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Antibody responses in *Plasmodium falciparum* malaria and their relation to protection against the disease

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SUMMARY

Protective immunity against *Plasmodium falciparum* may be obtained after repeated exposure to infection. Several studies indicate that immunity against the blood stages of the *P. falciparum* infection is mainly antibody mediated. Protective antibodies may act either on their own, mediate antibody-dependent phagocytosis and/or cell-mediated neutralization of parasites. This thesis describes several aspects of humoral immune responses to *P. falciparum* infection in individuals of different age groups, different genetic background and with different degrees of malaria exposure.

Several target antigens for antibody-mediated inhibition of parasite growth or invasion have been identified. One such antigen is Pf332, which appears on the surface of parasitized erythrocytes at late trophozoite and schizont stage. This surface exposure makes the antigen a possible target for opsonizing antibodies. We optimized an *in vitro* assay for studying cell- mediated parasite neutralization in the presence of Pf332-reactive antibodies. Our data demonstrate that, Pf332 specific antibodies are able to inhibit parasite growth on their own and in cooperation with human monocytes.

The *P. falciparum* parasites have evolved several mechanisms to evade the host neutralizing immune responses. In this thesis, we show that freshly isolated *P. falciparum* parasites from children living in a malaria endemic area of Burkina Faso were less sensitive for growth inhibition *in vitro* by autologous immunoglobulins (Ig) compared with heterologous ones. Analyses of two consecutive isolates taken 14 days apart, with regard to genotypes and sensitivity to growth inhibition *in vitro*, did not give any clear-cut indications on possible mechanisms leading to a reduced inhibitory activity in autologous parasite/antibody combinations. The frequent presence of persisting parasite clones in asymptomatic children indicates that the parasite possesses as yet undefined mechanisms to evade neutralizing immune responses.

Transmission reducing measures such insecticide treated nets (ITNs) have been shown to be effective in reducing morbidity and mortality from malaria. However, concerns have been raised that ITNs usage could affect the acquisition of malaria immunity. We studied the effect of the use of insecticide treated curtains (ITC) on anti-malarial immune responses of children living in villages with ITC since birth. The use of ITC did neither affect the levels of parasite neutralizing immune responses nor the multiplicity of infection. These results indicate that the use of ITC does not interfere

with the acquisition of anti-malarial immunity in children living in a malaria hyperendemic area.

There is substantial evidence that the African Fulani tribe is markedly less susceptible to malaria infection compared to other sympatrically living ethnic tribes. We investigated the isotypic humoral responses against *P. falciparum* asexual blood stages in different ethnic groups living in sympatry in two countries exhibiting different malaria transmission intensities, Burkina Faso and Mali. We observed higher levels of the total malaria-specific-IgG and its cytophilic subclasses in individuals of the Fulani tribe as compared to non-Fulani individuals. Fulani individuals also showed higher levels of antibodies to measles antigen, indicating that the intertribal differences are not specific for malaria and might reflect a generally activated immune system in the Fulani.

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III. Bolad, A., Nebie, I.,Esposito F. and Berzins k. The Use of Impregnated Curtains Does not Affect Antibody Responses against *Plasmodium falciparum* and Complexity of Infecting Parasite Populations. (Acta Trop., 2004).

IV. Bolad, A., Farouk, S., Dolo, A., Ogobara D., Nebie, I., Luoni, G., Sirima, BS., Modiano, D., Berzins, K. and Troye-Blomberg, M. Analyses of antibody responses to asexual blood stages of *Plasmodium falciparum* in ethnic tribes living in sympatry (Manuscript)

ABBREVIATIONS

ADCI**:** antibody-dependent cell-mediated inhibition

EIR**:** the product of the mosquito human biting rate times the proportion of sporozoite-infected mosquitoes.

Ig**:** immunoglobulin

IRBC**:** *Plasmodium falciparum* infected red blood cell

IFN: Interferon

ITC**:** Insecticide treated curtains.

ITNs**:** Insecticide treated nets, including bed nets and curtains.

IL: Interleukin

MHC: Major histocombatibility

MoAb**:** monoclonal antibody

MSP-1-2**:** merozoite surface proteins

NO**:** nitric oxide

PfEMP-1**:** the *P. falciparum* erythrocyte membrane protein-1

RBC**:** Red blood cell

VSA**:** variant surface antigens

Some definitions of significance for this study

Allele: Genes can exist in more than one form. Each different form of the same gene is called an allele.

Allelic family: alleles of a gene are grouped with regard to similar characteristics, e.g. the allelic families of *msp2*, Fc27- and IC1/3D7-allelic families, the allelic families of *msp1* gene, K1, MAD20 and RO33.

Genotype: a genetic characteristic of a parasite, the type of allele found at a polymorphic locus in an individual.

Isolate: freshly isolated parasites in primary culture (not cultured).

Laboratory Strain: If parasites have been cultured in the laboratory for extended time, they are referred to as strains.

Multiplicity of infection (MOI): number of infecting genotypes in an isolate.

1. INTRODUCTION

The parasite and the disease

Malaria is a life threatening parasitic disease annually causing 300–500 million clinical cases. The estimated morbidity due to malaria represents 2.3% of the overall global disease burden and 9% of that in Africa (WHO, 1996), ranking third among major infectious disease threats, after pneumococcal acute respiratory infections (3.5%) and tuberculosis (2.8%). According to recent data, there are 1.5 – 2.7 million deaths due to malaria each year, the bulk of which occurs in sub-Saharan Africa (Snow *et al*., 1999a).

Malaria is caused by the protozoan *Plasmodium,* transmitted to vertebrates by female *Anopheles* mosquitoes. In the vertebrate host, the asexual blood forms of the parasite are the life cycle stages that are exclusively responsible for morbidity and mortality of plasmodial infections. Four species of malaria parasites cause disease in humans, *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Whereas three species give rise to considerable malaria morbidity, only *P. falciparum* results in high mortality (WHO, 1997) as a result of its prevalence, virulence and drug resistance.

The disease is characterized by fever associated with symptoms, including headaches, chills, myalgia, malaise and joint pain that can be resolved into mild attack and run an uncomplicated course. In some cases, however, the disease can be resolved into life threatening complications, such as cerebral malaria or severe malaria anaemia.

Cerebral malaria is associated with the production of excessive levels of TNF-α (Grau *et al*., 1989; Kwiatkowski *et al*., 1990) and is thought to be, in part, due to mechanical obstruction of the cerebral microvasculature caused by a number of factors including the sequestration of parasitized erythrocytes to vascular endothelium (MacPherson *et al*., 1985; Pongponratn *et al*., 1991) and rosette formation (Carlson *et al*., 1990; Treutiger *et al*., 1992). Patients with cerebral malaria were shown to be more often febrile and presented earlier in the malaria season (Biemba *et al*., 2000).

Severe anaemia is another complication of *P. falciparum* malaria. Recently, Kurtzhals *et al.* (1997) showed that *P. falciparum* infection causes a rapidly reversible suppression of the bone marrow response to erythropoietin, while the severity of anaemia depends on the peripheral destruction of parasitized erythrocytes. The secondary hypersplenism may also contribute to increased red blood cells (RBCs) destruction (Sen *et al*., 1994). Cytokine levels, especially low plasma levels of the anti-inflammatory cytokine IL-10, were also shown to be associated with severe anaemia (Kurtzhals *et al*., 1998). Patients with severe anaemia are usually afebrile, with increased multiplicity of infection and late presentation of the disease in the malaria season (Mockenhaupt *et al.,* 2003).

The level of exposure to *P. falciparum* parasites has been shown to shape the pattern of host morbidity. This pattern can be used to describe a defined area as holoendemic or hypoendemic (see: Table 1, adapted from Molineaux (Molineaux, 1988).

Table (1): Endemicity levels classified by parasite prevalence*

Level	Prevalence
Holoendemic	Area with perineal high degree transmission Parasite rate in the one-year age group constantly over 75%, spleen rate in adults high or low, parasite density declining rapidly between 2-5 years of age and then slowly.
Hyperendemic	Area with intense but seasonal transmission Parasite rate in children of 2-9 years constantly over 50%.
Mesoendemic	Area with some transmission Parasite rate in children of 2-9 years as a rule 11-50% (may be higher During certain season of the year).
Hypoendemic	Area with little transmission Parasite rate in children of 2-9 years as a rule less than 10% (may be higher during certain season of the year).

*adapted from (Molineaux, 1988)

A high entomological inoculation rate (EIR) is often associated with increase in incidence of fever plus parasitaemia (Smith *et al*., 1998). In areas of high endemicity, the most frequent manifestation of severe malaria is severe anaemia, characteristically occurring in the first year of life (Snow *et al*., 1994; Kitua *et al*., 1996). Disease susceptibility rapidly declines after the first year of life, as anti-malarial semi-immunity is acquired.

In areas of lower endemicity and seasonal transmission (EIR 10-20), all age groups are susceptible to severe disease. Complications are most prominent in children below 1 to 4 years of age, with severe anaemia being a problem in children less than 1 year, while cerebral malaria is typically seen in older children (Snow *et al*., 1994; Greenwood *et al*., 1991).

The clinical disease is proposed to be mainly due to parasites expressing variant surface antigens (VSA) not recognized by pre-existing VSA-specific antibodies in that child (Ofori *et al*., 2002), and the disease episode results in an increase in levels of antibodies to VSA expressed by the infecting isolate (Bull *et al*., 2002; Dodoo *et al*., 2001; Marsh and Howard, 1986).

The distinction between infection and disease is particularly important in malaria, since infection with the parasite does not necessarily result in disease. In areas where malaria transmission from mosquitoes to human is intense, such as in many parts of sub-Saharan Africa, almost all of the children will have parasites in their blood constantly, without appreciable disease effects (Smith *et al*., 1993). These children have developed an anti-disease immunity (Playfair *et al*., 1991), while their anti-parasite immunity has not reached levels high enough to clear the infection (Greenwood *et al*., 1987). This suggests that asymptomatic, especially multiclonal, *P. falciparum* infections protect against clinical disease and provide with a status of preimunition (al-Yaman *et al*., 1997; Smith *et al*., 1999; Perignon and Druilhe, 1994; Färnert *et al*., 1999). The premunition is characterized by a decrease in the frequency and severity of disease episodes over several years, despite almost continuous infection, suggesting that immunity may develop through the acquisition of a repertoire of specific, protective antibodies directed against polymorphic target antigens. In such infections the phenotype of the parasites sometimes may remain stable over extended periods of time (Contamin *et al*., 1996).

Plasmodium *life cycle*

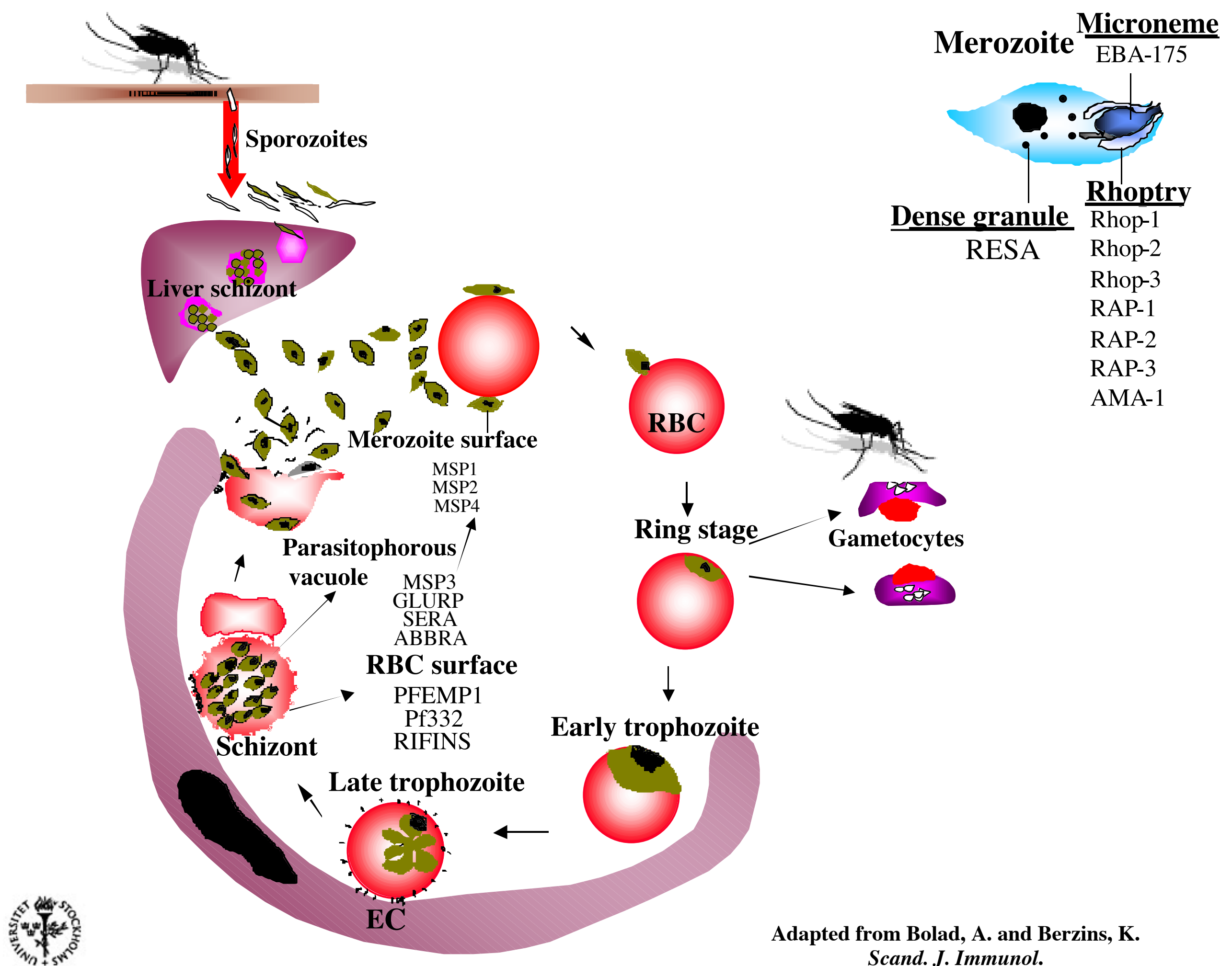
P. falciparum infection in human begins when an infected female *Anopheles* mosquito injects sporozoites during a blood meal. Within 30 minutes, sporozoites leave the circulation for the liver to initiate the infection. The co-receptor on sporozoites for

invasion involves, in part, the thrombospondin domains on the circumsporozoite protein (CSP) and on thrombospondin-related adhesive protein (TRAP). These domains bind specifically to heparan sulfate proteoglycans on hepatocytes in the region in apposition to the sinusoidal endothelium and Kuppfer cells (Frevert *et al*., 1993). After approximately 1–2 weeks, the infected liver cells (hepatocytes) burst, releasing thousands of merozoite-stage parasites, each of which is capable of invading RBCs.

The parasite has now started its asexual cycle, where it undergoes multiple rounds of invasion and replication inside the host erythrocytes. The invasion by the merozoite is complicated and is only partially understood. Four distinct steps in the invasion process take place: 1) initial merozoite binding to the surface of the RBC, 2) reorientation to allow the apical end to interact with the membrane of the host cells, 3) junction formation between the merozoite surface and the RBC membrane and 4) parasite entry. These processes involve specific interactions between parasite ligands on the merozoite surface and in the apical organelles, and receptors on the erythrocyte surface. High affinity ligands, such as erythrocyte binding antigen 175 (EBA-175) (Sim *et al*., 1994), which bind to sialic acid residues of glycophorin A on the surface of erythrocytes (Friedman *et al*., 1984), are responsible for tight junction formation. The interaction between these ligands and their receptors defines the major invasion pathway, which is dependent on sialic acid residues (Camus and Hadley, 1985). *P. falciparum* parasites may also use an alternative, sialic acid-independent pathway for invasion (Miller *et al*., 1977; Okoyeh *et al*., 1999).

Invasion is a remarkably rapid event, accomplished within 30 seconds of initial interaction with the erythrocyte (Dvorak *et al*., 1975). A membrane tight junction and an invagination are formed, and the junction moves along the surface of the merozoite until the membrane fuses at the posterior end of the parasite. This results in the formation of a parasitophorous vacuole containing the newly invaded merozoite with delicate cytoplasm and one or two chromatin dots (ring stage). The outer coat of the

merozoite is shed during invasion and it appears to accumulate posterior to the moving junction, and is eventually released into the extracellular surroundings. The ring stage grows and develops into a trophozoite, which undergoes an asexual division, erythrocytic schizogony. When the mature trophozoite starts to divide, separate merozoites are formed resulting in a schizont. Eventually the schizont bursts, releasing merozoites that can enter other erythrocytes and repeat the cycle. This cycle results in increasing numbers of RBCs being infected by the parasite (the asexual replication cycle). (Fig. 1, adapted from Bolad and Berzins (2000)).



Once inside of the erythrocytes, the parasite begins to modify both the internal and external structure of its host cell, in the process digesting haemoglobin, constituting the abundant source of amino acids required for parasite proteins synthesis. Digestion of hemoglobin releases toxic heme, which the parasite then detoxifies into a non-toxic crystalline form known as hemozoin (malaria pigment) (Goldberg, 1993). Morphological changes on the surface of the infected-RBC also include appearance of protrusions at the outer surface terms knobs (Kilejian, 1979). These knobs serve as a focal points for cytoadherence of late stage infected-RBC to endothelial cells. Proteins synthesized by the parasites and transported to the surface of eythrocytes are responsible for knob formation, in particular, histidine rich protein 1 (HRP-1), known also as knob associated histidine rich protein (KAHRP) (Taylor *et al*, 1987), malaria stage erythrocyte surface antigen (MESA), *P. falciparum* erythrocyte membrane protein-3 (PfEMP3) (Leech *et al*., 1984; Baruch *et al*., 1996; 1997). The adhesive changes in the infected-RBC are due to expression of PfEMP-1, which clusters on the external surface of knobs (Baruch *et al*., 1995). PfEMP-1 enables the infected-RBC to cytoadhere to various host cell receptors, including the scavenger receptor CD36, intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), thrombospondin (TSP) (reviewed by Sherman *et al*., 2003) and chondroitin sulfate A (CSA) (Fried and Duffy, 1996). These adhesions assist the parasite in escaping splenic clearance and promote sequestration of parasites in vital organs such as brain and placenta (Galbraith *et al*., 1980; Yamada *et al*., 1989; Aikawa *et al*., 1990).

To complete the cycle, some of the merozoites entering RBCs develop into gametocytes, the sexual stages, which are essential for transmitting the infection to others through female anopheline mosquitoes. The male and female gametocytes form a zygote in the insect's midgut. The zygotes develop into motile sporozoites through asexual

division in an oocyst attached to the intestinal wall of the mosquito. These sporozoites migrate to the salivary gland to continue the *Plasmodium* life cycle by infecting the next host during the next mosquito feeding.

Immune responses in malaria

In malaria endemic areas the inhabitants are usually infected repeatedly with malaria parasites and acquire immunity gradually. Such immunity includes a large variety of mechanisms that can neutralize the parasite. Liver schizonts express stage specific antigens, which are recognized by cytotoxic T lymphocytes (CD8+) as demonstrated both in animal model and humans exposed to malaria (Aidoo *et al*., 2000; Doolan and Hoffman, 1999; Nardin and Nussenzweig, 1993). The CD8+ T cells mediate the protection by secreting IFN-γ, which in turn induces nitric oxide dependent killing of parasites within the hepatocyte (reviewed by Nardin and Nussenzweig, 1993).

The merozoites that survive the pre-erythrocytic stage are released into the circulation where they become possible targets for antibodies. Merozoites invade erythrocytes and are then transformed into trophozoites and schizonts. The intraerythrocytic parasites are also targets for antibodies as some parasite antigens are expressed on the surface of infected-RBC. The antibodies can inhibit the development of intraerythrocytic as well as can mediate opsonization and phagocytosis of infected-RBC by blood monocytes.

The involvement of both B and T cells in immunity to malaria parasites has been indicated from studies in animal models of malaria (Meding and Langhorne, 1991). Experiments performed in B cells deficient mice have demonstrated that, infection with *P. yoelii* parasites was lethal, while it was nonlethal in normal mice (Cavacini *et al*., 1990). This indicates that the humoral immune system appears to play a major

role in protecting mice against this parasite. However, mice depleted of B cells were able to control their infections with *P. chabaudi* or *P. vinckei*, although they could not completely clear parasitemia. The involvement of T cells was demonstrated in mice depleted of both T and B cells, where transfer of normal or immune T cells protected mice from the lethal effect of *P. chabaudi*, whereas transfer of immune B cells led to complete clearance of parasitemia.

In humans, the existence of functionally distinct subsets of CD4+ T cell has been demonstrated by exposure *in vitro* of T-cells from malaria exposed individuals to crude or defined antigens, resulting in different responses (Troye-Blomberg *et al.*, 1990). These results suggest the occurrence of distinct immune responses that correspond to the Th1 and Th2 immune responses in mice to *P. chabaudi* (Langhorne *et al.*, 1989). In the *P. chabaudi* model of murine malaria, a Th1 type of response which is associated with IFN-γ/NO production switches to Th2 like response in the later phase of infection (Taylor-Robinson *et al.*, 1993).

Innate immunity and defense against malaria

The innate immune system is the evolutionarily older system, found in essentially all vertebrates. It provides with first line of defense and functions through immediate responses that use preexisting cells. Vertebrates also developed an adaptive immune system, however, the innate immune system is essential for instructing the cells of the adaptive system (T and B cells) by presenting antigen in the context of an appropriate co-stimulatory molecule. Cells of the innate immune system sense infection with a variety of pattern recognition receptors (PRRs). The best-known examples are, the

monocyte/macrophages mannose receptor and scavenger receptor. Another important functional class of PRRs is the Toll like receptors, initially described in *Drosophila*.

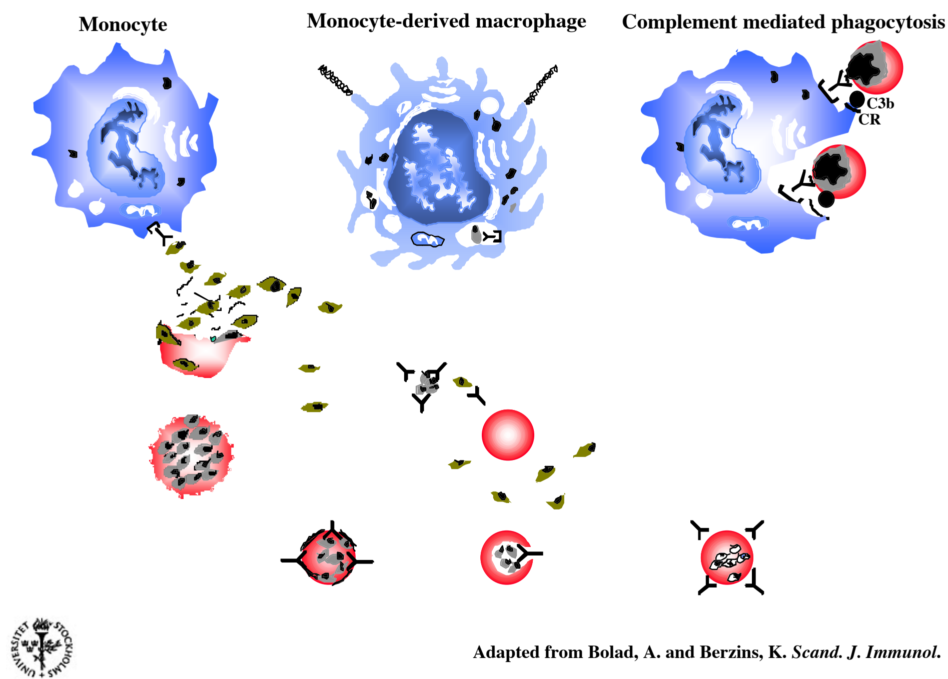
Monocytes (McGilvray *et al*., 2000), monocyte-derived macrophages (Jones *et al*., 1989), polymorphonuclear leukocytes (Kharazmi and Jepsen, 1984), NK-cells (Orago and Facer, 1991) are able to kill late stages of the intraerythrocytic parasite in the absence of antibodies. This killing may to some extent be attributed to the expression of PfEMP-1 on the surface of infected erythrocytes, containing binding sites for CD36 and/or ICAM-1, which may promote binding to leukocytes and enhance phagocytosis (Ruangjirachuporn *et al*., 1992; Serghides and Kain, 2001).

The innate immune system uses a series of PPR to detect the presence of pathogens, thus allowing for rapid host defense responses to invading microbes. Members of such receptors are the toll-like receptors (TLRs) (Gewirtz, 2003). Protozoan glycosylphosphatidylinositol (GPI)-anchor has the capacity to activate TLRs-mediated signalling (Campos *et al*., 2001). The GPI anchors in *P. falciparum* activate host innate immune responses and stimulate production of high levels of TNF-alpha by macrophages (Schofield *et al*., 1993). TLR-MyD88-mediated IL-12 production was shown to be associated with perforin-dependent liver injury induced by *P. berghei* infection (Adachi *et al*., 2001). However, production of IL-12 production by NK cells in appropriate dose has been shown to be useful in induction of protective immunity to *P. chabaudi* malaria infection (Stevenson *et al*., 1995).

Humoral immune responses

The functional background for malaria immunity is not fully understood, but several studies indicate that immunity against the blood stages of the *P. falciparum* infection is mainly antibody mediated. The exact mechanism of action of antibodies remains

incompletely explained. However, the efficacy of anti-malarial antibodies has been attributed to invasion/growth inhibition of *P. falciparum* parasites (Miller *et al.*, 1975; Wåhlin *et al.*, 1984), interference with binding of the parasite to the host cells (Udeinya *et al.*, 1983), antibody-dependent inhibition mediated by monocytes/macrophages (Groux and Gysin, 1990), or complement mediated opsonization of infected-RBC (Salmon *et al.*, 1986) (Fig. 2).



A number of studies have described associations between the presence of antibodies against certain *P. falciparum* antigens and reduced risk of clinical malaria (Taylor *et al.*, 1998; Ahlborg *et al.*, 2002; Metzger *et al.*, 2003). Target antigens in this context are, the merozoite surface proteins (MSP) (Metzger *et al.*, 2003; Polley *et al.*, 2003), antigens present in the apical organelles of the merozoites or expressed on the surface of infected erythrocytes, which hence all are considered as potential

vaccine candidates (Howard and Pasloske, 1993; Berzins and Perlmann, 1996; Bolad and Berzins, 1999).

The parasite inserts antigens into the surface of their host red cell that are polymorphic (Roberts *et al*., 1992; Brannan *et al*., 1994). The major antigen of this category is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1), which is encoded by the *var* multigene family comprising about 50 genes located on multiple chromosomes (Su *et al*., 1995). Most individuals can make humoral responses to these parasite-derived antigens on the erythrocytes surface (Marsh and Howard, 1986; Bull *et al*., 1998). Humoral immune responses to variant surface antigens may protect against infection in a variant specific manner (Ofori *et al*., 2002; Giha *et al*., 2000). Potentially conserved parts in PfEMP-1 have been indicated and antibody responses to these parts may be important for development of humoral neutralizing immunity against the parasite (Staalsoe *et al*., 1998; Marsh and Howard, 1986).

Clinical disease in children is correlated with an almost exclusive appearance of parasite variants corresponding to gaps in each child repertoire of anti-PfEMP-1 antibodies (Bull *et al*., 1998). However, recently, it was shown that antibodies from adult malaria patients may agglutinated heterologous isolates, suggesting that these antibodies recognize cross-reactive epitopes on the PfEMP-1 (Chattopadhyay *et al*., 2003).

Antibodies in P. falciparum *infection*

There is good evidence that IgG antibodies may have an anti-parasitic effect *in vivo,* as demonstrated by passive immunization with IgG from adult Africans to Gambian children (Cohen *et al*., 1961) or to adult Thai patients (Bouharoun-Tayoun et al., 1990). The cytophilic subclasses IgG1 and IgG3 subclasses were shown to predominate in protected individuals, while IgG2 and IgM could inhibit the *in vitro* effect of the former

(Bouharoun-Tayoun and Druilhe, 1992; Oeuvray *et al*., 1994). Rzepczyk *et al*., (1997) found that high proportion of individuals living in areas of high malaria transmission have antibodies to the MSP-2 antigen, these antibodies are primarily of IgG3 subclass. The levels to these antibodies directed to MSP-2 were associated with protection in The Gambia (Taylor *et al*., 1998), while in Senegal the protection was associated with IgG3 to *P. falciparum* extract (Aribot *et al*., 1996). Patients dying of severe malaria were found to have only trace amounts or no detectable levels of *P. falciparum* reactive IgG3 antibodies at the admission time, while a favourable outcome was observed in individuals when even limited levels of such IgG3 antibodies were detectable (Sarthou *et al*., 1997). Similarly, the levels of IgG1 antibodies to exoantigens were associated with clinical protection in patients from Madagascar (Chumpitazi *et al*., 1996). In another report, the balance between *P. falciparum* reactive IgG1 and IgG2 antibodies was found to be associated with protection from severe malaria in children from Kenya (Ndungu et al., 2002). However, recent data suggest that IgG2 antibodies may be involved in resistance to malaria in certain epidemiological settings (Aucan *et al*., 2000).

Although antibodies to *P. falciparum* parasite of all major classes are induced in malaria infected individuals, the possible protective role of IgE in malaria is unknown (Troye-Blomberg *et al*., 1999a). However, recently it was shown that the levels of IgE antibodies were lower in comatous children as compared to non-comatous ones with severe malaria (Calissano *et al*., 2003). IgE complexes with antigen or with IgG anti-IgE may induce release of TNF-α from certain cells with Fc receptors for this isotype (Dugas *et al*., 1995). The parasite induced TNF is known to contribute to pathogenesis of cerebral malaria (Clark *et al*., 1991). Thus, it seems that the balance between IgE complexed to antigen or IgG anti-IgE and the non-complexed IgE determines the outcome of the severity of the disease.

Although many functions have been described for IgM antibodies in infectious diseases, no specific function has been ascribed to IgM antibodies in malaria (Garraud *et al*., 2003). However, Scholander *et al*., (1996) found fibrils containing non-immune IgM extending from the knobs on the surface of *P. falciparum* infected erythrocytes. These fibrils were shown to be crucial for stable rosetting between the infected-RBC and non-infected ones, suggesting that IgM is important for the formation of rosettes.

Antibody dependent cell-mediated inhibition

Studies addressing the question of how IgG antibodies might mediate protection indicate that cytophilic subclasses act synergistically with monocytes in so-called antibody-dependent cellular inhibition (ADCI) (Bouharoun-Tayoun *et al*., 1995).

Several possible mechanisms whereby antibodies can confer protective immunity against malaria infection have been indicated (Ahlborg *et al*., 1996; Sabchareon *et al*., 1991; Bouharoun-Tayoun *et al*., 1990). Although, antibodies may by themselves inhibit parasite invasion/growth in *vitro,* it has been difficult to demonstrate any correlation between this activity and parasite neutralization or protection *in vivo*. Clinically effective IgG obtained from the sera of adults immune to *P. falciparum* was shown to suppress the parasite growth in co-operation with monocytes, although the IgG by itself did not inhibit invasion or intraerythrocytic parasite growth (Bouharoun-Tayoun *et al.*, 1990). Passive transfer experiments in squirrel monkeys and humans indicate that opsonic or cytophilic antibodies (IgG1 and IgG3 in humans) are associated with protective effect of antibodies (Bouharoun-Tayoun *et al*., 1992; Groux and Gysin, 1990; Shi *et al*., 1999). The mechanism responsible for this type of killing is the capture of antibodies on the surface of monocytes through receptors that bind the Fc part of the antibody, while the Fab part of the antibody molecule is bound to antigen/s on the surface of either merozoites (Bouharoun-Tayoun *et al*., 1990) or late infected

erythrocytes (Gysin *et al*., 1993). The cooperation between malaria-specific IgG1 and IgG3 and monocytes via the Fcγ receptors could induce cellular functions such as phagocytosis, antibody dependent cell-mediated inhibition (ADCI) (Shi *et al*., 2001) or secretion of monocyte-derived mediators (Tebo *et al*., 2001).

Fcγ receptor polymorphism and immunity to malaria

The FcγRs provide a bridge between the humoral and cellular arms of the immune system and thereby mediate phagocytosis or cytotoxicity (van de Winkel and Capel, 1993). In humans, there are three identified classes of Fc receptors for human IgG (FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). Among the FcγR, the low-affinity FcγRII class is the most broadly distributed. FcγRIIa shows genetic polymorphism, resulting in two distinct allotypes differing in one amino acid at position 131, histidine (H) or arginine (R), which is critical for binding of IgG. FcγRIIa-131H has higher affinity to bind IgG2 than FcγRIIa-131R (Warmerdam *et al*., 1991). FcγRIII has two isoforms, FcγRIIIa that exhibits a dimorphism with different affinity to IgG1 and IgG3 (Koene *et al*., 1997), while FcγRIIIb occurs in two allotypes being designated NA1 (Neutrophil antigen-1) and NA2. Particular allelic polymorphisms in FcγRIIa are associated with differential susceptibility to certain infections. Expression of the allele FcγRIIa-131R has been reported to be associated with protection against high-density *P. falciparum* infection in Kenya (Shi *et al*., 2001). Transfected phagocytic cells expressing FcγRIIa-131H tended to show high phagocytosis of infected erythrocytes opsonized with sera containing predominantly IgG3, while such phagocytosis with allotype FcγRIIa-131R tended to be higher with IgG1 containing sera (Tebo *et al*., 2002). In Thai individuals, the FcγRIIa-H/H131 genotype in combination with FcγRIIIB-NA2 was found to be associated with cerebral malaria (Omi *et al*., 2002).

Target antigens for neutralizing antibodies

Several target antigens for antibody-mediated inhibition of parasite growth or invasion have been identified (reviewed by Berzins and Anders, 1999). The protective effect is elicited by IgG antibodies to antigens on the surface of infected erythrocytes and to the antigens on the surface of the parasite or in parasitophorous vacuole.

Antibodies to some merozoite surface antigens (MSP-1 and MSP-2), to antigens present in the apical complex organelles of merozoites (EBA-175 in micronemes; Rhop-1-3, RAP-1-3 and AMA-1 in rhoptries) and to the dense granule antigen Pf155/RESA, show the capacity to inhibit merozoite invasion (reviewed by Berzins and Anders 1999).

Several antigens synthesized during trophozoite development (MSP-3, GLURP, SERA, ABRA and S-antigen) and are secreted into the parasitophorous vacuole, are found associated with the merozoite surface at the time of schizont rupture, as well as in a soluble form (exoantigens) in the supernatants of *P. falciparum* cultures (Jakobsen, 1995). Antibodies to several of these antigens have been shown to have high capacity to inhibit parasite growth or invasion *in vitro* (Perrin and Dayal, 1982; Banyal and Inselburg, 1985; Sharma *et al.*, 1998). Of the parasite-derived antigens expressed on the surface of infected erythrocytes, only Pf332 has been demonstrated to induce parasite-neutralizing antibodies, inhibiting the intraerythrocytic growth of the parasite (Ahlborg *et al.*, 1996).

Several *P. falciparum* antigens were demonstrated as targets for antibody dependent cell-mediated inhibition (ADCI) in *in vitro* including the merozoite surface protein 3 (MSP-3) (Oeuvray *et al.*, 1994), glutamate-rich protein (GLURP) (Theisen *et al.*, 1998) and serine repeat protein (SERP) (Soe *et al.*, 2002). Further more, we show in paper I of this thesis that also Pf332 is a target antigen in ADCI. However, while antibodies to Pf332 also proved able to inhibit parasite growth on their own

(Ahlborg *et al*., 1996), antibodies to MSP-3 (Oeuvray *et al*., 1994) and GLURP (Theisen *et al*., 1998) need the cooperation with monocytes in order to be inhibitory.

Cellular immune responses in malaria

It is well established that both humoral and cellular immune mechanisms contribute to the control of the asexual blood stages. Nevertheless, the acquisition and maintenance of protective immunity is largely T-cell dependent (Troye-Blomberg *et al*., 1994).

CD4+ T cells play an essential role in regulating the human immune responses to asexual blood stages of *P. falciparum* both with regard to production of cytokines and with providing help to the humoral component (Troye-Blomberg and Perlmann, 1994; Kabilan *et al*., 1987). The regulatory role of CD4+ T cells on immune responses to asexual stages of *P. falciparum* is indicated from *in vitro* experiments in which CD4+ T cells from malaria exposed individuals respond to diverse *P. falciparum* antigens by proliferation or by production of IFN-γ or/and IL-4 (Troye-Blomberg *et al*., 1990; 1994). The T cell production of IL-4, but not IFN-γ was found to be correlated to antibody levels in these individuals. Adults living in malaria endemic areas, may harbour parasites without showing symptoms and sometimes show no or low T cell responses to malaria antigen *in vitro,* in particular during acute infection (Theander *et al*., 1986). Moreover, a similar reduction in *in vitro* responsiveness to malaria antigens of T-cells from healthy individuals living in Madagascar was observed during the transmission period (Chougnet *et al*., 1990). Such lack of or low T cell responsiveness may be due to many factors, for example, host genetics (Jepson *et al*., 1997) or recruitment of antigen specific T-cell to other sites than the peripheral circulation (Hviid

et al., 1991), as also supported from studies in mice (Langhorne and Simon-Haarhaus, 1991: Sjolander *et al*., 1995).

CD4+ T cells also have important functions in anti-plasmodial immunity, including release of cytokines such as IFN-γ involved in the activation of mononuclear and polymorphonuclear leukocytes which phagocytose or lyse infected erythrocytes (Kharazmi and Jepsen, 1984; Orago and Facer, 1991). While, CD8+ T cells with a cytotoxic potential play an important role in immunity to the preerythrocytic stages of malaria parasites (reviewed by Nardin and Nussenzweig, 1993), these cells do not seem to take part in the clearance of asexual stages of the malaria parasite, as infected cells don not express MHC class I antigens. However, in contrast to the MHC-restricted αβ T cells, the MHC-unrestricted γδ T cells may have a direct cytotoxic potential on the asexual blood stages of malaria parasites, as demonstrated by their inhibitory activity in *P. falciparum* cultures (Troye-Blomberg *et al*., 1999b).

The Production of TNF-α may be essential for protection against *P. falciparum* malaria (Taverne *et al*., 1990). However, high levels of TNF-α have also been shown to be associated with severe complications (Shaffer *et al*., 1991). Studies in African children have shown that raised levels of the inflammatory cytokines TNF-α, interleukin (IL)-1β and IL-6 are associated with cerebral malaria (Kwiatkowski *et al.*, 1990).

Immune evasion mechanisms in malaria

One of the major questions in malaria research is why infections with *P. falciparum* malaria are often lethal as compared to those with the other malarial species. A possible explanation lies in the additional mechanisms that *P. falciparum* developed to evade the human immune responses and to avoid clearance by its host.

As mentioned above, sporozoites have a very short stay in the blood stream. During this period the host response is poor because of the relatively low density of sporozoites. The circumsporozoite protein, which is located at the surface of the sporozoites, contains multiple tandem repeats. These repeats may help the parasite to evade host immunity by exhibiting sequence polymorphism (Ramasamy, 1998). However, if an antibody response develops against sporozoites, the parasite tends to slough off the surface CSP coat. In minutes, sporozoites leave the circulation for the liver. Once inside the liver, each parasite multiplies giving rise to thousands new ones. These new parasites may constitute targets for CD 8+ T cells, as the infected hepatocytes express MHC class I molecules on their surface presenting parasite derived peptides (Schofield *et al.*, 1987; Klotz *et al.*, 1995a). To avoid this fate, the parasite tends to suppress T-cell immune responses. It has been shown that slight variation of the peptide bound to MHC molecules can reduce its binding affinity to either MHC or the T cell and may downregulate T cell responses, using altered peptide ligand antagonism (Gilbert *et al.*, 1998; Plebanski *et al.*, 1999).

In turn newly formed parasites burst out of the liver and home in on the red blood cells. Since the red blood cells express no or only very low amounts of MHC class I molecules (Botto *et al.*, 1990) and lack both the essential accessory molecules and antigen-processing machinery, malaria-infected red blood cells are not targets for CD8+ T-cells. However, circulating infected blood cells are targets for destruction in the spleen (Lee *et al.*, 1989; Ho *et al.*, 1990), but in order to avoid this, the parasite develops several mechanisms to escape the host immune responses.

One of the main mechanisms that allow the parasite to escape potentially neutralizing immune responses is the presence of antigens that vary between different strains of *P. falciparum* or change with time within strains (Hommel and Semoff, 1988; Mendis *et al*., 1991; Newbold, 1999; Staalsoe *et al*., 2002). The polymorphism is often caused by variations in sequence of the short tandem repeats of the antigens, which may constitute immunodominant epitopes. These repeats may help the parasite to evade host immunity by exhibiting sequence polymorphism or by preventing the normal affinity and isotype maturation of an immune response (Ramasamy, 1998).

The parasite antigens responsible for antigenic variation are mainly expressed on the surface of infected-RBC. These antigens serve to anchor the parasitized red cells (mature trophozoites and schizonts) to the lining of blood vessels in a range of different tissues (Udeinya *et al*., 1981). This phenomenon is called sequestration, and has evolved to bypass the destruction of infected-RBCs by the spleen. Infected-RBCs also have the ability to bind non infected-RBCs, leading to so called rosette formation and contributes to the sequestration of the parasites (Handunnetti *et al*., 1989; Udomsangpetch *et al*., 1989a). The advantage for the parasite to form such rosette may be to hide from the host immune responses, however, some studies suggested that rosetting also could enhance the invasion of uninfected-RBCs by merozoites (Sjoberg *et al*., 1991).

Most parasite lines and clones adhere to CD36, which has been considered to be an important receptor for infected-RBC adhesion (Barnwell *et al*., 1989). CD36 is found on the surface of several cell types including monocytes, dendritic cells and endothelial cell (EC). The presence of this receptor on the surface of dendritic cells may promote binding of late stage infected-RBC. Such binding has been suggested to inhibit the maturation of dendritic cells and modulate their function as antigen presenting cells and their subsequent activation of T cells (Urban *et al*., 1999).

Monocytes as well as dendritic cells may engulf the invading pathogen, process antigens and present them to T cells in the context of MHC-II molecules. The interaction between the antigens bound to the MHC-II and the T cell leads to the activation of those monocytes. *In vitro* studies have shown that monocyte functions including phagocytosis and generation of reactive oxygen intermediates (ROIs), are severely impaired following ingestion of malaria pigment (hemozoin) (reviewed by Sacks and Sher, 2002). Hemozoin may also interfere with the expression of MHC-II molecules and, thus, the parasite may impair antigen presentation and evade the host defences (Schwarzer *et al*., 1993; 1998).

However other mechanisms appear to exist enabling the parasite to escape immune pressure, for example as indicated from our field studies in Burkina Faso, where parasite field isolates were less sensitive to *in vitro* growth inhibition mediated by immunoglobulins (Igs) from the parasite donor than by those from other donors living in the same area. While this may reflect the effect of immune pressure *in vivo*, also other factors may be responsible for the decreased sensitivity of parasite isolates to autologous Ig and these will be discussed later in this thesis.

Antigenic polymorphism and variation in P. falciparum

P. falciparum parasites show a remarkably high degree of polymorphism at the various stages of their life cycle (Lockyer *et al*., 1989; Bull *et al*., 1998; Miller *et al*., 1993; Fenton *et al*., 1991; Konaté *et al*., 1999), which has important implications for the efficacy of parasite-neutralizing immune responses. Antigenic diversity in field populations of *P. falciparum* parasites may delay acquisition of protective immunity to malaria, the development of which may thus require repeated exposure to many different antigenic types or strains circulating in a given locality.

The antigenic diversity reflects polymorphisms in allelic gene products while polymorphisms in many antigens are caused by variations in the sequence of the short tandem repeats, which is a characteristic of many malaria antigens and which frequently constitute immunodominant regions. *msp1* and *msp2* genes are the best-studied antigens with regard to allelic polymorphisms (Snounou *et al*., 1999).

Antigenic variation is a process by which a clonal parasite population can switch its antigenic phenotype (Gardner *et al*., 2002). In *P. falciparum* the variant antigen/s are expressed at the surface of infected erythrocytes and the expression of these antigens can be modulated in a given parasite population either by immune pressure or transfer from intact to splenectomized animals. Antigenic variation is usually considered as a mechanism that allows parasite survival in an immune-competent host. However, antigenic variation of PfEMP-1 can also occur *in vitro* in the absence of immune pressure (Biggs *et al*., 1991; Roberts *et al*., 1992).

RELATED BACKGROUND

Plasmodium falciparum 332 antigen

The Pf332 antigen is synthesized as an approximately 750-kDa polypeptide (Wiesner *et al*., 1998), which is exported from the intracellular parasite to the erythrocyte membrane in vesicle-like structures and becomes expressed on the erythrocyte surface (Hinterberg *et al*., 1994). The gene product contains a large number of highly degenerated repeats rich in glutamic acid residues (29.8%) in its sequence, the whole Pf332 containing 29.8% glutamic acid (*P. falciparum* Genome Database, http://www.tigr.org/tdb/edb2/pfa1/htmls/index.shtml). The Pf332 from different parasite

isolates displays a marked restriction fraction length polymorphism (Mercereau-Puijalon *et al*., 1991), probably reflecting the location of the gene in the subtelomeric region of chromosome 11. The surface exposure of Pf332 makes the antigen a possible target for opsonizing antibodies (Gysin *et al*., 1993), which may mediate killing of parasites in cooperation with monocytes.

The human MoAb 33G2 has a high capacity to inhibit *in vitro* invasion/growth of erythrocytes by *P. falciparum* merozoites, as do other Pf332 reactive antibodies (Ahlborg *et al*., 1996). MoAb 33G2 cross-reacts with several *P. falciparum* antigens but shows the strongest reactivity with Pf332 (Iqbal *et al*, 1993a), suggesting that this antigen is the original target for the MoAb (Udomsangpetch *et al*., 1989b). The optimal epitope recognized by MoAb 33G2 is the pentapeptide VTEEI, which occurs more than 40 times in Pf332 (Kun *et al*., 1991; Mattei and Scherf, 1992).

Rabbit and human antibodies reactive with the VTTEI epitope were shown to interfere with schizont development by blocking the rupture of mature schizonts or, alternatively, by interfering with the development of parasite intraerythrocytically *in vitro* (Ahlborg *et al*., 1996). Individuals in malaria-endemic regions show a high prevalence of seroactivity to antigen Pf332 repeat sequences (Iqbal *et al*., 1993b), and the levels correlated inversely with parasite density in Tanzanian children (Warsame *et al*., 1997). Antibody reactivity with EB200 (a part of the Pf332 antigen) was prevalent in Senegalese individuals and correlated with lower incidence of clinical attacks of malaria (Ahlborg *et al*., 2002), suggesting that Pf332 may be target for potentially protective antibodies *in vivo* (Ahlborg *et al*., 1993).

The effect of immune pressure on parasites and their susceptibility to inhibition

The risk of developing clinical symptoms of malaria increases with increasing levels of *P. falciparum* parasitemia, but not uncommonly African children carry a high level of parasitemia without clinical symptoms (Marsh, 1992). These children have developed an anti-disease immunity, neutralizing the fever inducing malaria toxins, while their parasite-neutralizing immunity has not reached levels high enough to clear the infection.

The chronic persistence of parasites in a host despite the concurrent presence of potentially parasiticidal immune responses may require their adaptation to the immune pressure. Some of these parasites have the ability to antigenically vary molecules that are targets of anti-parasitic immunity and thus escape complete elimination from an immuno-competent host. However, other mechanisms appear to exist enabling the parasite to escape the immune pressure. An indication of such mechanisms was obtained by experiments where a laboratory strain of *P. falciparum* was grown *in vitro* in the presence of sub-optimal inhibitory concentrations of antibodies (Iqbal *et al.*, 1997). Using antibodies reactive with the relatively conserved antigens Pf332 and Pf155/RESA, parasites with a specific decreased sensitivity to antibody-mediated growth inhibition were readily generated. The relative resistance of the parasite to antibody-mediated growth inhibition developed successively against antibodies used in the culture, while the parasite remained sensitive to growth inhibition by other antibodies (Iqbal *et al.*, 1997). Continuing the culturing of the parasites after removal of the antibodies, the parasites gradually regained their sensitivity to growth inhibition. With antibodies to Pf332 in the culture, genotyping of the parasites showed that a new clone of parasites appeared, which, upon removal of the antibody pressure, was gradually replaced by parasites of the original genotype. Thus, the *P. falciparum* laboratory strain, which had been kept in culture for more than 20 years, contained at least two clones, one of which is dominating during ordinary culturing conditions. Thus, the immune pressure exerted by such antibodies appears to select for parasites

with low expression of a specific antigen from a heterogeneous parasite population. Alternatively, the antibody pressure may select for parasites the growth of which is promoted by the antibodies. In contrast, no genotypic change in the parasite population was detected in cultures grown in the presence of antibodies to Pf155/RESA. In this case the specific decrease in sensitivity to growth inhibition may be due to down regulation of either synthesis or expression of the specific antigen by antibody pressure and permits a means of immune evasion.

In a study performed in Burkina Faso (Wåhlin *et al*., 1997), it was observed that *P. falciparum* parasites may vary in their sensitivity to antibody-mediated invasion/growth inhibition *in vitro.* The isolates of *P. falciparum* were tested *in vitro* for their sensitivity to growth inhibition mediated by autologous and heterologous Ig fractions. The isolates were less sensitive to growth inhibition mediated by autologous Ig fractions compared to that mediated by heterologous Ig fractions. This lower sensitivity of isolates to autologous Igs may be due to the effect of immune pressure *in vivo,* selecting from a heterogeneous parasite population those with a low expression of antigens recognized by the host's antibodies. Several other mechanisms were also suggested to contribute to this lower sensitivity, including the following 1) the parasites cultured from each child may represent an expanding parasite population from a recent infection, mainly composed of parasite strains not seen earlier by the immune system of that specific child; 2) production of anti-idiotypic antibodies that could bind to inhibitory antibodies and thereby counteracts their parasite reactivity (Wåhlin *et al.*, 1990); 3) it is also possible that antibodies to the parasites in the ongoing infection have been partly consumed.

Dynamics of *P. falciparum* infections

Natural *P. falciparum* infections in areas with high transmission usually consist of multiple parasite clones (Färnert *et al*., 1999) of which there is a rapid turnover (Daubersies *et al*., 1996). Reappearance of asexual parasites in the peripheral circulation may be ascribed to either established chronic infection derived parasites or re-infection with new ones (Basco *et al*., 2000).

Population dynamic studies, which consider the genetic heterogeneity of *P. falciparum*, have shown fluctuations of different genotypes in space and time. The host immune response appears to play an important role in generating these dynamics (Day *et al*., 1992). Nevertheless, differences in parasitological profiles between single children might also reflect qualitative differences in protective immunity rather than differences in the genotypes of the infecting parasites (Färnert *et al*., 1999). However, the genetic diversity displayed by *P. falciparum* field isolates was found to be distinct in different geographic areas (Haddad *et al*., 1999; Babiker *et al*., 1999). Infections comprising multiple parasite clones appear mainly to be due to a single inoculation by a mosquito of an antigenically diverse parasite population, rather than multiple monoclonal inoculations (Babiker *et al*., 1999; Taylor, 1999).

It has become possible to study the dynamics of *P. falciparum* parasites by employing the techniques of molecular genetics, thus allowing the precise identification of target molecules for a rational design of vaccines. An understanding of these host-parasite interactions in the context of dynamics and immunity may reveal such target molecules. Genotyping makes it possible to analyse the dynamics of infections and to generate data on multiplicity of infection. Genotyping frequently is used to distinguish new from established infections (Cattamanchi *et al*., 2003).

msp1 and *msp2*, as polymorphic genes, are useful to study the dynamics of *P. falciparum* infections especially in samples collected from areas of intense transmission

and also beneficial to distinguish re-infection from established infections (Magesa *et al*., 2001).

Insecticide treated curtains and immunity to *P. falciparum* parasite

Malaria-related death rates are rising once again in Africa (WHO, 1999). This reflects the emergence of drug resistant strains of the parasite, changes in climate, population movements, highly efficient *Anopheles gambiae sensu lato* and *Anopheles funestus* vectors, a parasite population composed overwhelmingly of *P. falciparum*, poverty and lack of healthcare infrastructures. The result is widespread *P. falciparum* transmission at intensities tending to cause severe morbidity and mortality, especially in children below the age of 5 years.

Options to control the parasite include vaccines, drugs and impregnated nets. Difficult obstacles have been encountered in attempting to develop vaccines. The heterogeneity in parasites in different geographical areas, complex life cycle and protection requiring both antibody-mediated and cell-mediated immune responses represent an enormous technical challenge. In view of these obstacles, effective and approved vaccines are not yet available. In addition, drug resistant malaria has become one of the most important problems in malaria control in recent years. In the light of that, investigators realize the best approach for limiting the number of deaths caused by malaria depends on the basis of minimizing the human vector contact. One of the best-established methods for vector control has been the use of insecticide treated nets (ITNs).

Four standardized large scale mortality trials in Kenya, Ghana, Burkina Faso and The Gambia with differing transmission intensities showed that, as a result of using insecticide treated nets, child mortality was reduced by between 17% and 33%, while

treated curtains reduced child deaths by 14% in Burkina Faso (Alonso *et al*., 1991; D'Alessandro *et al*., 1995; Binka *et al*., 1996; Nevill *et al*., 1996; Habluetzel *et al*., 1997; 1999). The trials showed that the use of treated nets can prevent 6-8 deaths each year for every 1,000 children protected (Lengeler *et al*., 1998), providing first evidence for answering the main public health question, namely whether ITNs reduce mortality among children.

The ITNs possibly act in different ways to reduce human-vector contact. First, intact nets provide a physical barrier to mosquitoes. Second, the insecticide has toxic effects on mosquitoes that attempt to feed (Curtis, 1996). Third, the ITNs do not only guard against mosquito bites but also limit the spread of the disease by preventing mosquitoes from taking blood from infected individuals.

The use of insecticide treated nets (ITNs) was one of the main four strategies promoted by Roll Back Malaria (RBM), a global partnership founded in 1998 by WHO, UNICEF, the United Nations Development Programme, and the World Bank, to half the world malaria infection rate by 2010. The major obstacles for making ITN technology widely available are that the nets need regular re-treatment and they are charged with extra expenses for taxes and tariffs. In Abuja, Nigeria, in April 2000, 44 African leaders endorsed RBMs goals for 2010 and decided to abolish taxes and tariffs on ITNs.

It is a cause for concern that large-scale use of pyrethroid impregnated nets may delay the acquisition of immunity to malaria in individuals using them (Askjaer *et al*., 2001) and even that their use may merely lead to an increase in mortality and morbidity in the older age groups (Snow *et al*., 1994; Snow and Marsh, 1995; Trape and Rogier, 1996). However in some recent studies looking at the impact of ITNs use on the immunity against malaria, no difference was seen in antibody levels to *P. falciparum* crude extract or to certain asexual blood stage antigens between ITNs users and non-

users (Kariuki *et al*., 2003; Meraldi *et al*., 2002). Nevertheless, there is further need to monitor the long-term effects of ITNs usage in different malaria endemic settings on the acquisition of immunity against malaria.

Susceptibility to malaria infection

Several studies on malaria have investigated the association between the humoral immune responses, severity of the disease and several genes, including genes within MHC (Troye-Blomberg, 2002; Riley, 1996; Sjoberg *et al*., 1992). In recent years, substantial progress has been made in identifying the relative contribution of different immune mechanisms to protection against malaria infection and disease. Modiano *et al*., (1998) reported that the susceptibility to malaria infection and the ability to mount humoral immune responses to malaria varied between three African tribes living in sympatry in Burkina Faso (Fulani, Mossi and Rimaibé). The individuals of the Fulani tribe were less parasitemic and had higher levels of antibodies to defined epitopes in the two *P. falciparum* antigens, Pf332 and RESA, than the individuals of the neighbouring tribes, the Mossi and Rimaibe. Furthermore, the Fulani were less parasitaemic than the other tribes, despite the same level of exposure. This finding suggests that host genetic factors may at least in part play a role in determining the outcome of the immune response to infection with malaria (Luoni *et al*., 2001; Aucan *et al*., 2001).

Other factors involved in protection against the severity of the disease include 1) hemoglobinopathies, 2) erythrocyte polymorphisms 3) presence of a particular variant or subtype of HLA. The inherited disorders of haemoglobin (Hb) including HbS (Allison *et al*., 1954) and HbC (Modiano *et al*., 2001) have been associated with protection against malaria. Further studies show that some deficiencies in the red blood cell enzyme called

glucose-6-phosphate dehydrogenase may be associated with decreased susceptibility to severe disease through enhancing the phagocytosis of early stage infected erythrocytes (Cappadoro *et al*., 1998). In addition, presence of a particular variant of HLA-B was found to be associated with protection from developing severe disease (Hill *et al*., 1991). Recently, it has been shown that mutations in complement receptor 1 on the surface of erythrocytes may be associated with protection from severe malaria in individuals from Papua New Guinea (Cockburn *et al*., 2004).

THE PRESENT STUDY

Protective immunity against *Plasmodium falciparum* may be obtained after repeated exposure to infection. Several studies indicate that immunity against the blood stages of the *P. falciparum* infection is mainly antibody mediated. Protective antibodies may act either on their own, mediate antibody-dependent phagocytosis and/or cell-mediated neutralization of parasites. This thesis describes several aspects of humoral immune responses to *P. falciparum* infection in individuals of different age groups, different genetic background and with different degrees of malaria exposure.

Objectives

The principal objectives of this thesis were:

* To analyse the parasite neutralizing capacity of Pf332-specific antibodies with regard to efficiency and mechanisms, and to optimise an *in vitro* assay for studying the inhibition of *P. falciparum* growth inhibition by antibodies in cooperation with monocytes (ADCI).

* To investigate by which mechanisms the parasite escapes the immune response and to evaluate the stability of the decreased sensitivity of isolates to inhibition by autologous antibodies. The study also aimed at analyzing the effects of immune pressure on *P. falciparum* parasites with regard to antibody-dependent inhibition of parasite growth *in vitro*.

* To investigate the impact of long-term use of insecticide treated curtains (ITC) on children's immune responses for malaria in Burkina Faso, where ITC have contributed to a reduction in intensity of malaria infection. Furthermore, the multiplicity of genotypes in the infecting parasite was analysed.

* To assess possible correlates between the susceptibility to malaria and levels of *P. falciparum* reactive antibodies in plasma samples from ethnic tribes living in sympatry in two distinct endemic conditions; the Fulani/Mossi in Burkina Faso and Fulani/Dogon in Mali.

MATERIALS AND METHODS

Study area and populations

Burkina Faso (II, III & IV)

The study area in Burkina Faso is located in the vicinity of Ouagadougou (the capital), a typical zone of Sudanese savannah. Malaria has been documented to be as a top problem in Burkina Faso and accounts for 29% of deaths among children less than five years of age. The rainy season lasts from June to October, which corresponds to the high malaria transmission. The major vectors are *Anopheles gambiae s.s*, *Anopheles arabiensis* and to a lesser extent *Anopheles funestus*. The inoculation rate is high, each individual receiving several hundred infective bites/ year (300-500) (Cuzin-Ouattara *et al*., 1999), i.e. on the average more than one infective *Anopheles* bite per night (Esposito *et al*., 1988). The population of the study belongs to the Mossi and the Fulani tribes. The Mossi lives by subsistence farming, while the Fulani are cattle breeders.

Mali (IV)

The study area comprises four villages (Mantéourou, Naye, Binédama, and Anakédié) situated 850 km from Bamako, the capital of Mali, Our study was targeted to two ethnic tribes, the Dogon and the Fulani, who live in this area where the villages are less than 7 km from each other. In the study area malaria transmission is mesoendemic where the rainy season extends from July till October where an individual receives on average 4-20 infected bites/month (Coulibaly *et al*., 2002; Sagara *et al*, 2002). The major vectors are *Anopheles gambiae complex* and *Anopheles funestus*.

P. falciparum blood-stage extract preparation (I-IV)

The *P. falciparum* F32 strain (Tanzanian) was maintained in continuous culture and synchronized as previously described (Lambros and Vanderberg, 1979). When the parasitaemia reached 10% (schizont-infected red blood cells), cultures were washed twice in cold RPMI. 2.5 ml of culture at 10% hematocrit were layered on top of 60% percoll and centrifuged at 2000 rpm for 15 minutes (4°C). The interface layer containing late stage IRBCs schizonts was collected and sonicated on ice in phosphate-buffered saline. Sonicates were centrifuged at 2000 rpm for 8 minutes (4°C). The protein concentration was determined using Bradford method (Bradford, 1976). The *P. falciparum* crude extracts were aliquoted and stored at minus 20°C until use.

Enzyme linked immunosorbent assay (ELISA) (I-IV)

ELISA assays were performed for the detection of IgG class and subclass antibodies to *P. falciparum* parasite by coating 96-well round-bottom plates with 10 µg of *P. falciparum* crude extract/ml in sodium carbonate buffer (pH 9.6) overnight at 4°C. The wells were then blocked at 37°C with 100 µl of carbonate buffer containing 1% (w/v) BSA. After incubation for 4h, the plates were washed with saline containing 0.05% Tween 20. Serum dilutions were incubated overnight at 4°C (1:20 for IgG2 and IgG4, 1:400 for IgG1 and IgG3) or for 4 h at 37 ºC for IgG (1:1000). Total anti-malarial specific IgG were detected using alkaline phosphatase conjugated goat anti-human IgG (Fc fragment specific). Antibodies of IgG1, IgG2, IgG3 and IgG4 subclasses were detected using biotin conjugated mouse anti-human subclass specific monoclonal antibody and alkaline phosphatase conjugated streptavidine for each subclass. The assay was developed with *p*-nitrophenyl phosphate disodium salt as substrate and the optical densities were read at 405 nm.

The concentrations of IgG-subclasses of anti-malarial antibodies were calculated from standard curves obtained in a sandwich ELISA with six dilutions of myeloma protein of IgG1-4 isotypes or with highly purified IgG for total anti-malarial antibodies.

Cut-off values for seropositive samples were calculated as the mean optical density values at 405 nm plus 2 SD of the values obtained with sera from eight Swedish donors who had not been exposed to malaria. All tests were done in duplicate, and antibody levels were expressed as mean concentration units.

Isolation and preparation of peripheral blood mononuclear cells (PBMCs) (I & III).

Blood mononuclear cells from healthy donor were separated on Ficoll-Paque (Böyum, 1976). Briefly, Heparinized diluted whole blood is layered on top of a density gradient material (Ficoll-Paque) and subjected to a centrifugal force (1100 g) for 20 min at 20°C. Cells from the interface were collected and washed twice (10 min each) in Tris Hanks solution supplemented with 25-50% autologous serum and were then resuspended in 50% autologous serum ($2x10^6$ cells/ml). The preparations were performed at room temperature during the entire procedure, approximately (25-35°C), as it has been shown that monocyte tends spontaneously to aggregate at lower temperatures (Mentzer *et al.*, 1986) and platelets to be activated (Oliver *et al.*, 1999). It is also important that the peripheral blood or buffy coat should not be older than 8 hours and supplemented with anticoagulants. Platelet elimination can be easily done with low speed centrifugation (600-1000 rpm) after the separation on Ficoll.

Preparation of monocytes (I & III)

To separate adherent and non-adherent cells, mononuclear cells were suspended in autologus serum and then incubated at 37°C in 5% CO_2 atmosphere for one hour in Petri dishes. The non-adherent cells were removed by washing three times with Tris-Hank supplemented with 0.5% human serum albumin at room temperature. The adherent cells were removed by a cell lifter and their viability was detected by Trypanblue exclusion dye. The method permitted recovery of 97% of adherent cells.

***In vitro* parasite growth inhibition assay (I-III)**

The assay was performed as described earlier (Wåhlin *et al.* 1984). Parasite cultures were diluted with washed autologous red blood cells or group + O blood to give a parasitaemia of 1% and adjusted to a haematocrit of 4% using malaria culture medium supplemented with 20 % human AB+ serum. The cultures were set up in duplicate in flat-bottomed, cell culture, 96-well plates. Five serial dilutions of antibodies (Ab) were prepared in duplicate using malaria culture medium with no AB serum (incomplete medium). Aliquots of 100 µl cultures were then added to each well (resultant hematocrit of 2% and AB serum of 10%) and incubated at 37°C for 18–22 hours in a candle jar.

Antibody dependent cell-mediated invasion inhibition assay (I & III)

Antibody-dependent cell mediated inhibition of parasite growth *in vitro* experiments were performed as describes in paper I. Briefly, prior to the ADCI, we assessed the capacity of the purified IgGs or Igs alone to inhibit parasite invasion/growth. We then run the ADCI assay using an antibody concentration giving no or low inhibition by themselves (sub-optimal inhibitory concentration). Monocytes were then added at

$1.5x10^5$ to $2x10^5$/well in the presence of antibodies. Wells were carefully mixed and plates incubated at 37°C in 5% CO_2 atmosphere for 60 min. Thereafter, aliquots of 100 µl of adjusted culture added and plates were incubated at 37°C in 5% CO_2 atmosphere for 22 h-42 h.

After the incubation, cell suspensions were harvested and put into small centrifuge tubes for washing three times in Tris Hank. Monolayers of cells were prepared on glass 8-well multitest slides by fixing in 1% glutaraldehyde in PBS and air drying. Parasites were stained with acridine orange and the percentage of newly infected red blood cells was analyzed by counting infected erythrocytes in 25 microscopic fields per well, in a fluorescence microscope at a magnification of 100x. The percent parasitaemia represents the number of infected erythrocytes per 25 fields per well per 8 wells to a total number of both infected and non infected red blood cells in 8 wells (RBCs per 8 wells=$4x10^4$).

***P. falciparum* DNA preparation and PCR amplification (II & III)**

DNA was extracted as previously described (Snounou *et al.*, 1993a), briefly, about 300 µL blood was lysed by saponin (0.05%). After centrifugation, the parasite-containing pellet was resuspended in lysis buffer (40 mM Tris, pH 8.0, 80 mM EDTA, 2% sodium dodecyl sulfate) and incubated in proteinase K (125 µg/ml) for 4 to 15 h at 37 ºC. Then, the DNA was extracted with Tris-equilibrated phenol, pH 8.0 followed with phenol-chloroform and chloroform. The DNA from each sample was precipitated using 45 µl of a 3.0 M sodium acetate solution, pH 5.0 and 1 ml of cold absolute ethanol. The ethanol precipitation tubes were placed at -20º C for 2-4 h; storage for overnight is possible. The

DNA was recovered by centrifugation, washing by 1 ml of 70% ethanol and drying, the DNA pellet was resuspended in TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA).

To analyse the complexity of infecting parasite populations, isolates of *P. falciparum* parasite were genotyped by nested-PCR of *msp-1* block 2, and *msp-2* block 3 (Snounou *et al*., 1993b). Table (2) shows the oligonucleotides primers designed to amplify block 2 of *msp-1*, and block 3 of *msp-2*. The two genes were amplified by nested PCR, each amplification with conserved or family-specific primer pair being done separately. PCR products were electrophoresed on 1.8% agarose gels, and DNA visualized by ultraviolet trans-illumination after ethidium bromide staining. Bands obtained were compared by size.

Table 2: Sequences of oligonucleotide primers used to amplify merozoite surface protein (*msp*)-1 and *msp-2* polymorphic regions of *P. falciparum* isolates

Gene	Primer	Sequence	Remarks
msp-1	M1-OF	5'-CTA GAA GCT TTA GAA GAT GTA TTG -3'	In the first reaction (PFG-Nest1) the region spanning both 2and 4 is amplified.
	M1-OR	5'-CTT AAA TAG TAT TCT AAT TCA AGT GGA TCA-3'	
msp-1	M1-2MF	5'-AAA TGA AGG AAC AAG TGG AAC AGC TGT TAC-3'	MAD20 family-specific
	M1-2MR	5'-ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC3'	
	M1-2KF	5'-AAA TGA AGA AGA AAT TAC AAA AGG TGC-3'	K family-specific

	M1-2KR	5'-GCT TGCATC AGC TGG AGG GCT TGC ACC AGA-3'	
	M1-2RF	5'-TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG-3'	RO33 family-specific
	M1-2RR	5'-CAT CTG AAG GAT TTG CAG CAC CTG GAG ATC-3'	
Msp-2	M2-OF	5'-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA	Conserved
	M2-OR	5'-CTT TGT TAC CAT CGG TAC ATT CTT-3'	
msp-2	M2-FCF	5'-AAT ACT AAG AGT GTA GGT GCA $^{A}/_{G}$AT GCT CCA-3'	FC27family-specific
	M2-FCR	5'-TTT TAT TTG GTG CAT TGC CAG AAC TTG AAC-3'	
	M2-ICF	5'-AGA AGT ATG GCA GAA AGT AA$^{G}/_{T}$ CCT $^{C}/_{T}$CT ACT-3'	IC/3D7 family-specific
	M2-ICR	5'-GAT TGT AAT TCG GGG GAT TCA GTT TGT TCG-3'	

RESULTS AND DISCUSSION

Paper I. Parasite inhibitory activity of antibodies to *P. falciparum* 332 antigen in cooperation with monocytes.

One likely way for antibodies to confer protection against malaria is by opsonization of parasitized erythrocytes, thus, mediating neutralization of the parasites by antibody-dependent cell mediated killing.

All previous studies, which investigated the interaction between monocytes and antibodies in inducing killing of parasites, focused on antigens related to merozoites only (Oeuvray *et al*., 1994; Soe *et al*., 2002; Theisen *et al*., 1998). However, several other studies demonstrated a correlation between antibodies to antigens exposed on the surface of *P. falciparum* infected erythrocytes and protection against malaria infection (Marsh and Howard, 1986; Marsh *et al*., 1989; Bull *et al*., 1998; Ahlborg *et al*., 2002). One of these antigens is Pf332, which appears on the surface of infected erythrocytes at late schizogony, and thereby constitutes a possible target for opsonizing antibodies (Gysin *et al*., 1993). In view of these findings, we investigated the parasite neutralizing capacity of P332-reactive antibodies in conjunction with normal human monocytes. For this purpose, we optimized an *in vitro* assay to analyze the inhibition of *P. falciparum* growth by antibodies reactive to the Pf332 antigen in cooperation with normal human monocytes.

When testing the inhibitory activity of total IgG prepared from rabbits immunized with Pf332 derived sequences on parasite growth *in vitro*, the antibodies showed an unexpectedly low inhibitory capacity. Agglutination of the erythrocytes in the culture was observed, suggesting that the low inhibition was due to presence of haemagglutinins in the IgG preparation. Removal of haemagglutinins by absorption

increased the inhibitory capacity of IgG considerably. As haemagglutinins bind to both infected and non infected red blood cell, the reduced inhibition could be due to: 1) enhancement of invasion by bringing infected and non infected RBCs together; 2) haemagglutinin binding to the surface of infected RBCs may interfere with the binding of antibodies. Such an enhancement of parasite growth *in vitro* by specific Ig fractions, antibodies to certain *P. falciparum* antigens or malaria sera has been reported in several studies (Bouharoun-Tayoun *et al*., 1990; Shi *et al*., 1999; Brown *et al*., 1983; Franzén *et al*., 1989). Possibly, haemagglutinins may take part in such enhanced parasite growth *in vitro*.

To further analyze the specificity of antibodies of parasite growth inhibition and to circumvent the influence of haemagglutinin, specific antibodies were isolated on Sepharose column charged with peptides representing repeat sequences in Pf332. In accordance with previous studies (Ahlborg *et al.* 1996), our study demonstrated that antibodies to Pf332 were effective in blocking the parasite growth in terms of reduced numbers of newly infected red blood cells. Affinity purified IgG as compared to total IgG, inhibited parasite growth at considerably lower concentrations.

While infected erythrocytes incubated with Pf332 specific antibodies at sub-optimal inhibitory concentrations gave minimal or no inhibition, a marked synergistic inhibitory effect could be seen at 22 h when monocytes were added. However, increasing the incubation time to 42 h, increased the background inhibitory activity of monocytes alone, and no synergistic effect of the antibody monocyte cooperation could be seen. Monocytes alone gave some inhibition also at 22 h. Phagocytosed parasites were detected under the microscope in the presence or absence of antibodies, indicating that part of parasite growth inhibition was due to phagocytosis. Recently, a novel mechanism for nonopsonic phagocytosis of trophozoites and schizonts of *P. falciparum* was described, the phagocytosis being

mediated by an interaction between parasite ligands, including PfEMP-1, and CD36 on the surface of monocytes (McGilvray *et al*., 2000).

The monocytes used in our experiments were obtained from healthy non-exposed individuals. The inhibitory effects of the monocytes on parasite growth in the presence of Pf332-specific antibodies did not vary significantly from donor to donor. Monocytes collected from the same donor at different time points had not different effect on parasite growth. While this was consistent with the data of some studies (Tebo *et al*., 2001), the differences between the *in vitro* effects of antibodies in cooperation with monocytes as effector cells observed in some studies (Shi *et al*. 1999), could be attributed to a polymorphism in FcγRII. Functional consequences of this polymorphism *in vitro* are likely, since only the FcγRIIA-H131 allelic form is the only human FcγR that efficiently binds human IgG2 (Warmerdam *et al*. 1991).

In conclusion, our data are the first to demonstrate that Pf332, expressed on the surface of infected erythrocytes, is a target for parasite neutralization mediated by antibodies in cooperation with monocytes as effector cell. A part of the inhibitory effect on parasites was due to monocytes engulfing preferentially late stage infected erythrocytes, indicating that antibodies to the epitope VTEEI exert their antibody dependent monocyte mediated parasite killing at the late stages of the parasite cycle.

Paper II. The growth inhibitory effects of autologous and heterologous antibodies on wild isolates of *P. falciparum* parasite.

This study was aimed at defining mechanisms for *P. falciparum* parasites to evade neutralizing immune responses and is based on previous findings from this laboratory (Wåhlin *et al*., 1997). Wild isolates as well as plasma immunoglobulins were collected from asymptomatic randomly selected children 3-7 years of age either on one or two different time points (day 0 and day 14). The isolates were assessed for their sensitivity

to the growth inhibitory effects of autologous and heterologous antibodies using an assay developed in our laboratory (Wåhlin *et al*., 1984). To evaluate the introduction of new parasite populations by mosquitoes into asymptomatic children, differences in distribution in two unlinked single copy genes coding for parts of *msp1* and *msp2* (Snounou *et al*., 1993) were studied using PCR-based genotyping. We further correlated the *in vitro* findings to the complexity of parasite populations in blood samples taken at the two time points.

In accordance with previous findings (Wåhlin *et al*., 1997), our data demonstrate a decreased sensitivity of *P. falciparum* isolates to *in vitro* growth inhibition mediated by autologous host immunoglobulins compared to that mediated by heterologous ones. Testing the inhibitory activity of Ig fractions obtained on two occasions, days 0 and 14, from the same child, on autologous parasite growth, revealed that 4 out of 8 isolates obtained on day 14 were more sensitive to the day 14 Ig whereas, the remaining isolates were more sensitive to growth inhibitory effect mediated by the day 0 Ig.

Genotyping of highly polymorphic regions of *msp-1* and *msp-2* of *P. falciparum* in parasites from the blood of asymptomatic children revealed specific PCR patterns, involving in most cases appearance and disappearance of genotypes in day 14 samples as compared with day 0 samples. Moreover, the asymptomatic infections were complex since in some children up to 5-6 bands could be visualized in one sample with a single pair of primers.

As mentioned above, several possible mechanisms could contribute to the observed lower sensitivity of isolates to autologous Ig fractions. Although our analysis could not distinguish which of the mechanisms is dominating, the different patterns in inhibitory activity indicate that the day 14 parasites, at least in some individuals, are derived from a new infection, which was further supported by the genotyping.

To verify further this issue, asymptomatic children living in two villages underwent curative therapy (sulfadoxine plus pyrimethamine) for pre-existing malaria infection during the rainy season of 2002 (Bolad *et al*., unpublished). Blood samples were collected before treatment and on day 21 post-treatment in 95 asymptomatic children, of which only few come out with parasitemia within 21 days post-treatment. It is likely that these parasites represent new infection since malaria transmission in one village is 29 infected bites per year (Cuzin-Ouattara, personal comunication).

In accordance with other studies, appearance of new genotypes of parasites on day 14 samples may either be due to that the individual had acquired a new infection during the period between the sample collections (Daubersies *et al*., 1996) or that those parasites may have been sequestered at the time of the day 0 sampling (Färnert *et al*., 1997).

Paper III. The effect of the use of impregnated curtains on immunity to *Plasmodium falciparum* and on complexity of infecting parasite populations

As mentioned above, the use of ITNs has been proven effective in reducing morbidity and mortality from malaria. An important consideration is whether the use of the nets simply delays the onset of malaria immunity (Snow and Marsh, 1995; Trape and Rogier, 1996; Nebié *et al*., 2003; Kariuki *et al*., 2003b) and may lead to a change in both the clinical spectrum of severe disease and the overall burden of severe malaria morbidity (Snow *et al*., 1997).

To address this question, we analysed the impact of ITC-use on parasite neutralizing immune responses, complexity of infecting parasite populations and on the levels of anti-malarial antibody responses in children who had lived all their life in villages with or without ITC. The levels of parasite specific antibodies were determined with regard to IgG class and subclass. The capacity of these antibodies to inhibit

parasite growth alone or in co-operation with monocytes was tested *in vitro*. The effect of the ITC-use on the complexity of infecting parasite populations was studied using PCR-based genotyping of the parasite.

Our study shows that the use of ITC reduced the prevalence of infection significantly among the ITC-users, the ITC-users being more frequently aparasitemic than ITC non-users. Using PCR-based genotyping, the ITC-users were found to carry parasites giving multiple and different allelic bands of *msp2*, but the multiplicity of infection was not significantly different between the ITC-users and non-users. Screening of plasma samples from children living in villages with ITC, showed that the levels of parasite specific IgG1 and IgG3 antibodies were not affected by ITC-use. Antibodies from children of both groups proved able to inhibit parasite growth on their own or in conjunction with monocytes to similar degrees.

In accordance with other studies (Kitua *et al*., 1999; Meraldi *et al*., 2002; Branch *et al*., 2000; Kariuki *et al*., 2003a), the levels of antibodies were not affected in children using ITNs, suggesting that transmission-reducing interventions may have little effect on antibody levels in such individuals. In addition, other studies, in holoendemic settings, (Fraser-Hurt *et al*., 1999) indicated that ITC usage did not affect the multiplicity of infection, suggesting that the preimunition was not affected in ITC-users (al-Yaman *et al*., 1997; Färnert *et al*., 1999). However, other studies have reported lower prevalence of antibodies to variant surface antigens among children sleeping under treated bed nets as compared to those not using such nets (Askjaer *et al*., 2001). Similarly, infants sleeping under treated bed nets showed lower seropositivity of schizont reactive IgM antibodies (Snow *et al*., 1996). Probably the observed inconsistencies between the above mentioned data might be due to differences in endemic settings of the study areas, in the age range of the study populations and in the choice of antibody specificities assayed.

In areas of low malaria transmission, high rates of severe disease have been reported (Snow *et al*., 1997), suggesting that all cause mortality is saturated at relatively low transmission in children 0-4 years of age (Snow and Marsh, 2002). Thus, the ITNs may be working by reducing the frequency of severe and fatal infections and, thus, allowing immunity to develop. However, at all levels of transmission the overall balance of benefits, including reduced load on families and health services from non-life threatening malaria, favours the widespread introduction of ITNs in endemic areas of Africa (Snow and Marsh, 2002).

Taken together, while ITNs will undoubtedly save many lives from malaria, particularly in the short-term, their long-term use in areas of different degrees of transmission needs to be carefully monitored (Snow *et al*., 1996).

Paper IV. Antibody responses to *P. falciparum* in West African ethnic tribes living in sympatry.

There are well documented associations between host genetics and the response to infection in humans. These are associations evident particulary in the case of malaria, where many studies have demonstrated associations between malaria morbidity and/or infection and immune responses to specific malaria antigens, such as ring-infected erythrocyte surface antigen (RESA) (Petersen *et al*., 1990), merozoite surface protein 1 (Riley *et al*., 1992), merozoite surface protein 2 (Taylor *et al*., 1998). While enviromental factors, such as exposure may play an important role in shaping the immunity, other factors, for example host genetics, appear to control antibody and cell mediated immune responses to malaria infection (Sjoberg *et al*., 1992; Jepson *et al*., 1997; Aucan *et al*., 2001). Therefore there is considerable interest in identifying factors that are responsible for the regulation of the amount of protective antibodies produced.

In this study, we investigated the isotypic distribution of malaria specific serum antibodies to crude *P. falciparum* antigens in ethnic groups with similar or different genetic background, exposed to different parasite inoculation; the Fulani and Mossi in Burkina Faso and the Fulani and Dogon in Mali.

Previous studies compared the humoral immune responses to the two malarial antigens Pf332 and Pf155/RESA in three ethnic groups living in sympatry in Burkina Faso, the Fulani, Mossi and Rimaibé (Modiano *et al*., 1998). Fulani individuals were shown to be less susceptible to malaria infection and have the ability to mount markedly stronger antibody immune responses to malaria infection compared to the other ethnic groups, suggesting that the immune responses are at least in part genetically regulated (Luoni *et al*., 2001).

In the present study, the levels of antibodies reactive with *P. falciparum* asexual blood stage antigens were determined with regard to IgG and its subclasses in Fulani individuals living in Mali and Burkina Faso and were compared to levels of antibodies in neighbouring other ethnic tribes, the Dogon and Mossi, respectively.

Although the Fulani of Mali live under low transmission in a mesoendemic setting, the levels of parasite specific IgG, IgG1 and IgG3 antibodies were similar to those detected in Burkinabe Fulani, who are living in a high transmission setting. The levels of these antibodies were significantly higher in Fulani individuals of both countries than those detected in sympatrically living Mossi (Burkina Faso) and Dogon (Mali) individuals. Only low levels of parasite specific IgG2 and IgG4 were detected in the study populations and no significant differences were seen between the different tribes. The presence of significantly higher levels of the cytophilic antibodies, IgG1 and IgG3, in plasma samples from Fulani individuals, suggests that these antibodies may contribute to the lower susceptibility of this tribe for clinical malaria. Anti-malarial IgG1 and IgG3 antibodies are thought to be involved in parasite neutralization *in vivo*

by interacting with human monocytes to induce phagocytosis (Groux and Gysin, 1990) and cell mediated inhibition of parasite growth (Bouharoun-Tayoun, 1990).

In order to get an indication if serological differences seen between different tribes are specific for malaria or a more general feature of their humoral immune responses, the levels of IgG antibodies to a measles antigen were measured in plasma samples from Fulani, Mossi and Dogon individuals. Interestingly, the levels of measles specific IgG levels were significantly higher in samples from Fulani individuals of both countries compared to those in samples from Mossi and Dogon individuals. These results suggest that, host genetic factors may play a crucial role in determining general levels of antibodies. Whether the higher antibody levels in Fulani individuals is correlated with a lower susceptibility to other infections than malaria remains to be investigated.

Previous efforts aiming at linking genetic factors with higher antibody responses and with lower susceptibility to malaria infection did not give clear-cut results and just postulated the involvement of unknown immunological genetic factors (Riley *et al*., 1992; Modiano *et al*., 1998; Modiano *et al*., 1998; Modiano *et al*., 1998; Modiano *et al*., 1999). However, recent evidence suggests the presence of a locus on human chromosome 5q31-q33, which influences the intensity of infection, indicating that resistance/susceptibility genes in this region may influence the outcome of different immune responses (Luoni *et al*., 2001). The chromosome 5q31-q33 region contains numerous candidate genes encoding immunological molecules such as cytokines, growth factors, and growth-factor receptors (Chandrasekharappa *et al*., 1990) involved in the control of immunity to *P. falciparum* blood stages (Troye-Blomberg *et al*., 1994).

CONCLUDING REMARKS

This thesis describes several aspects of humoral immune responses to *P. falciparum* infection in individuals of different age groups and with different degrees of exposure. Antibodies from the study population were tested for their parasite inhibitory activity using *in vitro* invasion inhibition assay. The parasite neutralizing capacity of these antibodies was also assayed in conjunction with normal human monocytes. Plasma samples collected from asymptomatic children belonging to Mossi tribe living in Burkina Faso, where the transmission is hyperendemic, and from asymptomatic individuals belonging to two distinct tribes living Mali, where the transmission is mesoendemic, were screened for their content of parasite specific IgG class and subclass. Using the same method, the impact of impregnated curtains on the immunological status of children living all their lives in villages with ITC.

Target antigens of antibodies functional in ADCI remain to be defined. One of these antigens is the *P. falciparum* 332 antigen exposed on the surface of the late stage infected erythrocytes. We show in this thesis, Pf332 reactive antibodies are efficient in mediating neutralization of the parasite when tested alone or in co-operation with human monocytes. A part of the inhibition was due to phagocytosis of late stage infected erythrocytes. This study emphasizes the potential interest of Pf332-derived sequences for inclusion in a subunit vaccine against *P. falciparum* malaria. Although Pf332 is the first *P. falciparum* antigen expressed on the surface of infected erythrocytes to be identified as a target for ADCI, other antigens exposed on the surface of infected erythrocytes such as PfEMP1 and RIFINs also may constitute possible targets for opsonizing antibodies and are considered to be molecules of interest for vaccine development as they are involved in cytoadherence and resetting.

Previous studies have shown that freshly isolated *P. falciparum* parasites isolated from children living in Burkina Faso were less sensitive to growth inhibition mediated by autologous Ig as compared with heterologous Ig fractions. These may either be due to downregulation of the synthesis or expression of the target antigens by antibody pressure *in vivo* or, that the antibody pressure selects for parasites with low expression of a specific antigen from a heterogeneous parasite population. Alternatively, lower sensitivity of parasites isolates to autologous Ig may be due to a recent infection with parasites not previously seen by the immune system of an individual. Analyses of two consecutive isolates taken 14 days apart, with regard to genotypes and sensitivity to growth inhibition *in vitro*, indicates that the parasite possesses as yet undefined mechanisms to evade neutralizing immune responses. However, our unpublished data (Bolad *et al.*, unpublished) suggest that the lower sensitivity to autologous Ig was due to the presence of a recent infection with isolates not previously encountered by the immune system of the donor. In concordance with our previous study, the results reinforce the concern about *Plasmodium* antigenic diversity as a major obstacle towards the development of an effective malaria vaccine.

Efficacy trials have proved that pyrethroid impregnated bed nets and curtain are effective in reducing morbidity and mortality from malaria. However, it has also been argued that the use of impregnated nets might delay the acquisition of immunity in children and even may lead to loss of already acquired immunity. We show in this thesis that while the use of the ITNs in Burkina Faso, where the transmission is high, resulted in a significant reduction in the levels of infection were not significantly affected the levels of *P. falciparum* specific antibodies or the multiplicity of infection. It is hypothesised that the additional impact of ITNs by reducing exposure may be greatest where the intensity of transmission is low. Thus, it would be interesting to carry out

further studies to identify the effectiveness of intervention strategies in areas characterised by low and unstable transmission.

Analysis of antibody immune responses in different ethnic groups living with different endemic settings revealed apparent heterogeneity in immune responses to asexual *P. falciparum* antigens. Although the Fulani of Mali and the Fulani of Burkina Faso live under two distinct epidemiological settings, the levels of antibodies to *P. falciparum* asexual blood stage antigens and to a measles antigen did not differ significantly between the two groups. The Fulani of both groups showed significantly higher levels of cytophilic antibodies than the other ethnic groups living under the same epidemiological settings. However, further analyses using a panel of other pathogens are needed for exploring the basis for the lower susceptibility of Fulani individuals to malaria.

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