”. Under the influence of factors such as erythropoietin, haematopoietic stem cells in the bone marrow or spleen

(during effective erythropoiesis) multiply and differentiate to produce young and fully functional RBCs, i.e. reticulocytes, which are the earliest RBCs to be released into circulation (Schofield and Grau 2005). Thus, the reticulocyte count reflects an increased bone marrow output to replace the red cell losses. Otherwise, anaemia will inevitably develop when the accelerated removal of erythrocytes is not compensated for by an adequate bone marrow response as is often the case in malaria. Inadequate erythropoiesis can result from reduced production of erythroblasts (erythroid hypoplasia) or from normal or increased production of erythroblasts but with an intramedullary cell death (ineffective erythropoiesis).

In malarial anaemia, bone marrow suppression has been described in all malaria patients as well as in asymptomatic infections (Helleberg *et al.*, 2005), and is thought to be responsible for both the degree of and the delayed recovery from anaemia (Abdalla *et al.*, 1980). In acute malaria, there is a reduced total erythropoietic activity, as indicated by a normal or reduced marrow cellularity combined with reduced erythroblast proportions. Meanwhile, in chronic malaria, there is an increase in total erythropoietic activity, as evidenced by an increase in marrow cellularity (erythroid hyperplasia) together with an increased proportion of erythroblasts accompanied by inappropriately low levels of reticulocytosis, suggesting that this is associated with a greater ineffectiveness of erythropoiesis than in acute malaria (Wickramasinghe and Abdalla, 2000, reviewed by Chang and Stevenson, 2004). In addition, RBC iron utilisation, a measure of effective erythropoiesis is reduced in both acute and chronic malaria. It is thought that during malaria, there is a shift of iron distribution from functional compartments, comprising metabolically active iron that is required for normal function, toward storage compartments, that constitute an iron reserve, and thus suggesting a relative deficit in erythropoietin production or bone marrow unresponsiveness to erythropoietin (Verhoef *et al.*, 2002). Functional studies on dyserythropoietic bone marrows confirmed that there was an abnormal cell cycle distribution of early polychromatic erythroblasts in both acute and chronic malaria, with an increased proportion of cells in the G2 phase of the cell cycle, and a reduction in the ratio of the number of cells in the DNA synthesis (S) phase to the number in G2 (S/G2 ratio) (Wickramasinghe *et al.*, 1982). Further kinetic studies of erythroblasts suggested that all classes of erythroblasts had a prolonged S phase and the rate of production of polychromatic erythroblasts was reduced by about 50% of normal. This was thought to be as a result of a high death rate in this cell compartment (Dörmer *et al.*, 1983). Based on morphological abnormalities of erythroblasts in the bone marrow of patients, the basis for the ineffective erythropoiesis was thought to be due to apoptosis (Abdalla and Wickramasinghe 1988). Morphological abnormalities of erythropoiesis (dyserythropoiesis) include multi-nucleated erythroblasts, rupture of the cell nucleus with disintegration of chromatin (karyorrhexis), as well as incomplete mitosis. In a study of Gambian children with malaria, the disturbance in erythropoiesis was found to be confined to the morphologically recognisable erythroblast population (early polychromatic erythroblasts). The prevalence of these abnormalities was more marked in children with chronic than acute malaria

(Abdalla and Wickramasinghe, 1988, reviewed Wickramasinghe and Abdalla, 2000, reviewed Chang and Stevenson, 2004).

The mechanisms underlying the perturbation of erythropoiesis and ineffective erythropoiesis are not fully clarified. Several factors have however been implicated including direct and indirect effects of factors produced by the parasite.

Effect of soluble mediators (cytokines /chemokines)

Following clinical observations, the nature of the host's response critically influences the pathologic manifestations of malaria infection. Severe malaria is known to be associated with an acute inflammatory response characterized by elevated levels of pro-inflammatory cytokines and elevated responses have been linked to the etiology of severe malarial anaemia (Lyke *et al.*, 2004; Cordery *et al.*, 2007).

The macrophage migration inhibitory factor (MIF) is produced by activated T cells and macrophages, and has a wide range of biological activities including the induction of tumour necrosis factor alpha (TNF- α). MIF also inhibits the anti-inflammatory activity of glucocorticoids. It is released from macrophages in response to *Plasmodium* infected red cells. Due to its prominent expression in plasma, spleen and bone marrow during experimental malaria, it has been implicated in the development of malarial anaemia through erythropoietic suppression (Martiney *et al.*, 2000, Mcdevitt *et al.*, 2006). In *in vitro* studies, MIF was found to inhibit the formation of burst forming unit-erythroid (BFU-E) cells (Martiney *et al.*, 2000). MIF-knockout mice infected with *P. chabaudi* developed less severe anaemia had better erythroid development (as evidenced by increases in colony forming unit-erythroid (CFU-E) and BFU-E and demonstrated an improved survival relative to controls (Mcdevitt *et al.*, 2006).

Tumour necrosis factor (TNF)- α is an important immunoregulatory molecule in malaria which on one hand plays an important role in limiting malaria parasitaemia; but on the other hand is responsible for the development of the life-threatening complications of severe malaria. In patients with malarial anaemia high levels of serum TNF have been reported which were correlated with the severity of the anaemia (Gandapur and Malik, 1996). Children with severe anaemia were observed to have a depressed reticulocyte response and gross morphologic abnormalities of erythropoietic cells in the bone marrow. In rheumatoid arthritis patients an increased local production of TNF- α is associated with a low frequency and increased apoptosis of bone marrow erythroid progenitor and precursor cells, and the cytokine is responsible for the anaemia seen in these patients (Papadaki *et al.*, 2002).

The chemokine Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES: CCL5), has been implicated in malarial anaemia. Known roles of RANTES include promotion of

the migration of erythroid precursors into hematopoietic tissues as well as prevention of erythroid progenitor cell apoptosis. Hence, suppression of RANTES may lead to an ineffective erythropoietic response. In a study of Kenyan children RANTES was observed to decrease during severe malaria anaemia and was associated with the suppression of erythropoiesis (Were *et al.*, 2006).

Hemozoin

During infection, the concentration of Hz after erythrocyte rupture may be as high as 100 µg/ml, but it is rapidly cleared from the circulation by the liver and spleen because of its particulate nature. As a result of its high concentration in immune tissue, Hz has been suggested to contribute to the systemic inflammatory immune responses seen during malaria infection. It has been shown that Hz purified from *P. falciparum* activates macrophages to produce pro-inflammatory cytokines, chemokines, and nitric oxide as well as enhance maturation of human myeloid dendritic cells (DC) (Coban *et al.*, 2005, Parroche *et al.*, 2007). In a study of Kenyan children with malaria, circulating free and intraleukocytic Hz was found to be associated with anaemia and ineffective erythropoiesis, probably as a consequence of lipid peroxidation (Casals-Pascual *et al.*, 2006). Furthermore, this association was independent of levels of the proinflammatory cytokines TNF- α and IFN- γ which have also been implicated in the suppression of erythropoiesis during anaemia (Casals-Pascual *et al.*, 2006). *In vitro*, 4-hydroxynonenal (4-HNE), a final product of lipid peroxidation generated by hemozoin was found to inhibit the growth of progenitor erythroid cultures. Thus, 4-HNE, in addition to playing a role in erythrocyte deformability and consequent cell destruction, may also be involved in dyserythropoiesis and anaemia (Giribaldi *et al.*, 2004).

The spleen and reticuloendothelial hyperactivity

The spleen plays a very important role in the pathophysiology of malaria. One of its functions is in the removal of infected, opsonised or damaged cells in the circulation (Urban *et al.*, 2005). A rapid splenic enlargement in malaria has been associated with its increased capacity to clear both damaged and infected red cells from the circulation, both by Fc-receptor mediated immune mechanisms and by recognition of the reduced deformability, thus limiting the acute expansion of infection. In addition, splenomegaly has been associated with macrophage hyperplasia during malaria.

Diagnosis of malarial anaemia

Microscopy

Microscopy is the most common method used to confirm the presence of *Plasmodium*, quantification of parasitaemia, and in the determination of reticulocyte counts. The absolute

reticulocyte count is a useful reflection of erythroid activity in response to anaemia as to whether the erythroid response is appropriate to the degree of anaemia.

Haemoglobin/haematocrit:

Other useful algorithms include haemoglobin (Hb) estimation or packed cell volume (haematocrit) by microhaematocrit centrifugation.

Clinical signs

Clinical correlates of anaemia commonly used in diagnosis particularly where resources are limited include: skin (palm and nail bed), mucous membranes (conjunctival), pallor, and hepatosplenomegaly.

Haptoglobins

The glycoprotein haptoglobin (Hp), an acute phase protein, is one of the body's main tools for removing circulating, toxic free hemoglobin during intravascular hemolysis. Hp binds rapidly and with high affinity to free hemoglobin (Hb) with the resulting Hp/Hb complexes taken up by macrophages so that the heme iron is recycled. When large amounts of Hp/Hb complexes are formed in plasma, the Hp levels fall, following the clearance of complexes. Hp is therefore useful in diagnosing the presence of intravascular and extravascular hemolysis in malaria (Rogerson, 2006).

Bone marrow appearance

In malaria, the bone marrow varies in appearance depending on the duration of infection with malaria. In acute *falciparum* malaria, the bone marrow may be hypocellular, normocellular or hypercellular in non-immune adults and children with malaria, and may show evidence of dyserythropoiesis, iron sequestration and erythrophagocytosis. Maturation defects may be present in the bone marrow for 3 weeks after the clearance of parasitemia. Large, abnormal looking megakaryocytes have been found in the marrow and the circulating platelets may also be enlarged, suggesting dysthrombopoiesis. Meanwhile, in chronic malaria, the bone marrow is extremely hypercellular, with erythroid hyperplasia demonstrating dyserythropoiesis with functional ineffective erythropoiesis, as indicated by an inadequate reticulocyte response and perturbation of erythroblast cell kinetics (Wickramasinghe and Abdalla, 2000).

Management of malarial anaemia

Use of antimalarials

The treatment of malarial anaemia is fundamentally directed at use of anti-malarial agents to eliminate or control the underlying infection, taking into account the pattern of drug resistance in the given area where infection was contracted. Prompt treatment of malaria infections with effective, fast-acting anti-malarial drugs has been shown to rapidly reduce symptomatic high density parasitaemia and clear parasites from the blood, allowing erythrocyte numbers to be restored thus reducing the risk of anaemia (Crawley, 2004, Weatherall *et al.*, 2002, Carneiro *et al.*, 2006).

Transfusion

In the cases of severe malarial anaemia, urgent blood transfusion has proven to be life saving but an important drawback is the risk of contamination with HIV (Weatherall *et al.*, 2002). Exchange transfusion has been thought to benefit patients with high parasite counts (Njuguna and Newton, 2004). Exchange transfusion is believed to reduce the parasite load, remove toxic substances, reduce microcirculatory sludging, and increase the oxygen-carrying capacity of the blood (Riddle *et al.*, 2002).

Iron

Malaria and anaemia, especially that due to iron (Fe) deficiency, are two leading causes of morbidity worldwide (Menendez *et al.*, 1997), and iron deficiency is particularly found in areas where malaria is endemic. Malaria may contribute to iron deficiency and thus anaemia, by reducing iron absorption during acute episodes and through sequestration of iron in malaria pigment. The consequences of iron deficiency include depression of cell-mediated immune responses and impairment of normal motor and cognitive development (Menendez *et al.*, 1997). The role of iron in the prevention and treatment of anaemia in malaria-endemic regions is a highly contentious issue. There is controversy regarding the fact that iron deficiency may protect against malaria and that Fe repletion may lead to recrudescence of malaria. However, Fe supplementation was commonly seen to improve Hb levels in children with malarial and anaemia. In a study of Tanzanian children, iron supplementation was effective in preventing severe anaemia without increasing susceptibility to malaria (Menendez *et al.*, 1997).

Anthelmintics

Helminthes such as hookworm and whip worm are common in many malaria endemic areas and can lead to anaemia in their own right due to gastrointestinal blood loss. Hence treatment of malaria anemic patients with anti-helminthics is considered appropriate.

Micronutrient supplementation

Areas endemic for malaria often have a high prevalence of micronutrient malnutrition. Studies suggest that vitamin A can improve hematologic indicators and enhance the efficacy of iron supplementation (Fishman *et al.*, 2000). Vitamin A is thought to positively influence Hb levels by 1) stimulation of human erythroid precursors 2) enhancing iron availability to the bone marrow by mobilizing it from storage forms such as ferritin, 3) decreasing risk of infection and infection-related anaemia, and 4) enhancing absorption of iron from the gut (Tolentino and Friedman 2007). Hematologic improvement, including increased Hb and serum iron concentration, has been shown to occur with vitamin A supplementation among children (Fishman *et al.*, 2000). Vitamin A supplementation was observed to reduce episodes of clinical malaria in children in Papua New Guinea (Tolentino and Friedman 2007).

Vitamin B12 is necessary for the synthesis of red blood cells and deficiencies of this micro-nutrient are associated with megaloblastic anaemia (Tolentino and Friedman, 2007).

It is thought that recurrent malaria hemolysis stimulates the production of red blood cell precursors, increasing the demand for folate and this can lead to folate depletion. Normally, folic acid is necessary for cell growth and repair, and is also essential for the formation and maturation of red blood cells. Deficiency of folate leads to slowing of DNA synthesis and impaired cell proliferation. This, can in turn, lead to intramedullary death of many of these abnormal cells and a shortened lifespan of circulating red blood cells and hence anaemia (Menendez *et al.*, 2000, Tolentino and Friedman, 2007).

Prevention

Community-based malaria control interventions, including antimalarial chemoprophylaxis, use of insecticide-treated nets (ITNs), and indoor residual spraying, consistently improved anaemia-related outcomes in young children (Carneiro *et al.*, 2006, reviewed in Casals-Pascaul and Roberts 2006). Regarding chemoprophylaxis, it is argued it may delay the development of naturally acquired immunity in children, and there is also the risk of drug resistance (Menendez *et al.*, 1999). In addition, intermittent preventive treatment (IPT) is further recommended for children and pregnant women.

The rhoptry and rhoptry proteins

To date, several *P. falciparum* antigens belonging to the intraerythrocytic stages of the parasite have been identified and studied as vaccine candidates. Most of these antigens are mainly located on the merozoite surface or in the apical organelles, the rhoptries, micronemes and dense granules (Figure 3) (Mongui *et al.*, 2007). The rhoptries are a pair of organelles at the apical end of the parasite. Ultra structural and biochemical studies suggest that the rhoptries play an important role in the process of erythrocyte invasion (López *et al.*, 2004). They contain many lipids and proteins which are released during the merozoite invasion of erythrocytes. The rhoptries are connected to the surface of the apical end of the merozoite by a duct-like structure (López *et al.*, 2004) and they discharge their content onto the erythrocyte membrane at the point of initial contact between the merozoite and the erythrocyte. (Aikawa *et al.*, 1981).

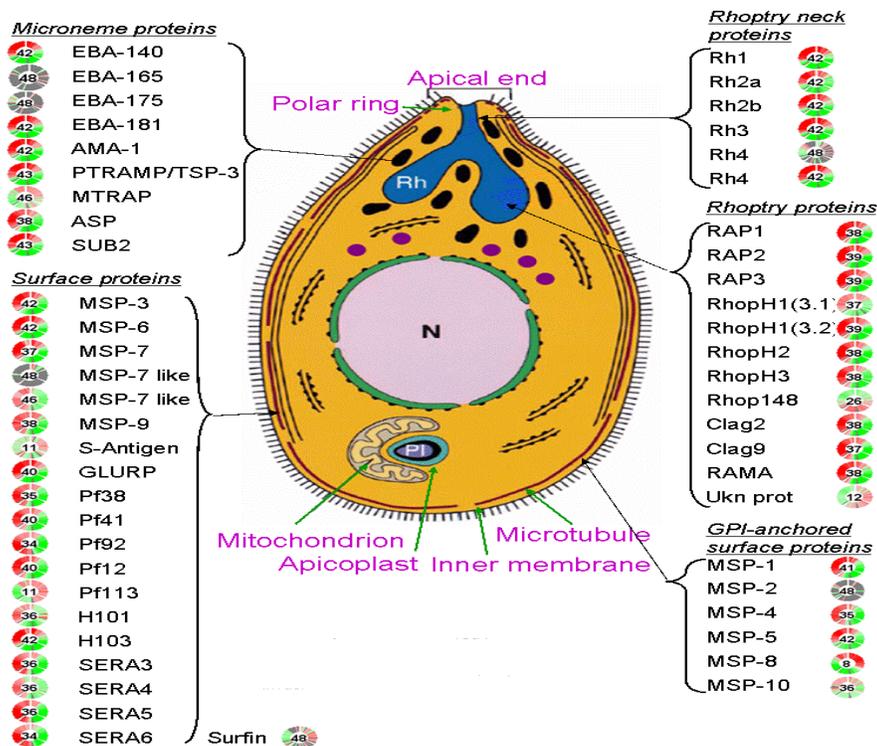


Figure 3. Sub-cellular localization of merozoite proteins. Adapted from ([www. Plasmodb.org](http://www.Plasmodb.org))

The described constituents of this organelle include the non-covalently associated low- molecular weight complex (LMW, designated Rhop-L), the high-molecular weight protein rhoptry protein

complex (HMW, Rhop-H), the apical membrane antigen-1 (AMA-1), MAEBL, a serine protease, and the recently identified homologues of the reticulocyte-binding proteins of *Plasmodium vivax*, PfNBP1 and -2 (Sam-Yellowe 1996, Yang *et al.*, 1996, reviewed by Blackman and Bannister 2001).

In *P. falciparum* at least 20 rhoptry proteins have been characterized and these proteins are involved both in binding to the exterior of RBCs and in the later stages of invasion in formation of parasitophorous vacuole (reviewed in Kats *et al.*, 2006). The rhoptry-resident proteins appear to be predominantly secreted with soluble proteins present in the rhoptry neck and lumen. However, a small number of proteins are membrane-associated either by glycosylphosphatidylinositol (GPI) anchors e.g. (RAMA) or integral membrane anchors-Rhop148 (Lobo *et al.*, 2003; Topolska *et al.*, 2004a).

Invasion is an important step in the parasites' life cycle during which time merozoites are briefly exposed to the host immune system between RBC egress and reinvasion. Thus proteins on the surface of merozoites or those that are secreted during invasion are thought to serve as attractive targets for neutralizing antibodies and as potential vaccine candidates (Proellocks *et al.*, 2007).

The low molecular weight rhoptry complex (Rhop-L)

The Rhop-L complex is formed by the rhoptry associated proteins (RAP)-1, -2 and -3. The precise role of the RAP proteins is not clear, but it has been speculated that the complex may be important in the erythrocyte invasion of merozoites (Baldi *et al.*, 2000, reviewed in Preiser *et al.*, 2000). RAP-1, -2 and -3 exist in complexes and these complexes are maintained even after erythrocyte invasion and early parasite maturation (Howard *et al.*, 1984, reviewed in Preiser *et al.*, 2000).

Rhoptry-associated protein- 1 (RAP- 1)

RAP-1 is a soluble protein with an apparent molecular weight of 82kDa. It is expressed during schizogony, and it is first detected at 38 hrs post infection (Jaikaria *et al.*, 1993). It exhibits minimal genetic polymorphism and is synthesized as an 86kD precursor, which subsequently is N-terminally cleaved to generate an 82kD molecule (p82) (Moreno *et al.*, 2001). In late schizogony; a fraction of p82 is further processed at amino acid residue 119 to yield a 67k-Da molecule (p67). As part of their maturation the processed RAP-1 products associate non-covalently with either p39 (RAP-2) or p37 (RAP-3) to form heterooligomeric complexes (Howard *et al.*, 1990, Jacobson *et al.*, 1998, Moreno *et al.*, 2001). Both p82 and p67 are in schizont/segmenter stages, and in free extracellular merozoites, the p82 protein is also detected in ring stage parasites (Moreno *et al.*, 2001). Merozoites containing a form of RAP-1 truncated from residue 345 are equally viable, display normal

erythrocyte invasion and grow at similar rates as the wild-type parasites. This truncated form still expresses the N-terminal region and is detected in rhoptries, suggesting that normal trafficking signals exist in this region (Baldi, 2000). However, it was not detected in newly invaded rings. Here, RAP-2 was detected in the endoplasmic reticulum rather than in the rhoptry organelles, suggesting that the C-terminal region is important for binding and trafficking of RAP-2 indicating that RAP-2 lacks its own rhoptry targeting signal and is probably not involved in the invasion process (Baldi, 2000). Antibodies to RAP-1 have been shown to block merozoite invasion *in vitro*. In addition, monkeys immunized with RAP-1 and -2 are partially protected against parasite challenge (Clark *et al.*, 1987, Ridley *et al.*, 1990).

Rhoptry-associated protein- 2 (RAP- 2)

RAP-2 is a soluble protein of 42 KDa (39 KDa has also been reported) (Ridley *et al.*, 1990, Baldi *et al.*, 2002). Its expression pattern coincides with that of RAP-1, and there is no evidence for proteolytic cleavage of the protein. Subsequent to erythrocyte invasion, RAP-2 can be detected in ring-stage parasites, along with RAP-1, but the rhoptry localization of RAP-2 is not essential for erythrocyte invasion (Baldi *et al.*, 2000). The ring surface protein (RSP)-2 has been shown to be identical to RAP-2 (Douki *et al.*, 2003). It is present on the surface of ring infected erythrocytes for 16-20 h post merozoite invasion. Between approximately 16-20 h it progressively becomes replaced by PfEMP-1 (Douki *et al.*, 2003). The protein has been identified as a target of ring stage-reactive monoclonal antibodies (Douki *et al.*, 2003). Monoclonal antibodies produced against RSP-2 have been shown to specifically bind to conformational epitopes located in the first 200 amino acids of the N-terminal region of the protein (Sterkers *et al.*, 2007). RSP-2 is discharged onto the membrane of ring infected erythrocytes (rIEs) as well as normal erythrocytes (nEs) (probably due to delayed or aborted invasion) at the point of contact with the merozoite and gradually spreads on the red cell surface (Layez *et al.*, 2005). The number of RSP-2-tagged rIEs depends on the parasite load and is always much lower than the number of RSP-2-tagged nEs. RSP-2 has been noted as a key molecule in the sequestration of young blood stage forms of the CSA phenotype and normal erythrocytes to endothelial cells, thus evading possible destruction in the spleen (Douki *et al.*, 2003). Previous and recent data suggest that the antibody response against RSP-2 can induce phagocytosis and complement binding to RSP2-tagged erythrocytes, leading to the destruction of uninfected erythrocytes, which could be partly responsible for the anaemia observed in *P. falciparum* infected individuals (Layez *et al.*, 2005, Awah *et al.*, unpublished data, Paper I).

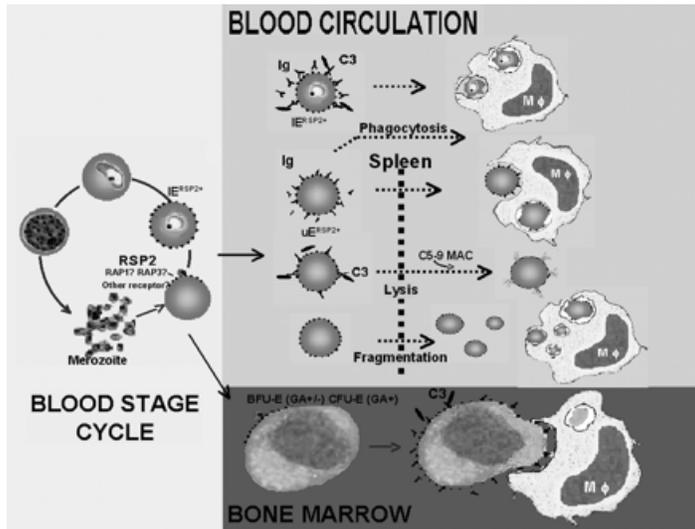


Figure . Schematic representation of the role of RSP-2 in the development of severe anaemia during *P. falciparum* infection. Adapted from Layez, *et al. Blood* 2005; 106:3632-3638.

Rhoptry-associated protein- 3 (RAP- 3)

RAP-3 has a molecular weight of 40-kDa (37 has also been reported) and is encoded by the RAP-3 gene (Baldi *et al.*, 2002) and forms a complex with RAP-1. RAP-3 is similar to RAP-2 (44% amino acid identity) and it has been observed to co-precipitate with RAP-1 and RAP-2. Its expression coincides with that of RAP-1 and RAP-2, which is during schizogony, and it can also be detected in ring-stage parasites after erythrocyte invasion. Targeted gene disruption experiments have demonstrated that RAP-3 is not essential for parasite survival. It was observed that, the loss of RAP-3 had no effect on parasite growth or on trafficking of RAP-1 and RAP-2 into rhoptry organelles, lending credence to the notion that the loss of RAP-3 can be complemented for by RAP-2. This is consistent with the fact that RAP-2 and -3 form separate heterodimers with RAP-1 rather than a trimeric complex (Baldi *et al.*, 2002).

The high molecular weight rhoptry complex (Rhop-H)

This complex is composed of three distinct high molecular weight proteins (140 k-Da Rhop-1, 130 k-Da Rhop-2 and 110 k-Da Rhop-3) encoded by unrelated genes. RhopH2 and RhopH3 are encoded by single-copy genes, whereas RhopH1 is encoded by the multi-copy cytoadherence-linked asexual gene (CLAG), constituting a family of genes (CLAG genes). At least three of the five (and probably all five) CLAG genes in *P. falciparum* are expressed, and each is capable of forming a complex with RhopH2 and RhopH3. Thus, it is suggested that there are potentially five variant RhopH complexes, which all appear to be expressed in the same merozoite at the same time.

The role of the HMW complex in invasion remains largely unknown. It binds to RBCs via a trypsin- and chymotrypsin-sensitive GPI-anchored receptor, and is subsequently transferred to the cytoplasmic face of the parasitophorous vacuole membrane. Given that there are variant forms of the HMW complex and that antibodies against the complex are partially protective, it is thought that it acts as an adhesin during invasion.

Rhoptry associated membrane antigen (RAMA)

RAMA is a GPI anchored protein synthesized as a 170-kDa protein in early trophozoites, between 15 and 20 h post-infection, several hours before rhoptry formation, and is transiently localized within the endoplasmic reticulum and Golgi within lipid-rich micro-domains. It is expressed in late rings, trophozoites and schizonts, and it has been shown to interact with the LMW and HMW complexes. RAMA is secreted during invasion and binds to the RBC surface. It is thought to have a possible role in rhoptry biogenesis as regions of the Golgi membrane containing RAMA bud to form vesicles that later mature into rhoptries, and in host cell invasion. A correlation between anti-RAMA antibodies with protective immune responses has been observed (Mongui *et al.*, 2007).

Rhop148

RHop148 is single pass transmembrane protein which is expressed in late rings, trophozoites and schizonts. It is suggested to have possible role in rhoptry biogenesis (Mongui *et al.*, 2007).

Immune responses to the LMW RAP proteins

The complex life cycle of *Plasmodium* provides quite a number of potential vaccine targets. Several proteins expressed by the parasite during the erythrocytic cycle are currently under investigation, including rhoptry-associated proteins, as possible vaccine candidates. Proteins of the Rhop-L complex are, to a varying degree, quite immunogenic as observed from several studies, and considerable amounts of data exists on the efficacy of *in vivo* human humoral/cellular immune responses to these antigens as well as from *in vitro* and experimental animal studies (reviewed by Preiser *et al.*, 2000). Hence antibodies to the RAP complex may play a role in immunity to *P. falciparum* infections.

Merozoite proteins are known to be exposed to the immune system both as surface proteins on merozoites and as exoantigens circulating in blood. Although the contents of the rhoptries are only transiently accessible to antibodies, seroepidemiological studies have demonstrated the development of antibody responses to RAP-1, RAP-2 and RAP-3 following infection in different

endemic regions (Alifrangis *et al.*, 1999, Fonjungo *et al.*, 1999, Johnson *et al.*, 2000, Howard *et al.* 1993, Jakobson *et al.*, 1993, Jakobsen *et al.*, 1997).

Monoclonal antibodies directed against epitopes of *P. falciparum* RAP-1 inhibited merozoite invasion of red cells *in vitro*, one of the epitopes detected being highly conserved, and the other more variable between *P. falciparum* isolates (Schofield *et al.*, 1986). *In vitro* growth inhibition assays have indicated that antibodies directed against RAP-1 and RAP-2 proteins have inhibitory effects on *P. falciparum* growth in *in vitro* cultures (Moreno *et al.*, 2001).

Experimental immunisation of *Saimiri* monkeys with a mixture of RAP-1 and -2 induced specific merozoite invasion inhibitory antibodies, which protected the animals against challenge with *P. falciparum* (Ridley *et al.*, 1990). Although denatured RAP-2 seemed to be relatively immunogenic in *Saimiri* monkeys, the protein was only weakly recognised by antibodies in human immune serum (Ridley *et al.*, 1990, Stowers *et al.*, 1997). This implies that either RAP-2 is not that immunogenic in man, or that the assays used to screen for reactivity were of low sensitivity. Mice immunised with a recombinant form of RAP-2 produced antisera which recognised the native protein by indirect immunofluorescence and immunoblotting (Stowers *et al.*, 1997). Since both RAP-1 and RAP-2 are relatively non-polymorphic antigens they are considered to be good vaccine candidates. Immune responses to RAP-3 following natural infections in humans and or experimental models to the best of our knowledge have so far not been fully characterised.

Phagocytosis in malaria

Phagocytosis has long been recognized to play a crucial role in the defense of the host during malaria infections. It is regarded as an important effector mechanism in malaria. Rapid phagocytosis of parasitized RBCs not only prevents merozoite invasion of RBCs but also reduces the toxic effects of malaria GPI (Kumaratilake and Ferrante 2000). However, increased erythrophagocytosis also contributes to the development of severe anaemia. Indirect signs of erythrophagocytosis are splenomegaly and the presence of malaria pigment in monocytes/macrophages resulting from the phagocytosis of parasitized erythrocytes and free hemozoin.

The complement system and malaria

Complement is part of the innate immune system and underlies one of the main effector mechanisms of antibody-mediated immunity. Its physiologic activities include: defending against bacterial infection, bridging innate and adaptive immunity and disposing of immune complexes and

the products of inflammatory injury (Walport, 2001, reviewed in Carroll, 2004). The complement system consists of the sequential activation of many pro-enzymes that, in turn, can catalyze the activation of other enzymes. There are three pathways of activation of the complement system: the classical, the mannose-binding lectin and the alternative pathway. All three pathways share the common step of activating the central component C3, but differ according to the nature of recognition (reviewed in Carroll, 2004). A key part of the complement cascade is the formation of C3b from C3 following cleavage by enzyme complexes called C3 convertases. C3b can then bind to cell surfaces (foreign and non-self antigens) tagging them for uptake by phagocytic cells (macrophages) (Walport 2001, reviewed in Carroll, 2004) or retention on follicular dendritic cells (FDCs) for recognition by cognate B lymphocytes (Carroll, 2004). It is noteworthy that the activation of the complement cascade is carefully regulated by several proteins in plasma and on cell surfaces including Factor I, CR1 (CD35), decay-accelerating factor (DAF, also known as CD55) and membrane inhibitor of reactive lysis (MIRL, also known as CD59). These three proteins are found on the erythrocyte surface and are responsible for its complement-regulatory properties. Thus, in addition to functioning as oxygen carriers; erythrocytes perform an important function as regulators of the complement cascade to prevent autologous complement attack (Stoute, 2005). CR1 on erythrocytes serves to remove immune complexes (ICs) from the circulation by binding to ICs containing C3b. In the liver and spleen, these ICs are removed by macrophages and in this process, CR1 molecules are continuously lost but the erythrocytes are recycled. In addition, CR1 accelerates the degradation of C3 convertases and promotes the inactivation of C3b. CD55 functions mainly to accelerate the decay of C3 convertases. CD59 prevents the assembly of the membrane attack complex (MAC) that forms a pore in the cell membrane, leading to lysis by binding to the C5b-8 complex and preventing the polymerization of C9 (Walport, 2001, Stoute, 2005).

Studies in human malaria suggest that the complement system, in particular the classical pathway, may play a role in the host defense against malaria, and may also be associated with disease pathology (reviewed by Chang and Stevenson, 2004). Components of the classical pathway were demonstrated to be depressed, whereas components of the alternative pathway remained unaffected. Complement activation was found to occur during or soon after schizont rupture, if the parasite density was sufficiently high, and if complement fixing antibodies were present. Furthermore, the degree of hypocomplementemia was found to correlate with various complications of malaria, such as disseminated intravascular coagulation jaundice, anaemia and cerebral malaria (Wenisch *et al.*, 1997). During childhood malarial anaemia, the RBCs remaining in the circulation show reduced levels of CR1 and CD55 expression and increased C3b deposition, which may result in increased destruction of RBCs and ensuing anaemia (Waitumbi *et al.*, 2000, Owuor *et al.*, 2008, Odhiambo *et al.*, 2008). In addition, the deficiency in CR1 and CD55 expression has been shown to vary with the age of the host, but irrespective of endemicity; being (high in neonates, decreasing after 6 months (and increasing sometime after into adulthood) low in young children and increasing with age in

both endemic and non-endemic countries (Waitumbi *et al.*, 2004, Stoute 2005).

Apoptosis

Cell death may be described either as apoptosis or non-apoptotic cell death, the latter traditionally called 'necrosis'. Apoptosis, is a form of programmed cell death, and is involved in development, elimination of damaged cells, and maintenance of cell homeostasis. Deregulation of apoptosis may cause diseases, such as cancers, immune diseases, and neurodegenerative disorders. Apoptosis is executed by a subfamily of cysteine proteases known as caspases. Caspases are the central components of the apoptotic response. Caspases belong to a conserved family of enzymes that irreversibly commit a cell to die. Once an initiator caspase is activated, it can trigger a cascade to activate downstream executioner caspases. Subsequently, the activated executioner caspases cleave numerous cellular targets to destroy normal cellular functions, activate other apoptotic factors, inactivate anti-apoptotic proteins, eventually leading to apoptotic cell death (reviewed by Jiang & Wang 2004). Morphological features of apoptosis include, extensive plasma membrane blebbing followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called "budding." Apoptotic bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes thus preventing any inflammatory reactions (reviewed by Jiang & Wang 2004, and Elmore 2007). In mammalian cells, the apoptotic response is mediated through either the intrinsic pathway (mitochondrial) or the extrinsic pathway (death receptor pathway), depending on the origin of the death stimuli. The intrinsic pathway is triggered in response to a wide range of death stimuli that are generated from within the cell, such as oncogene activation and DNA damage. The extrinsic pathway is initiated by the binding of an extracellular death ligand, such as FasL, to its cell-surface death receptor, such as Fas. An additional pathway is the perforin/granzyme pathway (reviewed by Riedl & Shi, 2004, reviewed by Elmore, 2007). Ferrokinetic studies have shown that as many as 50% of the erythroid precursors die in the marrow. In malaria, the bone marrow response is suppressed in anaemic individuals who present with a deficit in erythroid cells numbers thought to be due to the death of erythroid cells in the erythroblast compartment. The mechanism of cell death in this context is however not clear. Based on morphological abnormalities in the bone marrow of patients, apoptosis had been suggested to be responsible for the observed dyserythropoiesis in patients with anaemia (see previous section on Mechanisms of anaemia).

THE PRESENT STUDY

The blood stages of *P. falciparum* inevitably cause anemia, which continues to be a major threat to life in the developing world (Miller *et al.*, 2002). The pathogenesis of malarial anaemia is multifactorial and incompletely understood, but is thought to arise from both decreased red blood cell production and increased red blood cell destruction with the destruction of normal RBCs being paramount (Jakeman *et al.*, 1999). Normal RBCs destruction may occur by immunological mechanisms, involving antibodies and complement binding to the RBC surface, causing complement mediated lysis and phagocytosis (Looareesuwan *et al.*, 1987), The target antigens in this context may be parasite derived antigens non-specifically adsorbed to the RBC surface (Layez *et al.*, 2005, Evans *et al.*, 2006, reviewed by Casals-Pascaul and Roberts 2006) or RBC antigens binding auto-antibodies (Facer, 1980). Earlier studies by Layez *et al.* (2005) showed that the tagging of normal RBCs with the parasite antigen RSP-2/RAP-2 resulted in the destruction of these cells following opsonisation with RSP-2/RAP-2 reactive antibodies.

The present study was therefore undertaken to:-

- ❖ To determine whether RSP2/RAP2 and other members of the low molecular weight rhoptry associated protein complex (RAP-1 & -3) maybe associated with erythrocyte destruction and bone marrow suppression associated with malarial anaemia in *falciparum* infections.
- ❖ To investigate the frequency and functional capacity of naturally acquired anti-RSP2/RAP2 antibodies in the sera of anaemic and non-anaemic malaria infected children residing in a malaria endemic region.

Materials and methods

The methods for the included investigations are described in the separate manuscripts

Results and discussion

Study I

Severe malaria is an inevitable consequence of *falciparum* infection and anaemia is a constant feature of the disease. The causes of anaemia are complex and multifactorial. Summarily, its pathogenesis is thought to be a result of increased red cell destruction as well as decreased red cell production (probably due to bone marrow suppression) of which the destruction of normal erythrocytes is considered paramount (Jakeman *et al.*, 1997). However, the mechanisms and molecular components involved in RBC destruction remain elusive. Recently, the ring surface protein-2 (previously described as RAP-2 and now referred to as RSP-2/RAP-2) has been implicated in the destruction of erythroid cells during anaemia (Layez *et al.*, 2005). In this study, we have confirmed and extended these findings. We have shown that antibodies to RSP-2/RAP-2 and other low molecular weight rhoptry proteins recognize the surface of RSP-2/RAP-2-tagged erythroid cells in a parasitaemia-dependent manner, and, furthermore, can induce the destruction of these cells either by opsonophagocytosis or complement activation. This was also true for other proteins of the low molecular weight complex. We further showed that antibodies to RSP-2/RAP-2 and RAP-1 mediated the death of erythroblasts in the presence of monocytes/macrophages *in vitro*. Previous studies had shown that in response to acute malaria, there is proliferation and hyperactivity of the reticulo-endothelial system, resulting in the phagocytosis of both parasitized and unparasitized RBCs, which have abnormally rigid membranes (reviewed by Menendez *et al.*, 2000). It had been suggested that the presence of RSP-2 on the surface of normal and infected RBCs might lead to the rigidification of these cells and hence an enhanced clearance from the circulation (Layez *et al.* 2005). The deformed membrane may also encourage the deposition of complement components thus targeting them for clearance by the reticuloendothelial system or lysis (Waitumbi *et al.*, 2000, 2004).

Bone marrow suppression is generally present in all malaria patients (Kurtzhals *et al.* 1997), and is characterised by ineffective erythropoiesis and dyserythropoiesis (morphological abnormalities of erythropoiesis) and is considered an important aggravating factor in the pathogenesis of malarial anaemia (Abdalla *et al.* 1980, Philips and Pasvol 1992, Wickramasinghe and Abdalla 2000). This is thought to be as a result of cell death in the erythroblast cell compartment (Dörmer *et al.* 1983, Wickramasinghe and Abdalla, 2000)

We showed that antibodies to RSP-2/RAP-2 and RAP-1 may mediate the death of erythroblasts in the presence of monocytes/macrophages *in vitro*. The mechanism of cell death is not yet known. However preliminary data indicates that, the cells are to some extent dying by apoptosis. In support of our results, Anantrakulsil *et al.* (2005) detected some apoptosis in bone marrow aspirates from Thai patients. However, the data from each subject could not be interpreted in the same way, as the

percentages of apoptotic erythroid cells in bone marrow from each patient and controls varied from low to high, and there was no association with parasitaemia.

Study II

In order to complement our *in vitro* findings, we sought to determine the frequency, specificity and functional capacity of naturally acquired anti-RSP-2/RAP-2 antibodies in the sera of infected children. All sera investigated recognised RSP-2/RAP-2 irrespective of the clinical status of the subjects. The anaemic group of children had higher levels of IgG than the non-anaemic controls; the meanwhile levels of IgM were similar in both groups. With regard to IgG subclasses, there were generally very low levels detected of IgG1 and IgG3 antibodies, while the levels of IgG2 and -4 antibodies were below the detection limit in all individuals. A possible reason for the absence of anti-RSP-2/RAP-2- specific IgG subclasses in our study is that the assay for antibody detection was not sensitive enough. To the best of our knowledge, no study has looked at the subclass distribution of anti-RSP2/RAP2 antibodies in the sera of patients. However, previous studies with RAP-1 peptides found that individuals had predominantly IgG1 and only few sera contained traces of IgG2, 3, or 4; only 2 individuals had IgG3 reactive to one of the antigens used in the study (Fonjungo *et al.*, 1998).

RAP-1 is known to form hetero-oligomeric complexes with RAP-2 and related RAP-3 (Baldi *et al.*, 2000, Baldi *et al.* 2002. Experimental immunisation of *Saimiri* monkeys with purified RAP-1 and RAP-2 conferred partial protection against *P. falciparum* infection (Ridley *et al.*, 1990). In addition, we do not know much about the dynamics of anti-RSP2 responses in natural infections. At the moment, there is paucity of data regarding the dynamics of antibody responses to RSP-2/RAP-2. In endemic areas, acquired immunity to malaria builds up with age following several repeated attacks and is not sterile. Previous studies by Johnson *et al.* (2000) indicated that levels of antibodies to RAP-2 do not reach a maximum until the age of 30 years, while levels of antibodies to RAP-1 increased significantly above the age of 15 years. In another study, of Papua New Guinean natives, maximum levels of antibodies to both RAP-1 and RAP-2 were not reached until after the age of 30 years (Stowers *et al.* 1997), implying that, there might be a slow acquisition or build up of antibodies to RSP-2/RAP-2 during natural infections. This could explain the low levels or absence of antibodies of the different IgG subclasses in our study population of four and five year olds. Another plausible explanation could be that, like for RAP-1, antibodies to RSP-2/RAP-2 are short-lived (Fonjungo *et al.*, 1999), or might be a reflection of exposure, as suggested by several authors with regard to other antigens as well (Fonjungo *et al.*, 1999, Perlmann *et al.*, 2000, reviewed by Preiser *et al.*, 2002).

In addition, sera from the non-anaemic group with lower levels of IgG activated complement more in terms of the proportion of cells binding C3b. This implies that, the effector functions of these antibodies are more important than their amounts. Indeed, the non-anaemic individuals showed higher levels of IgG3 antibodies than the anaemic ones, and this IgG subclass is the most efficient complement activating IgG. Studies in human malaria suggest that the complement system, particularly the classical pathway, may play a role in host defense against malaria, even though it may also be associated with disease pathology (reviewed by Chang and Stevenson, 2004). *P. falciparum* reactive antibodies have previously been shown to play a critical role in immune protection against the asexual blood stages of the parasite (Cohen *et al.*, 1961, McGregor *et al.*, 1963, Sabchareon *et al.*, 1991), and the antibody isotype and subclass appears to more critical in this context (Warmerdam *et al.*, 1991, Bouharoun-Tayoun and Druilhe 1995, reviewed by Wipasa *et al.*, 2002, Garraud *et al.*, 2003, Tangteerawatana *et al.*, 2007).

Concluding remarks and future perspectives

Taken together, we might surmise that during SMA, an inadequate bone marrow response due to erythroblast cell death may overlap with the accelerated destruction of normal erythrocytes, either by opsonisation or complement activation, further aggravating the anaemia which may become fatal. In addition, high levels of anti-RSP-2/RAP-2 antibodies may be detrimental to the host. This could therefore have implications in the design, development and deployment of further interventions against malaria.

We observed the death of RSP-2/RAP-2-tagged erythroblasts in the presence of anti-rhoptry antibodies, but the mechanism of cell death is still unknown. Previous studies had suggested apoptosis to be responsible for the death of bone marrow erythroid cells (Abdallah and Wickramasinghe, 1988). Our data indicates that cells might be dying by apoptosis and necrosis.

SMA has been shown to have an inflammatory basis and the cytokines TNF- α , IFN- γ and MIF have been implicated in the bone marrow dysfunction of dyserythropoiesis. To elucidate further the mechanism of cell death, trans-well experiments will be performed to study the effect of monocyte contact on the erythroblasts as well as the effect of any soluble mediators/cytokines.

Severe malaria anaemia is also a major cause of morbidity and mortality in pregnant women; with severe consequences on their unborn fetuses. Using maternal sera and sera from cord blood, we will proceed by investigating the frequency of anti-RSP-2 antibodies in these individuals and their role in the development of anaemia during malaria.

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