

**Investigation into *Plasmodium falciparum*
multiplication rates, selectivity and invasion
pathways in relation to malaria severity**

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Abstract

Plasmodium falciparum, a protozoan parasite, causes more than 500 million clinical cases of malaria every year. It is a disease that is on the increase due to factors such as drug resistance and political instability in affected regions. Although differences in virulence between *P. falciparum* strains were described more than sixty years ago, how parasite virulence factors may contribute to the development of severe malaria has been largely neglected in malaria research and is not well understood. In this thesis I describe my work on potential *Plasmodium falciparum* virulence factors related to parasite invasion and multiplication during the asexual blood stage of infection.

Parasite multiplication rates and red blood cell (RBC) selectivity of Thai field isolates have previously been shown to be associated with severe malaria. In contrast, I found that clinical isolates from Malian and Kenyan children diagnosed with uncomplicated or severe malaria did not differ in their multiplication rates or RBC selectivity. Furthermore I show an unexpected finding that isolates from hyperparasitaemic children had significantly lower multiplication rates than isolates from children with uncomplicated malaria. These results raise the possibility of differences in malaria virulence factors between sub-Saharan Africa and Asia.

I investigated RBC selectivity as a potential virulence factor further in a rodent malaria model using *Plasmodium chabaudi*. The experimental design allowed me to look at the selectivity index in congenic lines, which significantly differed in their virulence to the host. Supporting my *P. falciparum* data, no association between the selectivity index and virulence was seen. This is the first description of the selectivity index in *P. chabaudi* and RBC invasion was found to be very unselective as expected for a malaria parasite which does not exhibit a reticulocyte preference.

The Kenyan field isolates were also typed for their RBC invasion pathway profile by measuring their invasion into enzyme-treated RBC. Although invasion profiles of

field isolates have been reported previously, as far as I am aware, this is the first report looking at invasion profiles of field isolates in relation to disease severity. In line with previous results, field isolates invade RBC by multiple invasion pathways. No difference between the invasion profiles of isolates from uncomplicated or severe malaria patients was seen.

Finally, I looked at rosetting, another potential virulence factor, in laboratory strains to examine its possible role in enhancing parasite invasion and evading host immunity. I found that isogenic rosetting and non-rosetting parasites were inhibited to the same degree by invasion-inhibitory antibodies, indicating that rosetting does not enhance invasion in the presence of host immunity.

Overall my thesis presents a detailed study of potential *P. falciparum* virulence factors related to invasion, working both with clinical isolates and laboratory strains. Severe malaria in Africa was not found to be associated with parasite multiplication rates, RBC selectivity or specific invasion pathways. Other virulence factors such as rosetting and platelet-mediated clumping may be of major importance in this region. This work suggests the possibility of differences in malaria virulence factors between sub-Saharan Africa and Asia, which may need to be taken into consideration for drug and vaccine design.

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Finally, Paul, thank you for all your love and patience and for keeping a smile on my face!

Abbreviations

ANOVA	analysis of variance
CR1	complement receptor 1
cRPMI	complete RPMI
CT	chymotrypsin
DBL	Duffy-like binding domain
EBP	erythrocyte binding protein
GPA	Glycophorin A
GPB	Glycophorin B
GPC	Glycophorin C
Hb	haemoglobin
HEPES	4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid
Ht	haematocrit
IgM	immunoglobulin M
incRPMI	incomplete RPMI
IQR	interquartile range
iRBC	infected red blood cell
Nm	neuraminidase
PCR	polymerase chain reaction
Pcv	packed cell volume
PMR	parasite multiplication rate
PNA	peanut (<i>Arachis hypogaea</i>) lectin
Pt	parasitaemia
R	resistant
RBC	red blood cell
RF	rosette frequency
S	sensitive
SI	selectivity index
SRC	spearman rank correlation

Stdev	standard deviation
STI	soybean trypsin inhibitor
T	trypsin
VSA	variant surface antigen

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CHAPTER 1: Introduction

1.1 Severe malaria

1.1.1 Malaria

In over 100 years of malaria research, despite major advancements in our understanding and treatment of the disease, parasite drug resistance and political instability have led to a recent increase in malaria cases (World Health Organization 2003). There are an estimated 500 million clinical cases annually (Snow *et al.* 2005) and all efforts are needed to further enhance our current knowledge about the causes, pathology and treatment of this disease to prevent a further increase of cases and discover new strategies to combat it.

1.1.1.1 World distribution

Although in the distant past malaria occurred throughout Europe, malaria has been eradicated from large parts of the world in a major effort in the 20th Century and now is predominant throughout the tropical regions of the world (figure 1).

Figure 1:

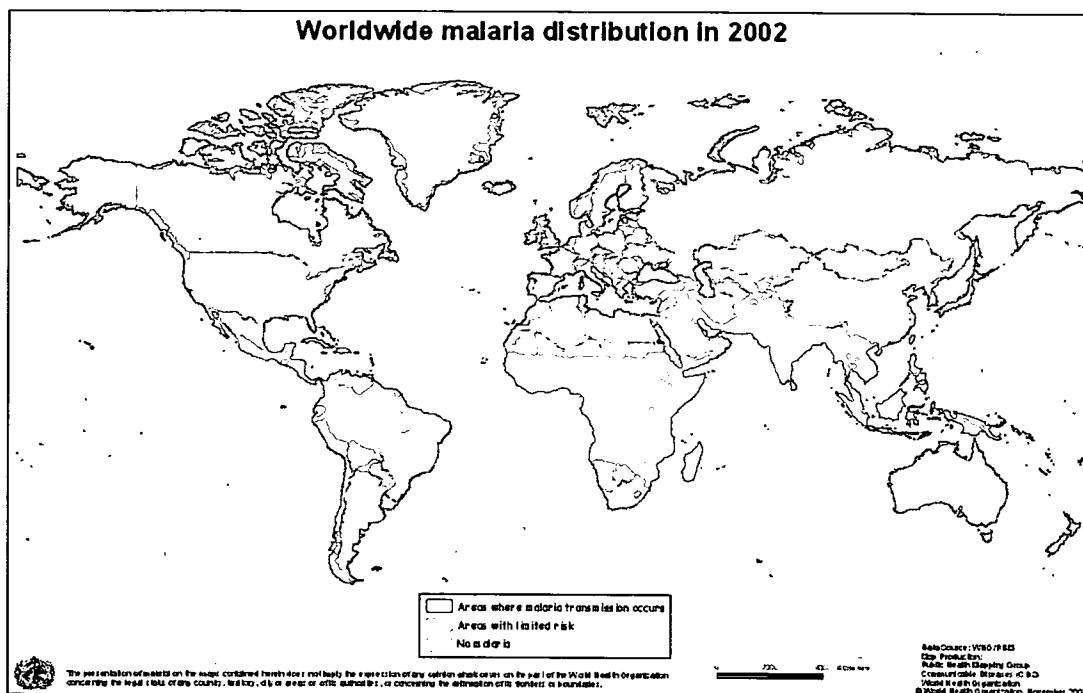


Figure 1: Malaria distribution in 2002.

(From: <http://globalatlas.who.int/globalatlas/InteractiveMapping>).

1.1.2 Malaria – The parasite and disease

1.1.2.1 *Plasmodium*

Malaria is caused by a parasite of the genus *Plasmodium*, which belongs to the Apicomplexa phylum, as characterised by their apical complex. The apical complex typically contains electron dense polar rings, two or more rhoptries and also micronemes. Four of the *Plasmodia* species infect humans: *P. vivax*, *falciparum*, *ovale* and *malariae*. But it is known that other primate species may infect humans and have done so in the past – for example *P. knowlesi* (Singh *et al.* 2004). Although *P. falciparum* is the commonest species in the tropics and causes most of the malaria morbidity and mortality, *P. vivax* is an important infection in subtropical regions and can have a considerable impact on the economy (Harinasuta and Bunnag 1988). Although not the subject of this thesis, it is interesting that *P. falciparum* has evolved

to be so virulent to the human host, in contrast to the other three species. Virulence throughout this thesis is defined as the damage done to the host. It is separate from studies defining virulence as high growth rates in rodent malaria models (Walliker *et al.* 1976).

1.1.2.2 *Plasmodium falciparum* – life cycle

The *P. falciparum* life cycle has both an asexual haploid stage in the human host and a sexual replication stage in the mosquito vector (figure 2). *P. falciparum* is transmitted by female mosquitoes of the genus *Anopheles*. The life cycle in a new human host starts with sporozoites being injected from the salivary glands of the vector, the mosquito (figure 2). The sporozoites travel to the liver and infect hepatocytes, where they will develop into schizonts. After 5-6 days an estimated 30,000 merozoites are released from each schizont as a result of asexual fission. After being released from the liver the merozoites invade erythrocytes. Intraerythrocytic development takes approximately 48 hours, during which the ring stage parasites mature to pigmented trophozoites and schizonts.

The mature stages (pigmented trophozoites and schizonts) are rarely seen in the peripheral circulation as they express variant surface antigens, which mediate adherence of the parasite infected red blood cell (iRBC) to endothelial cells (Sherman *et al.* 1993). This is known as cytoadherence and to date a number of different forms of cytoadherence of *P. falciparum* iRBC have been described: the binding to endothelial cells, the binding to uRBC (known as rosetting), the binding of iRBC to other iRBC (known as autoagglutination) and finally, the binding to chondroitin sulfate A in the placenta (Newbold *et al.* 1999). These different phenotypes, in particular binding to endothelial cells, leads to sequestration in blood vessels and organs, and prevents clearance by the spleen (Barnwell *et al.* 1983). Cytoadherence in small blood vessels of the brain, placenta and other major organs is thought to be a major factor involved in severe disease (Beeson and Brown 2002).

At schizogony the merozoites are released, which reinvade new RBC to complete the asexual cycle. Ring stage parasites can alternatively develop into male or female gametocytes. These are ingested by the mosquito vector with the blood meal. It is in the vector that the sexual cycle of *P. falciparum* occurs. In the midgut of the mosquito the gametocytes develop into gametes, which fuse to form a zygote. These elongate into motile ookinetes, which then migrate through the midgut wall and round up as oocysts. The oocyst divides and matures over 7 to 15 days to finally produce sporozoites that migrate to the salivary glands, ready to complete the *P. falciparum* life cycle (Garnham 1988).

Figure 2:

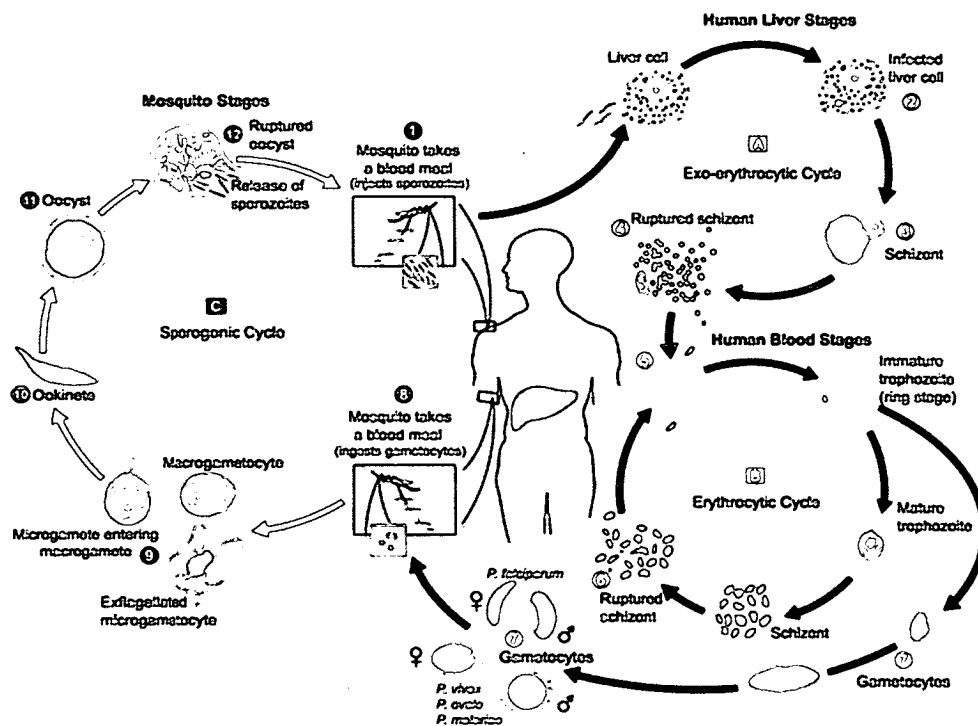


Figure 2: Overview of the malaria life cycle. Blue arrows indicate the stages in the human host and red arrows the stages of development in the mosquito host. The erythrocytic cycle is shown in B. Reproduced from Centres for Disease Control and Prevention (http://www.cdc.gov/malaria/biology/life_cycle.htm).

1.1.2.3 Malaria – the disease

The clinical features in malaria result from the asexual blood stage of the infection. Most malaria infections cause fever, malaise, headache and lethargy, they are however not lethal. But in some cases severe disease develops with the main clinical features being unrousable coma, anaemia, respiratory distress and organ failure (Marsh and Snow 1997). Respiratory distress, caused by metabolic acidosis, alone or in connection with other symptoms has a particularly high fatality rate of around 25% (Marsh and Snow 1997). To date no approved vaccines exist to protect from malaria and the spread of parasites resistant to anti-malarial drugs makes the treatment difficult (Walliker 2000).

In areas of high transmission severe malaria is a feature seen mainly in young children, as increased exposure enhances immunity and so protects older children and adults from severe malaria and maternal antibodies protect infants (Gupta *et al.* 1999b). However, immunity to *P. falciparum* is not known as sterile immunity as parasites are still frequently found in the blood of adults living in malaria endemic areas. Interestingly, the age at which different severe malaria pathologies occur varies, for example in Kenya the average age at which children present at hospital with severe anaemia is 22 months and for cerebral malaria is 40 months (Marsh 1992; Marsh and Snow 1997). It is not understood why only a small proportion, that is around 2%, of *P. falciparum* infections result in severe malaria, and what the factors that may cause severe malaria are (Greenwood *et al.* 2005) (see below).

In lower transmission areas, such as Southeast Asia, severe malaria is as likely to occur in adults as it will in children (Luxemburger *et al.* 1997) as the population does not build up a protective level of immunity. It is important to emphasise that severe malaria in adults much more frequently has different manifestations of the disease with renal failure being an often observed complication (Waller *et al.* 1995; Luxemburger *et al.* 1997; Nacher *et al.* 2001). Cerebral malaria also occurs in adults with malaria and has a very high mortality rate of around 22% (White 1987).

1.1.3 Factors affecting severe malaria

1.1.3.1 Overview

As discussed earlier on, severe clinical malaria occurs in only around 2% of infected individuals (Greenwood *et al.* 2005). To prevent severe malaria cases occurring it is necessary to understand the factors that may predispose an individual to develop severe malaria. The contribution of the different factors is currently unknown and is likely to vary with different locations and environments. The factors can be grouped into three categories: host factors, parasite factors and geographic and social factors (figure 3).

Figure 3:

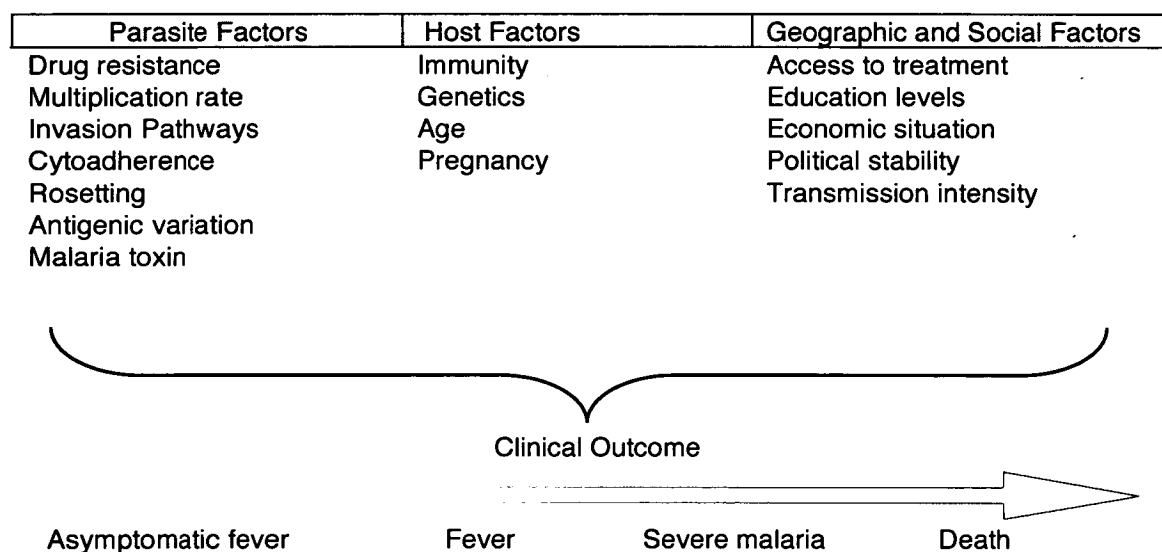


Figure 3: Many factors may lead to severe malaria as the outcome of an infection with *P. falciparum*. Factors are grouped as parasite, host and geographic and social factors. Adapted from (Miller *et al.* 2002).

1.1.3.2 Non-parasite factors

The geographic and social and host factors encompass factors such as the availability of health care, maternal education levels, economic factors and political stability (Marsh 1992; Miller *et al.* 2002; Greenwood *et al.* 2005). Unfortunately, political instability has been named as one of the main reasons for the deterioration of malaria control (Greenwood *et al.* 2005). Host factors that have been associated with the outcome of a *P. falciparum* infection are age, previous exposure to the parasite and host genetics. Host genetics plays a major role and accounts for around 25% of total variation in the susceptibility of individuals to malaria disease in Kenya (Mackinnon *et al.* 2005b). Malaria resistance genes, such as the sickle cell anaemia gene, have reached higher frequencies in malaria endemic areas, where they provide protection from severe malaria (Haldane 1949). Interestingly, the sickle cell gene and the alpha-thalassaemia polymorphisms, which are among the best known blood polymorphisms to confer protection from severe malaria, were found to only explain 2.5% and 2% of the protection from severe malaria, respectively (Mackinnon *et al.* 2005b). This indicates the complexity of the relationship between host genetics and resistance to malaria.

1.1.3.3 Parasite virulence factors

In general parasite biology, parasite virulence has been described by theories looking at the trade-off between parasite virulence and host fitness. It is assumed that a parasite virulence factor will enhance the survival and/ or transmission of the parasite. The trade-off means that a virulence factor, though advantageous to the parasite, by definition will decrease host fitness or in other words increase pathogenesis (Anderson and May 1982).

Differences in virulence between *Plasmodium* parasite strains have been shown in the past. The strongest evidence has been provided from rodent malaria models. It has been shown that different *P. chabaudi* strains differ in virulence and virulence can be selected for by passaging isolates through immune mice (Mackinnon and

Read 1999). Furthermore, more virulent *P. chabaudi* strains out compete the less-virulent strains and show enhanced transmission to the mosquito vector (Mackinnon and Read 2004a; de Roode *et al.* 2005a; de Roode *et al.* 2005b). There is also good evidence for differences in virulence of *P. falciparum* strains. Nearly 80 years ago, differences in virulence between *P. falciparum* strains were described: In the past, neurosyphilis was treated with fevers induced by carefully monitored malaria infections and it was found that “various geographical races [of *P. falciparum*] can be recognised as being distinct by their clinical virulence, immunological reactions and other biological properties” (James *et al.* 1932).

Similarly, more recent work investigated the agglutination phenotypes of *P. falciparum* field isolates from children with severe or uncomplicated malaria. The aim was to further unravel whether isolates causing severe malaria express rare or commonly recognised surface antigens. In detailed studies the agglutination responses to variant surface antigens (VSAs) expressed on the surface of *P. falciparum* iRBC was examined. Parasite isolates taken from children of various ages and clinical manifestations were tested for agglutination by sera from the child the parasite isolate was collected from (homologous) and sera from a different individual (heterologous). It was found that the VSA expressed by isolates in children with severe malaria are commonly recognised by heterologous sera (Bull *et al.* 1998; Bull *et al.* 1999; Bull *et al.* 2000). Similar results were found by another group, again reporting the association of a high VSA recognition of parasite isolates with severe malaria (Nielson *et al.* 2002). This does not seem to be due to affected children being immuno-compromised with low antibody titres, as sera from children with severe malaria have similar antibody levels as children protected from severe malaria (Dodoo *et al.* 1999) and will agglutinate parasites isolated from other children (Bull *et al.* 1998). Therefore, it has been suggested that children who develop severe malaria have a ‘gap’ in their acquired VSA antibody repertoire (Bull *et al.* 1998) or lack the ability to maintain a broad profile of anti-VSA antibodies (Tebo *et al.* 2002). Independently of the disease status the occurrence of common isolates is inversely associated with age (Nielson *et al.* 2002), indicating that acquired-immunity may select for parasite isolates expressing rare VSA not yet seen

by the immune system. So acquired immunity may select against a commonly recognised, more virulent *P. falciparum* phenotype. This is supported by the fact that immunity may be gained after as little as one or two infections with *P. falciparum* (Gupta *et al.* 1999a). Further analysis of the age distribution of cerebral malaria led to the most plausible explanation being that a distinct antigenic type of *P. falciparum*, which is common, causes cerebral malaria and that these antigenic types induce strong strain-specific immunity and postnatal protection provides a relatively long period of protection (Gupta *et al.* 1999a). A possible VSA that is predicted to have a high switching rate during the initial stage of infection is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Gatton *et al.* 2003) (Roberts *et al.* 1992). So most children may encounter the common virulent strains or commonly expressed VSA during the time where they still have postnatal protection and can build up immunity towards them. The few children that are infected later will consequently develop severe malaria. These commonly recognised virulent strains may explain why parasitisation in non-immune travellers generally leads to disease and has a higher case-fatality rate (Marsh 1992) (Muhlberger *et al.* 2003).

So the question is what makes the commonly recognised virulent strains so virulent? The clinical manifestations associated with human malaria are mainly due to the erythrocyte stage of the *P. falciparum* life cycle. Therefore, parasite virulence factors, associated with intraerythrocytic development are of particular interest. Potential *P. falciparum* virulence factors are drug resistance, cytoadherence, immune-suppression, autoagglutination, growth as defined by the multiplication rate and RBC selectivity of isolates, invasion pathways used by the merozoite to enter the RBC and finally rosetting (Marsh 1992; Miller *et al.* 2002). I will talk about the latter three virulence factors in more detail below as they were investigated in this study. The other factors, cytoadherence, immune suppression and autoagglutination will not be discussed here, but are the current focus of research by other groups (see (Urban *et al.* 1999; Roberts *et al.* 2000; Pain *et al.* 2001; Sherman *et al.* 2003)).

1.2 *P. falciparum* multiplication rate and selectivity index

1.2.1 Parasite multiplication rate

Parasite multiplication rate during the asexual cycle, as a measure of how many RBC are newly infected when a schizont bursts, is an intriguing subject. Generally it is assumed that 8-32 merozoites are released at schizogony (Garnham 1988). The multiplication rate indicates how many of these re-invade. Although a parasite multiplication rate (PMR) of 5-fold is often quoted for laboratory strains, how many merozoites reinvade *in vivo* is not well understood and experimental studies *in vivo* are difficult to design as *P. falciparum* infections require treatment to prevent the development of severe malaria.

There are a few available estimates of parasite multiplication rates *in vivo*. One source has been derived from the meticulously collected data generated during neurosyphilis treatment programs early in the last century, where fevers induced by malaria parasites were used to cure neurosyphilis as described above. All Giemsa counts were regularly collected and carefully monitored, allowing a retrospective analysis of the parasite growth rates. From the available data the multiplication rate has been estimated to be around 9-fold during the expansion phase and interestingly to abruptly be reduced to a value of 1-fold or less by an unknown mechanism (Simpson *et al.* 2002). Unfortunately, no further references or information was given in regards to this postulated decrease in the parasite multiplication rate and the proposed control mechanism (Simpson *et al.* 2002). Fever has been shown to effect density-dependent regulation of parasite growth in a non-immune host (Gravenor and Kwiatkowski 1998). Additionally, in this retrospective analysis it was shown that the PMR varies across parasite strain and patient, even when patients are infected with the same parasite strain (Simpson *et al.* 2002). Vaccine studies have designed PCR based methods to investigate the early growth phase of the parasite in the volunteer, before any symptoms arise. These experiments are generally carried out with Caucasian volunteers being infected with a laboratory strain, such as 3D7.

The measured multiplication rates have been estimated to be around 12-15-fold (Cheng *et al.* 1997; Lawrence *et al.* 2000; Bejon *et al.* 2005), but the peak parasitaemias in these early stages of the infection are very low (10 parasites per microL)(Lawrence 2000). Furthermore, there does seem to be considerable variation in the early growth rates in naïve individuals, ranging from around 2-fold to 30-fold (Bejon *et al.* 2005). It is important to note that these studies were carried out in malaria naïve individuals and none of the above studies characterised how the multiplication rate may change after the early expansion phase. A different approach that has been taken to study the parasite multiplication rate is to look *in vitro* at the first cycle of invasion of field isolates. This has been carried out in Thailand.

In vitro multiplication rates of parasite isolates from Thai patients, diagnosed with uncomplicated malaria, multiplied at a mean invasion rate of 2.8 (Chotivanich *et al.* 2000). However, in the same study, parasite isolates from patients with severe malaria had a multiplication rate of around 8-fold. This was significantly higher than the group of isolates from patients with uncomplicated malaria (Figure 4) and provided evidence that more virulent isolates with faster growth rates may cause severe malaria. There is supporting data from rodent models, where *P. chabaudi* growth rates have been associated with the level of virulence caused to the host (Mackinnon and Read 1999; Mackinnon and Read 2003, 2004b). Whether severe malaria in children in Africa is also in part caused by *P. falciparum* isolates with high parasite multiplication rates is unknown.

Figure 4:

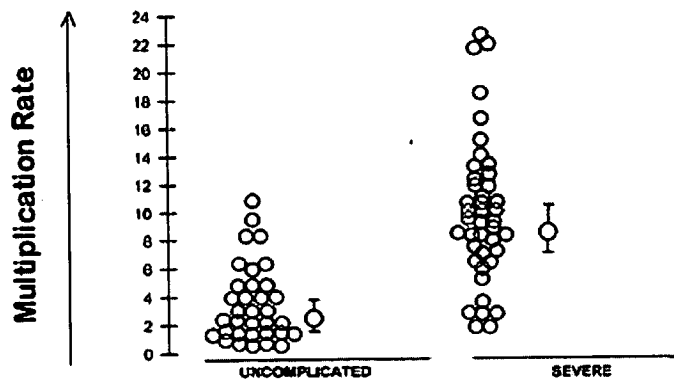


Figure 4: Multiplication rate of Thai field isolates from patients diagnosed with uncomplicated (left hand) or severe (right hand) malaria. The median multiplication rates are 2.8 and 8.3 respectively. Adapted from (Chotivanich *et al.* 2000).

1.2.2 RBC selectivity

Further insight into parasite multiplication *in vivo* can be gained by understanding whether red blood cell (RBC) invasion is a random, unrestricted process, or whether some selection occurs, with only a subset of RBC being accessible for invasion. A preference to invade reticulocytes by a number of different *Plasmodium* species is well documented, for example *P. berghei* and *P. vivax* merozoites are known to have a large preference to invade reticulocytes (Ott 1968; Mons 1990). A careful study looking at the selectivity index (SI) of a range of different *Plasmodia* species confirmed these preferences and gave a good overview of the RBC selectivity of different *Plasmodia* species (Simpson *et al.* 1999). The SI is a measure of whether merozoites can invade all RBC randomly (figure 5). It is defined as the number of observed multiple-infected RBC in relation to the number expected by chance, as calculated with a Poisson distribution. A SI of 1 indicates random invasion. A SI, which is larger than 1, indicates that more multiple-infected RBC are seen than expected and that invasion is not random. Non-random invasion may be explained by

factors such as parasite receptor preference, age-related changes in host RBC or host merozoite-agglutinating antibodies.

Figure 5:

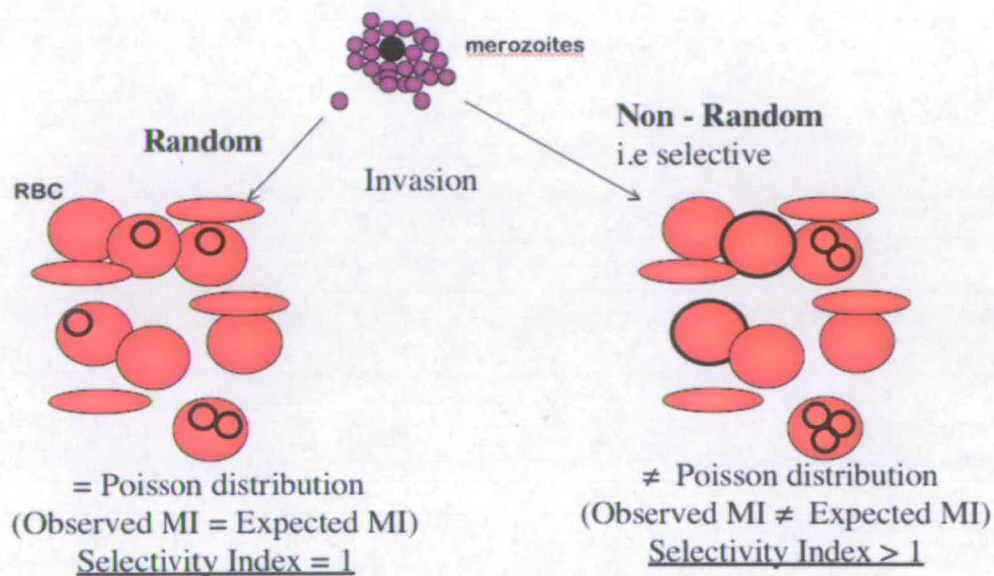


Figure 5: The selectivity index (SI) is a measure of merozoite invasion. If invasion is a random process (left side), then the number of multiple-infected RBC equals the expected number and the $SI=1$. If invasion is a non-random process (right side) and some of the RBC cannot be invaded (indicated by the thick black RBC membranes), then one will observe more multiple-infected RBC than expected and the $SI>1$.

Importantly it was found that Thai *P. falciparum* isolates from patients with severe malaria were less selective than isolates from patients with uncomplicated malaria (Simpson *et al.* 1999). This correlated well with the PMR data from Thailand (Chotivanich *et al.* 2000). One may speculate that parasites that can invade a larger population of the RBC, that is they are less selective, and multiply at a higher rate, cause severe malaria. As far as I am aware neither the PMR nor the SI has been studied for field isolates from patients with severe malaria in Africa, where malaria is often highly endemic and mainly children are diagnosed with severe malaria.

1.3 *P. falciparum* invasion pathways

1.3.1 Basic description of invasion

Following from the question as to what proportion of RBC are invaded, as measured with the SI, it is also necessary to understand how merozoites invade erythrocytes. From video microscopy studies it is known that merozoites go through the stages of reversible attachment to a RBC, reorientation so that the apical end of the merozoite is in contact with the erythrocyte surface, tight junction formation and finally internalisation (figure 6).

Figure 6:

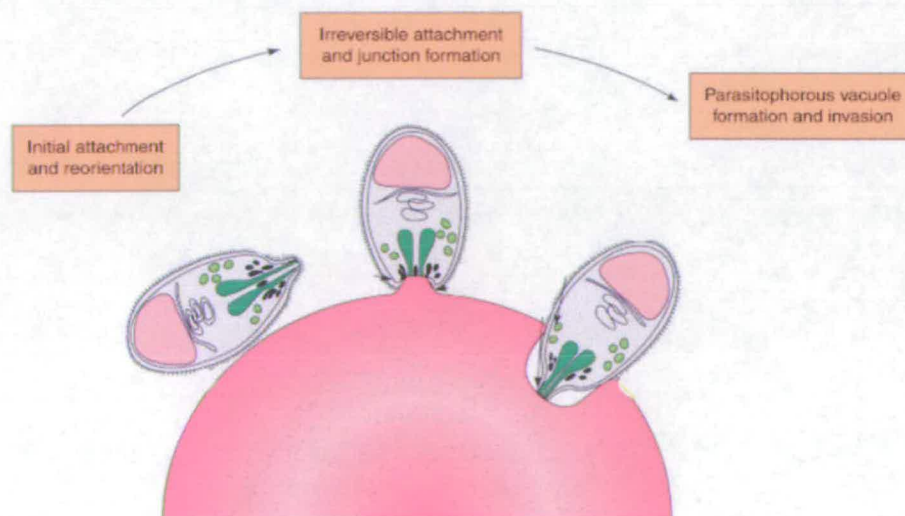


Figure 6: Merozoite invasion into the RBC comprises a number of steps. Adapted from (Chitnis and Blackman 2000).

In contrast to *P. vivax*, which is restricted to erythrocytes expressing the Duffy blood group antigen and to reticulocytes, multiple pathways have been described for invasion by *P. falciparum* merozoites. In the following paragraphs I will first of all give a description of the parasite proteins that have been postulated to be involved in merozoite invasion, then move on to describe potential RBC receptors and finally group the pathways by their enzyme-sensitivity profile.

1.3.2 *P. falciparum* merozoite antigens

1.3.2.1 The EBP protein family

Identified originally in *P. vivax*, there is now a well-defined family of genes, known as the erythrocyte binding protein (EBP) family (also known as the ebl family or the DBL-EBL family). The EBP family encodes proteins involved in specific recognition of host cell receptors and invasion (figure 7).

Figure 7:

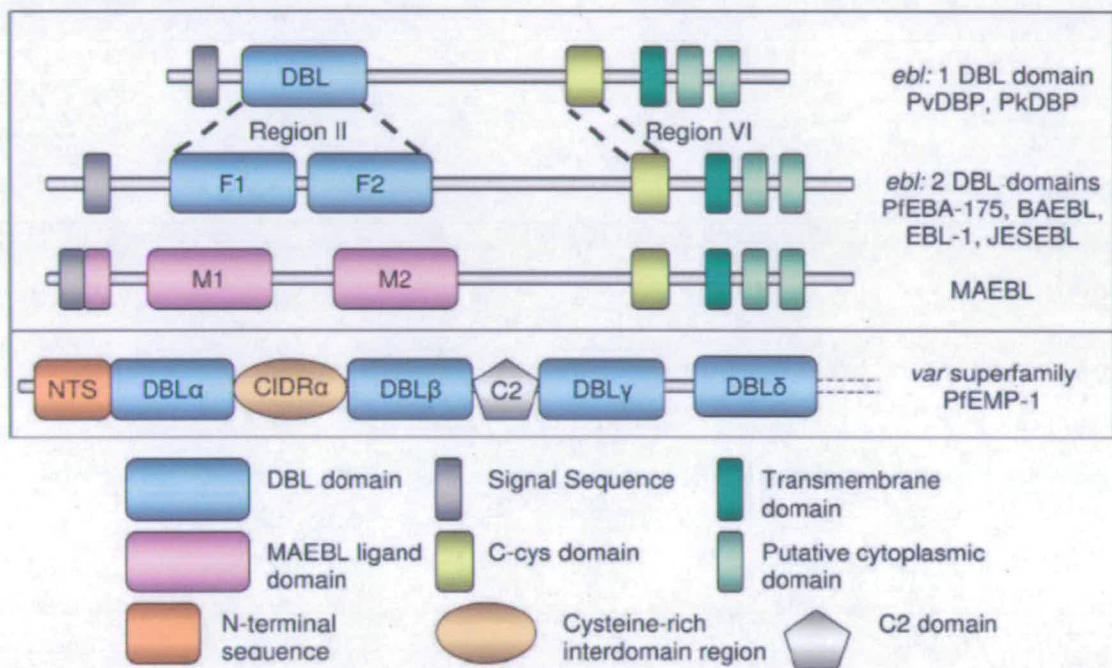


Figure 7: Protein structure of the EBL protein family. Reproduced from (Tolia *et al.* 2005).

The genes are usually single-copy and have a multi-exon structure. The proteins generally have a tandem copy of the DBL domain in *P. falciparum* although the *P. vivax* protein only has one copy and the *P. falciparum* antigen MAEBL also forms an exception with a unique domain, which is more similar to AMA1 (Blair *et al.* 2002).

The EBP gene family, because of the characteristic DBL domain, falls within the larger DBL superfamily. Another well-known DBL family are the *var* genes encoding PfEMP1 (*P. falciparum* erythrocyte protein 1), which mediates antigenic variation and cytoadhesion (discussed below under 1.4 Rosetting). The EBP family is distinct from the *var* genes as the exon structure differs; furthermore EBP proteins lack the integral membrane domain and the acid cytoplasmic tail (Peterson and Wellemms 2000).

As described the EBP family consists of several family members, which have largely been identified through the genome project (figure 7). EBA-175, the name originating as it is a 175 kDa erythrocyte binding antigen, was the first EBP protein identified in *P. falciparum* (Camus and Hadley 1985). EBA-175 binds sialic acids and the backbone of glycophorin A for invasion (Sim *et al.* 1994). Recently, the binding domain has been found to form dimers in an antiparallel fashion. Dimerisation may occur when two EBA-175 domains assemble around glycophorin A, which exists as a dimer on the erythrocyte surface (Tolia *et al.* 2005). PEBL (also known as EBA-165) was identified in the genome due to its close homology to EBA-175, but is thought to be a transcribed pseudogene (Triglia *et al.* 2001). EBL-1 is also similar to EBA-175, yet little is known about its possible role in RBC invasion (Peterson and Wellemms 2000). BAEBL (also known as EBA-140 or EBP2) is another EBP family protein identified through its homology. Interestingly different RBC binding specificities were initially reported for BAEBL (Mayer *et al.* 2002; Narum *et al.* 2002), and it was found that there are four polymorphisms that lead to different RBC binding specificities (Mayer *et al.* 2002). Another EBP family protein, JESEBL (also known as EBA-181), has also been described to show different RBC binding and perhaps receptor specificity due to allelic polymorphism (Mayer *et al.* 2004). JESEBL is not essential for invasion and can be disrupted (Gilberger *et al.* 2003).

1.3.2.2 The RBL protein family

Two proteins mediate recognition of reticulocytes by *P. vivax* the reticulocyte binding protein 1 and 2 (PvRBP-1 and PvRBP-2). Orthologues to both of these

proteins have been identified in *P. falciparum*. This family forms a separate family from the EBP proteins and similarly has several family members. So far five proteins have been identified, of which one PfRh3 (*P. falciparum* reticulocyte binding protein homolog 3) is thought to be a pseudogene as no translated product has been found in asexual stage parasites (Taylor *et al.* 2001). PfRh2a and b, orthologues to PvRBP-2, were described first in 2000 (Rayner *et al.* 2000). Two more family members PfRh1 and PfRh4 have also been identified (Taylor *et al.* 2002). Since then several studies have shown that different levels of these proteins may mediate different receptor specificities (Taylor *et al.* 2002; Duraisingh *et al.* 2003; Stubbs *et al.* 2005). Furthermore, although these genes were all thought to be single copy genes, it has been found that there are PfRh1 gene amplifications in laboratory strains and that this may affect the invasion profile (Triglia *et al.* 2005). Most recently it has been described that PfRh4 activation may be involved in a pathway switch to sialic acid-independent pathways (Stubbs *et al.* 2005). In this study, W2mef parasites either selected to grow on neuraminidase treated RBC or where EBA-175 was disrupted had increased expression levels of PfRh4 and the pseudogene PEBL, which are located in a head-to-head orientation on chromosome 4 (Stubbs *et al.* 2005). Furthermore, as it was found that disrupting EBA-175 led to the expression of PfRh4 and sialic acid –independent invasion, this indicated some overlap of the PfRh and EBP protein family as independently of each other they can mediate parasite invasion, albeit by different invasion pathways (Stubbs *et al.* 2005).

1.3.2.3 MSP1

Another family of proteins are the merozoite surface proteins (MSPs), of which only MSP1, the best-characterised protein, will be discussed here (details to proteins MSP2 to MSP9 in: Tonhosolo *et al.* 2001; Mills *et al.* 2002; Oeuvray *et al.* 1994; Marshall *et al.* 1997; Marshall *et al.* 1998; Trucco *et al.* 2001; Black *et al.* 2001; Vargas-Serrato *et al.* 2002). MSP1 is highly abundant on the surface of merozoites (Blackman *et al.* 1991a; Blackman *et al.* 1991b). Although the exact function of MSP1 is unknown, there is considerable evidence for its role in invasion. Knockouts of MSP1 are not possible, indicating that this protein is essential (Gaur *et al.* 2004).

Furthermore, antibodies to the protein can inhibit invasion (Blackman *et al.* 1990; Uthaipibull *et al.* 2001). *In vivo* evidence that MSP1 antibodies protect from severe malaria is slightly more conflicting with evidence for a protective effect of human MSP1 antibodies in some (Egan *et al.* 1999; John *et al.* 2004), but not all studies (Dodoo *et al.* 1999; Corran *et al.* 2004). The protein itself consists of a precursor, which is processed twice leaving a 19kDa C-terminus attached to the merozoite surface by a GPI-anchor (McBride and Heidrich 1987). MSP1-19, as it is referred to, contains two EGF-like domains, indicating a possible role in receptor-ligand interactions (Holder and Blackman 1994). A possible receptor for MSP1-19 has been suggested and will be discussed further below.

1.3.2.4 AMA1

Apical membrane antigen 1 (AMA1) is another essential merozoite surface protein. This protein is expressed from a single copy gene at the schizont stage of the parasite, localised to the rhoptries and spread on the merozoite surface after schizont rupture (Narum and Thomas 1994). It is not possible to knock the gene out, demonstrating that it is essential for blood-stage growth (Triglia *et al.* 2000). Furthermore, antibodies to AMA1 can inhibit parasite invasion (Hodder *et al.* 2001), and antibodies to AMA1 are associated with protection from clinical disease (Polley *et al.* 2004). Although, there does seem to be a strong strain-specific component to this immunity as heterologous antibodies are not as effective at inhibiting parasite invasion (Kennedy *et al.* 2002; Healer *et al.* 2004; Malkin *et al.* 2005), which complicates the use of AMA1 as a vaccine candidate (Malkin *et al.* 2005).

1.3.2.5 Overview:

Altogether the above description of *P. falciparum* invasion proteins shows an amazing complexity and it seems like numerous strategies have evolved to enhance the chance of successful invasion in the face of erythrocyte polymorphisms and host immune responses. Examples for the strategies to enhance successful invasion are the complexity of proteins that mediate invasion, as listed above, the different levels

of expression of invasion ligands, such as PfRh1 and PfRh2a/2b, and the evolution of allelic variants, such as seen for BAEBL and JESEBL. Furthermore, although the *P. falciparum* invasion proteins, which have been associated with specific invasion pathways, were described in the above paragraphs, there are a number of other proteins associated with merozoite invasion, such as the serine repeat antigen multigene family (SERA), glutamate-rich protein (GLURP) and subtilisin-like protein, which to date have not been associated with a specific invasion pathway and are described elsewhere (Li *et al.* 2002, Miller *et al.* 2002, Dziegiel *et al.* 1993, Blackman *et al.* 1998).

1.3.3 Erythrocyte receptors

Identifying possible receptors on erythrocytes is of course of major importance to understand merozoite invasion. Yet, despite numerous parasite proteins having been described as having a role in invasion, the number of receptors identified is still very limited. The main RBC receptors for *P. falciparum* merozoite invasion, which have been shown to mediate some of the invasion process or pathway, are the glycophorin proteins A, B and C (Pasvol and Wilson 1982; Dolan *et al.* 1994; Sim *et al.* 1994; Maier *et al.* 2003). Glycophorin A is highly abundant on the RBC surface, although its exact biological role is not clear. The glycophorins, as their name suggests, sugar residues on their extracellular domains and these contain sialic acid residues, which are important for binding of some of the parasite proteins. Two other described erythrocyte surface proteins, Band 3 and Kx, the latter being a blood group antigen which is not present on RBC of the McLeod phenotype, have been suggested to be involved in merozoite invasion (Goel *et al.* 2003; Kato *et al.* 2005). However, there is no strong evidence for those receptors. Finally, five other receptors that differ in regards to the invasion pathway they mediate are proposed to be present – they have been termed receptor A, E, X, Y and Z (Dolan *et al.* 1994; Rayner *et al.* 2001; Duraisingh *et al.* 2003; Gaur *et al.* 2003; Gilberger *et al.* 2003; Cortes *et al.* 2004). Besides their sensitivity to a number of enzymes (see below), little is known about these putative receptors.

1.3.4 Invasion pathways

The receptors involved in invasion, as described above, were identified by looking at invasion into mutant erythrocytes or enzyme treated RBC. Enzymes, which are used to study invasion pathways are trypsin, neuraminidase and more recently chymotrypsin. Trypsin removes GPA, GPC and Receptor X (Pasvol *et al.* 1982, Maier *et al.* 2003, Dolan *et al.* 1994). Neuraminidase removes sialic acids, which are linked to glycoproteins on the RBC surface (Pasvol *et al.* 1982). Finally, chymotrypsin removes GPB, Band 3, Receptor E and Z (Dolan *et al.* 1994, Goel *et al.* 2003, Gilberger *et al.* 2003, Duraisingh *et al.* 2003). The invasion pathways identified to date with these enzymes are shown in table 1. Five different invasion profiles can currently be distinguished, and these may point to one or more invasion pathways. It is still unfortunately a relatively crude method, but a number of *P. falciparum* invasion characteristics have been established. The invasion phenotype of laboratory strains has been found to be stable (Binks and Conway 1999). Parasites can adapt to invade by a different pathway, either upon long-term culturing on, for example, neuraminidase treated RBC or when a particular parasite invasion ligand is disrupted or knocked out (Mitchell *et al.* 1986, Dolan *et al.* 1994, Stubbs *et al.* 2005). These findings indicate that *P. falciparum* can adapt to selective pressure induced by blood polymorphisms and similarly may be able to switch invasion pathways, if invasion-inhibitory antibodies target and inhibit a pathway. It would be interesting to study whether invasion-inhibitory antibodies do cause laboratory isolates to invade by a different pathway *in vitro*.

Table 1: Invasion profiles described for *P. falciparum* laboratory strains

Invasion Profile type ^a	Parasite Ligand	RBC receptor	Reference
Nm _S T _S CT _R	EBA-175	GPA	(Pasvol 1982)(Camus 1985) (Sim 1994)
	BAEBL	GPC	(Maier 2003)(Mayer 2003)
Nm _S T _R CT _S	?	GPB	(Dolan 1994)
	JESEBL	Receptor E	(Gilberger 2003)
Nm _S T _R CT _R	PfRh1	Receptor Y	(Rayner 2001)(Triglia 2005)
Nm _R T _S CT _?	?	Receptor X	(Dolan 1994)
Nm _R T _R CT _S	PfRh2b	Receptor Z	(Duraisingh 2003)
	MSP1	Band 3	(Goel 2003)
	AMA1	Kx ^b	(Kato 2005)
Nm _R T _? CT _?	PfRh4	?	(Stubbs 2005) ^c

a Invasion profile type abbreviations: Nm = neuraminidase, T = trypsin, CT = chymotrypsin, S = sensitive and R = resistant

b Erythrocyte membrane protein, Kx (Kato 2005)

c PfRh4 has so far only been associated with the sialic-acid independent pathway in some but not all sialic-acid independent laboratory *P. falciparum* isolates (Stubbs 2005)

1.3.5 Field Isolate Data

As it became possible to assess which invasion pathways are used by laboratory strains, this has also been taken to field studies. The first question to ask was whether field isolates use a large variety of invasion pathways or invade via one major pathway. This is of importance for any vaccines that are designed to target merozoite antigens and inhibit invasion. To date three studies have looked at the invasion of field isolates into enzyme treated RBC (Okoyeh *et al.* 1999; Baum *et al.* 2003; Lobo *et al.* 2004). Okoyeh *et al.* found that out of 15 Indian isolates 12 could invade by an alternate invasion pathway to the EBA-175/GPA pathway (Okoyeh *et al.* 1999). This was an important finding indicating that vaccines based on EBA-175 may not be efficient at inhibiting invasion and parasite growth. In contrast, the study on 38 isolates in The Gambia concluded that a vaccine eliciting antibodies against EBA-175 may be effective initially, as glycophorin A, which EBA-175 binds to, was the primary receptor for the majority of the Gambian field isolates studied (Baum *et al.* 2003). A large variety of invasion pathways were used by field isolates collected in

Brazil (Lobo *et al.* 2004). In the Brazilian study half of the isolates (7 of 14) invaded via the neuraminidase and trypsin sensitive pathway, although they conclude from studies with mutant erythrocytes, which lack glycophorin A, that 5 of those isolates still invade via an alternative pathway to the EBA-175/GPA pathway (Lobo *et al.* 2004). Hence, these three studies gave an excellent insight into the invasion pathways used by *P. falciparum* in the field. However, no studies to date have looked at invasion pathways used by field isolates from patients diagnosed with severe malaria.

1.4 *P. falciparum* rosetting

1.4.1 Association with severe malaria

Rosetting is a cytoadherence phenotype of *Plasmodia*, where RBC bind to the surface of iRBC (figure 8). It was first described in *P. fragile* (David *et al.* 1988) and subsequently was identified in *P. falciparum* (Handunetti *et al.* 1989).

Figure 8:



Figure 8: Image of a rosette.

The parasites are stained with ethidium bromide (red).

Rosetting is one of the best-studied parasite virulence factors, and it has been repeatedly associated with severe malaria in Africa (Rowe *et al.* 1995) (Rowe *et al.* 2002b) (Carlson *et al.* 1990) (Ringwald *et al.* 1993) (Kun *et al.* 1998) (Heddini *et al.* 2001) (Pain *et al.* 2001) (Treutiger *et al.* 1992) (Newbold *et al.* 1997). Despite the

fact that the rosetting frequency can only be assessed *in vitro*, there is evidence supporting the notion that rosettes form *in vivo* and may be a causative for malaria pathogenesis. First of all, it is convincing that the finding that isolates from patients with severe malaria rosette with a higher frequency than isolates from patients with mild malaria has been repeated numerous times (see above). Furthermore, in a malaria endemic region of Papua New Guinea there is an unusually high frequency of a gene, which is linked to a human blood group polymorphism that reduces parasite rosetting. This gene allele, leads to a decrease of the complement receptor 1 (CR1) levels on RBC and is associated with protection from severe malaria (Cockburn *et al.* 2004). CR1 is a rosetting receptor, and lack of CR1 on RBC reduces the rosetting frequency (Rowe *et al.* 1997; Rowe *et al.* 2000).

1.4.2 Molecular Basis of rosetting

The parasite rosetting ligand is PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), a member of the DBL family. PfEMP1 is expressed by *var* genes, of which 50 to 60 copies exist per parasite isolate (Su *et al.* 1995). It is thought to be the major parasite protein mediating variant surface antigens for immune evasion and cytoadherence, whether to endothelium lining the blood vessels (Newbold *et al.* 1997), white blood cells (Urban *et al.* 1999), other iRBC causing autoagglutination (Roberts *et al.* 2000; Pain *et al.* 2001) or other RBC causing rosetting (Rowe *et al.* 1997). The complex domain structure of the different PfEMP1 types allows this large variation in receptor recognition (Su *et al.* 1995). The DBL α domain has been shown to bind RBC to form rosettes (Rowe *et al.* 1997) and there is ongoing work to establish whether there is a conserved sequence over a large number of parasite strains to identify possible epitopes for vaccine design (Alex Rowe, personal communication).

One identified RBC receptor involved in the formation of rosettes is CR1 on the RBC. RBC deficient in CR1 show a reduced rosetting phenotype compared to normal RBC (Rowe *et al.* 1997) and antibodies to CR1 can reverse rosetting of both laboratory strains and field isolates (Rowe *et al.* 2000). IgM is also a required serum

factor, which has to be present at the time of rosette formation (Clough *et al.* 1998a) and binds to the surface of rosetting iRBC (Rowe *et al.* 2002b). Other RBC receptors are CD36 (Handunnetti *et al.* 1992), heparan sulphate-like molecules (Chen *et al.* 1998) and the ABO blood group sugars (Carlson and Wahlgren 1992). The preference of rosette formation with blood group A RBC (Carlson and Wahlgren 1992; Rowe *et al.* 1995; Barragan *et al.* 2000) also associates rosetting with severe malaria, as blood group A has been independently associated with severe malaria (Fischer and Boone 1998; Lell *et al.* 1999; Pathirana *et al.* 2005) and also increased invasion rates of laboratory parasites (Chung 2005).

1.4.3 Why rosette?

The underlying mechanism associating the rosetting of *P. falciparum* isolates with severe pathological symptoms is unknown. Rosettes are found in other *Plasmodia* species, which do not cause severe malaria, so there must be something unique to *P. falciparum*. Previous work shows a possible involvement of rosettes in obstructing microvascular blood flow (Kaul *et al.* 1991; Dondorp *et al.* 2004) and it may be the formation of rosettes together with other cytoadherence phenotypes of *P. falciparum*, which cause severe malaria pathogenesis. Rosettes have also been associated with higher parasitaemia levels in the host (Rowe *et al.* 2002a), suggestive of rosetting somehow promoting parasite growth and/or survival. This has been shown not to be due to an increase in the parasite multiplication rate of rosetting isolates compared to non-rosetting isolates (Clough *et al.* 1998b). The correlation between rosetting and parasitaemia is not seen in children and adults with uncomplicated malaria (Rowe *et al.* 2002b) (Iqbal *et al.* 1993). Alternatively, the combination of rosetting and a high multiplication rate may lead to severe disease. The lack of any association between rosetting and the multiplication rate *in vitro*, does not rule out such an association occurring *in vivo* under conditions, where host immunity is present. Rosettes may enhance parasite survival by mediating immune evasion and is investigated in this study.

1.4.3 Rosetting as a mechanism to evade invasion inhibitory antibodies

Rosetting may allow merozoites to directly target the attached RBC without exposure to serum antibodies (figure 9). In semi-immune and immune hosts parasite growth is inhibited by antibodies directed to merozoite surface antigens (Pasvol 1982). An example is merozoite-surface-protein 1, the most abundant merozoite surface protein. Seropositive children have been shown to be more resistant to clinical malaria in several studies, and human antibodies inhibit parasite invasion *in vitro* (Egan *et al.* 1999). Although other studies show no correlation between MSP1 antibody levels and protection from clinical malaria (Dodoo *et al.* 1999). If rosetting does allow merozoite release and RBC invasion without prolonged exposure to the host sera, the invasion inhibitory or growth inhibitory antibodies could not bind and be active, and higher invasion rates of rosetting parasites would be seen than non-rosetting parasites in the presence of inhibitory antibodies.

Figure 9:

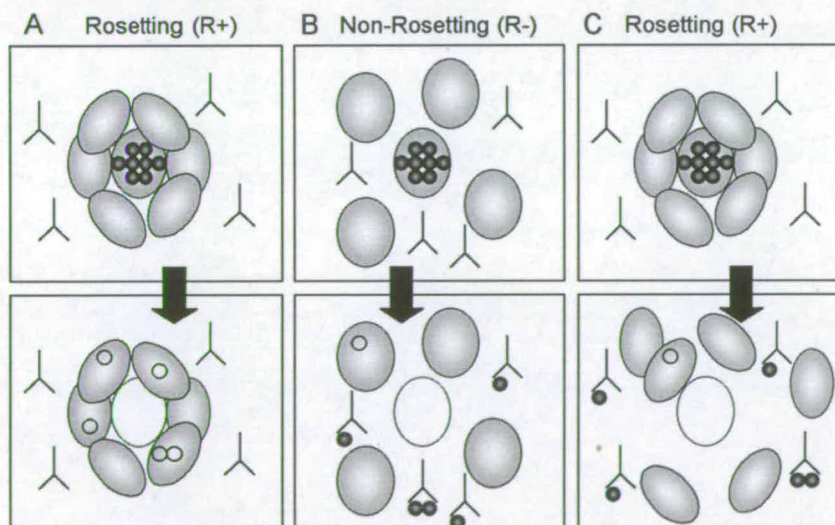


Figure 9: Diagram showing how merozoites released from rosetting and non-rosetting *P. falciparum* iRBC could be differentially inhibited by invasion-inhibitory antibodies. A) Rosetting might reduce the effect of invasion-inhibitory antibodies if merozoites invade directly into the RBC in rosettes, thereby avoiding prolonged exposure to antibodies in the host's plasma. B) Merozoites derived from non-

rosetting parasites would be exposed to antibodies in the plasma and hence would show lower invasion rates than merozoites from rosetting parasites. C) If merozoites from rosetting parasites do not preferentially invade the RBC in the rosette but are exposed to plasma antibodies in a similar way to merozoites from non-rosetting parasites, they would be expected to show reduced invasion rates in the presence of host antibodies.

1.5 Aim of Thesis

Parasite factors that may contribute to the pathogenesis of severe malaria are not well understood. There is evidence from Thailand, that parasites with higher multiplication rates and a decrease in RBC selectivity may cause severe malaria in patients. However, it is unknown whether these results from Southeast Asia, where malaria transmission is low and severe malaria affects patients of all ages, are representative for Africa, where mainly children below 5 years of age have severe malaria and often some degree of immunity is present. This study concentrates on *P. falciparum* field isolates from children in two African study sites and addresses the following questions related to potential parasite virulence factors:

1. Are parasite multiplication rate and selectivity index virulence factors associated with severe malaria in African children? (Chapter 2)
2. Is the selectivity index affected by immune selection, host immunity, the day of infection and/or mosquito transmission in *P. chabaudi*? (Chapter 3)
3. What are the invasion profiles of Kenyan clinical isolates from children with uncomplicated and severe malaria, and is there an association between invasion pathways and disease severity? (Chapter 4)
4. Do rosettes protect from invasion-inhibitory immunity? (Chapter 5)

CHAPTER 2: Low multiplication rates of African *Plasmodium falciparum* isolates and lack of association of multiplication rate and red cell selectivity with malaria virulence

A version of this chapter has been submitted as: Deans, AM *et al.*. Low multiplication rates of African *Plasmodium falciparum* isolates and lack of association of multiplication rate and red cell selectivity with malaria virulence. *American Journal of Tropical Medicine and Hygiene*. In press 2005.

2.1 Abstract

Two potential malaria virulence factors, parasite multiplication rate (PMR) and red cell selectivity (measured as selectivity index, SI) were assessed in *Plasmodium falciparum* clinical isolates from Mali and Kenya. At both sites PMR were low (Kenya median 2.2, n=33, Mali median 2.6, n=61) and did not differ significantly between uncomplicated and severe malaria cases. Malian isolates from hyperparasitaemic patients had significantly lower PMR (median 1.8, n=19) than other Malian isolates (uncomplicated malaria median 3.1, n=23; severe malaria median 2.8, n=19, P=0.03, Kruskal Wallis). Selective invasion occurred at both sites (Kenya geometric mean SI 1.9, n=98, Mali geometric mean SI 1.6, n=104) and there was no significant association between the SI and malaria severity. Therefore, in contrast to previous results from Thailand, I found no association of PMR and SI with malaria severity in African children. This raises the possibility of differences in the mechanisms of malaria virulence between sub-Saharan Africa and Asia.

2.2 Introduction

There is a poor understanding of factors causing severe malaria, although host genetics, environmental determinants and parasite properties are all thought to play an important role (Miller *et al.* 2002). As far back as the early 20th century when malaria therapy was used to treat neurosyphilis, differences in *Plasmodium*

falciparum strain virulence were recognised (James *et al.* 1932). In parasite evolutionary theory, differences in strain virulence can be explained by the trade-off hypothesis, which describes how parasites evolve to balance the fitness advantages of high growth and transmission with the fitness cost of host death (Anderson and May 1982). In other words, parasites that grow faster transmit more efficiently, but are also more virulent to the host. The relationship between parasite growth rate and virulence has been shown in rodent malaria models with *P. chabaudi* (Mackinnon and Read 1999; Mackinnon and Read 2003, 2004). In human malaria, *P. falciparum* virulence was associated with *in vitro* parasite multiplication rate (PMR) in a study carried out in Thailand (severe malaria isolates median PMR 8.3, uncomplicated malaria isolates median PMR 2.8) (Chotivanich *et al.* 2000). Whether this relationship between PMR and virulence is seen in other malaria endemic countries is unknown.

Further insight into parasite multiplication *in vivo* can be gained by understanding whether red blood cell (RBC) invasion is a random, unrestricted process, or whether some selectivity occurs, with only a subset of RBC being accessible for invasion. The selectivity index (SI) compares the observed number of multiple-infected RBC (containing more than one ring-stage parasite) to the number expected from a random Poisson distribution (Simpson *et al.* 1999). A SI of 1 indicates random, non-selective invasion of RBC, whereas a SI of, for example, 2, indicates two-fold more multiple-infected RBC than expected by chance alone. Factors that may lead to non-random invasion ($SI > 1$) include parasite receptor preference, host RBC polymorphisms or host merozoite-agglutinating antibodies (Miller *et al.* 1984; Ramasamy *et al.* 1999; Ramasamy *et al.* 2001). A SI of < 1 could occur if invasion by one merozoite rendered a RBC refractory to further invasion.

In all human Plasmodium infections multiple-infected RBC occur (Simpson *et al.* 1999). In *P. vivax* the SI is high (about 8), which reflects the preference of *P. vivax* for reticulocytes and young red blood cells (Simpson *et al.* 1999). In *P. falciparum* infections with parasitaemias above 2%, multiple-infected RBC are largely due to the high parasite load, and the SI is around 1 (Simpson *et al.* 1999). *P. falciparum*

infections with lower parasitaemias (<2%) show a tendency towards higher RBC selectivity, with a mean SI of 2.44 (Simpson *et al.* 1999). Importantly, after accounting for parasitaemia effects, red cell selectivity of *P. falciparum* isolates has been associated with disease severity in Thailand (Simpson *et al.* 1999). Severe malaria isolates show random, unrestricted invasion of RBC (geometric mean SI 1.35), whereas uncomplicated malaria isolates show restricted, non-random invasion with a higher number of multiple-infected cells (geometric mean SI 2.31) (Simpson *et al.* 1999).

Together with the PMR results (Chotivanich *et al.* 2000), the above data indicate that there may be more virulent isolates causing severe malaria in Thai patients due to rapid, unrestricted growth and subsequent high parasite burdens in the host. The influence of these factors on malaria severity in African children has not previously been studied. Therefore I investigated PMR and red cell selectivity in relation to malaria virulence in two diverse sites in Africa.

2.3 Materials and Methods

2.3.1 Parasite isolates

Parasite isolates were collected from Bandiagara, Mali and Kilifi, Kenya. Malaria transmission is seasonal at both sites, with infective bites ranging from 20-60 per month in Mali (Lyke *et al.* 2004b) and 10-30 per year in Kenya (Kinyanjui *et al.* 2004). In Mali there is a single malaria season every year (July-December), whereas in Kenya there are two malaria seasons per year (June-August and December-February). The Malian samples were collected as part of the Bandiagara Malaria Project case-control study in which uncomplicated malaria controls were age-, residence- and ethnicity-matched to severe malaria cases (Lyke *et al.* 2003; Lyke *et*

al. 2004b). The WHO criteria for severe malaria were applied (World Health Organization 2000), although patients with hyperparasitaemia (>500,000 parasites per microlitre of blood) and no other symptoms or signs of severe disease were analysed as a separate group, as previous studies indicate a good prognosis for these children (Marsh *et al.* 1995; Lyke *et al.* 2003). Uncomplicated malaria cases were all those recruited into the study with fever and *P. falciparum* parasitaemia who did not have severe malaria or hyperparasitaemia (Lyke *et al.* 2003; Lyke *et al.* 2004a). In Kenya, children with cerebral malaria (unrousable coma with a Blantyre score ≤ 2), prostration (inability to sit unaided or in babies, to breast feed) or respiratory distress (abnormally deep breathing) were considered to have severe malaria. The simpler clinical definition applied in Kenya identifies approximately the same group of children at risk of life-threatening malaria as those identified by the more comprehensive WHO criteria (Marsh *et al.* 1995). In Kenya, uncomplicated cases were children with malaria presenting at hospital with no symptoms or signs of severe disease, who were treated as outpatients with oral therapy. Blood samples were collected after obtaining informed consent from the children's parents or guardians, and protocols were approved by ethical review boards in the UK, USA, Mali and Kenya.

2.3.2 Parasite culture

After collection the blood samples were spun down for 5min at 400g to remove the Buffy Coat layer. Subsequently, lymphocytes were removed from the blood samples by diluting the blood sample with incRPMI to 50% haematocrit and layering it over 5ml pre-warmed Lymphoprep (BioWhittaker). The sample was spun at 400g for 20min. After centrifugation the white blood cells were removed and the pellet washed twice in incRPMI. The Malian isolates were then frozen in glycerolyte and shipped to Edinburgh. The isolates were thawed as follows. The isolates were diluted in a series of salt solutions (0.2 ml of 12% saline added slowly, followed by 10 ml of 1.8% saline followed by 10ml of 0.9% saline/0.2% glucose) and washed in RPMI 1640 medium (BioWhittaker) containing 2mM glutamine (BioWhittaker) 37.5mM Hepes (BioWhittaker), 20 mM glucose (Sigma) and 25 µg/ml gentamicin (Gibco)

(incomplete RPMI). The cells were resuspended in complete RPMI (incomplete RPMI supplemented with 10% human AB serum), gassed with 3% CO₂, 1% O₂, 96% N₂ and incubated at 37°C. Kenyan isolates were collected into heparinised tubes and put into culture within 12 hours of collection. Lymphocytes and the buffy coat were removed as described above, the RBC washed three times in incomplete RPMI and resuspended in complete RPMI, then gassed and incubated as above. Cultures were monitored by Giemsa (BDH) smears for 18-36 hours, and only those with normal morphology that matured to the schizont stage were included in the study. Thai samples were frozen and shipped to Edinburgh and then thawed and cultured in the same way as the Malian samples.

2.3.3 Thin blood smears for selectivity index calculations

Thin smears were prepared from blood collected from the patient on arrival at hospital and were stained with Giemsa. A subset of the slides for SI calculations are from the isolates used to collect *in vitro* PMR data. The remaining slides in both Kenya and Mali were collected from children recruited into other studies of factors affecting severe malaria (Rowe *et al.* 1995; Rowe *et al.* 2002; Lyke *et al.* 2004a).

2.3.4 Selectivity Index

300 ring-infected RBC were counted per slide and the number of rings in each RBC was recorded (Leica microscope). The parasitaemia was estimated as the proportion of RBC infected with at least one parasite from a count of 1000 RBC. The SI was calculated as described by Simpson *et al* (Simpson *et al.* 1999). The observed number of multiple-infected RBC (i.e. 2 or more rings per RBC) was determined by counting. The expected number of multiple-infected RBC was determined with a Poisson distribution ($e^{-\mu} \mu^x/x!$), where μ is $(-\ln(1-\text{parasitaemia}))$ and x is the number of parasites per erythrocyte (i.e 1, 2, 3, 4 or 5). These values were multiplied by the number of cells counted (i.e 300) to obtain the number of RBC with 1,2,3,4 or 5 parasites per erythrocyte and therefore also the total number of expected multiple-

infected RBC. To obtain the selectivity index the number of observed multiple-infected RBC was divided by the number of expected multiple-infected RBC.

2.3.5 *In vitro* PMR assay

The assay to determine PMR in the first cycle of *in vitro* culture was set up as described by Chotivanich *et al* (Chotivanich *et al.* 2000). All parasites were cultured as described above until the mature schizont stage. The schizonts were enriched by centrifugation through Percoll. For this the schizonts were spun down for 2min at 250g. The pellet was resuspended at 50% haematocrit in cRPMI and carefully layered over 5ml pre-warmed Percoll (Sigma). The Percoll concentration was 60% for Malian samples, but had to be adjusted to 55% whilst working in Kenya to enrich a pure schizont population. A 90% Percoll stock was prepared from Percoll (Sigma) and 10X RPMI (Sigma), for example to prepare 30ml of 90% Percoll, 27ml Percoll were diluted with 3ml of 10X RPMI. From this stock the working concentrations were prepared with incRPMI, generally 10ml were prepared at a time, so for a 60% Percoll concentration 6.67ml of 90% Percoll were diluted with 3.33ml incRPMI. The sample was spun for 20min at 2000rpm. After centrifugation the schizont band, which is visible above the percoll layer as a dark brown layer, is separated from the RBC and younger iRBC. The band is isolated and washed twice with 5ml incRPMI for 2min at 250g. The schizont pellet is then resuspended in cRPMI and aliquoted into 5 1.5ml Eppendorf tubes. The schizonts were diluted to 2% haematocrit and a control smear was made to check the parasitaemia. The samples were then adjusted to around 1% parasitaemia with normal group A (Malian samples) or group O (Kenyan samples) red blood cells, each from one single healthy Caucasian donor respectively. To each of the 5 samples either control or enzyme-treated RBC (see chapter 4) were added. They were incubated in 96-well plates (Corning) at a volume of 50µl at 2% haematocrit with duplicate wells for each isolate. The 96-well plate was placed within a gas modulator chamber (Billups-Rothenberg) to allow culturing in 3% CO₂, 1% O₂, 96% N₂ for 24hrs at 37°C. Thin blood films of the pre-invasion sample and the ring-invaded RBC after 24hrs were made and stained with Giemsa to determine the parasitaemia. At least 1000 RBC from each slide of the duplicate wells

per sample were counted. The PMR is the number of ring-infected RBC after invasion divided by the pre-invasion parasitaemia. The multiplication rate can also be calculated using the total number of ring-stage parasites present in all infected RBC after invasion (Chotivanich *et al.* 2000). Analysis of the data using total number of ring-stage parasites rather than the number of ring-infected RBC did not materially affect any of the results shown here; therefore only data referring to the number of ring-infected RBC are given.

2.3.6 Rosetting

Rosette frequency was assessed by staining an aliquot of culture suspension with 25 µg/ml of ethidium bromide. A wet preparation of the stained culture suspension at 2% haematocrit was viewed with a fluorescence microscope and the percentage of 200 mature-infected erythrocytes binding 2 or more uninfected erythrocytes was counted (the rosette frequency).

2.3.7 Statistical analysis

Statistical analysis was done with StatView 5.0.1 software and Minitab 14.1. The power calculation was determined using S-plus software. Non-parametric tests (Mann-Whitney U or Kruskal Wallis) were used for most analyses due to the non-normality of the data, and correlations were assessed by Spearman rank correlation. The PMR data were square-root transformed to conform to normality and homogeneity of variance assumptions. They were then analysed using a general linear model for the effects of disease severity, parasitaemia, country of origin, and the interaction between disease severity and country of origin. Repeating the non-parametric analysis of the PMR with parametric tests using the transformed data did not materially alter the results (data not shown). The SI data were normalised by log transformation. Geometric mean SI (95% confidence intervals) for disease severity groups were calculated and compared by one-way analysis of variance. Correlations with SI were assessed by simple regression.

2.4 Results

2.4.1 Growth of field isolates *in vitro*

During the field work in Kenya a total of 69 samples were collected, 34 from severe malaria patients and 35 from uncomplicated malaria patients. I found that nearly 50% (16 samples) of isolates from severe malaria patients failed to grow to the mature trophozoite stage in culture. Whereas, only 2 isolates from mild malaria patients failed to grow in culture. This difference may partly be explained by previous drug treatment. Analysis of the severe malaria patients' medical files showed that 9 (56%) out of the 16 samples that failed to grow had been from patients who had had previous drug treatment. It is likely that this occurred in more cases, as there were a number of samples with no entry in this section of the medical file. In contrast of the 18 samples from children with severe malaria that grew only 4 (22%) children had previous drug treatment recorded. Unfortunately, for the samples from children with uncomplicated malaria there is no existing record on possible previous drug treatment. 24 samples could not be included in the study for other reasons; these were that the parasitaemias were too low by the schizont stage, contamination or due to schizont rupture prior to percoll isolation. I collected multiplication rate data for 33 samples as described below. The Malian samples were collected as part of a large case-control study described elsewhere (Lyke *et al.* 2003; Lyke *et al.* 2004b). Samples selected for inclusion in this study generally had >1% parasitaemias, which grew well to the schizont stage.

2.4.2 Clinical and laboratory details of patients in the PMR study

The laboratory data for the patients in the PMR study are shown in Table 1. Children with severe malaria tended to have higher parasitaemias, higher white blood cell counts and lower haemoglobin levels than children with uncomplicated malaria (Table 1). The Kenyan children with severe disease are younger than those with uncomplicated malaria; however, this age difference is not seen in the Malian dataset because age-matching was part of the study design (Table 1). The parasites from severe malaria cases showed higher rosette frequencies than those from uncomplicated malaria cases as described previously (Rowe *et al.* 1995; Rowe *et al.* 2002), although this was not statistically significant in Kenya where the sample size was small (Table 1). Of the 13 Kenyan children with severe malaria, 3 had cerebral malaria, 5 had respiratory distress and prostration, 4 had prostration alone and 1 had respiratory distress alone. Of the 19 Malian children with severe disease, 5 had cerebral malaria, 10 had other neurological impairment (prostration, obtundation or repeated seizures), 2 had respiratory distress, 1 had severe anaemia and 1 had jaundice. One Malian child with respiratory distress died, and all the other children survived.

Table 1: Laboratory data and parasite multiplication rates for patients included in the PMR study in Kenya and Mali.

	N ^a	Age ^{bc} (months)	Hb ^{bc} (g/dL)	Pt ^{bd} (%)	WBC ^{bce} (x 10 ⁹ /L)	RF ^{cig}	Parasite Multiplication rate ^g
Kenya							
All patients	33	33 (30) [30]	8.7 (2.6) [27]	6.0 (4.1)	13.7 (12.0) [27]	1.3 (0- 5.5) [20]	2.2 (1.1-3.3)
Uncomplicated	20	44 (35) [17]	9.8 (1.3) [14]	4.0 (2.3)	9.0 (3.6) [14]	1.0 (0-3) [12]	2.3 (1.2-3.6)
Severe ^h	13	20 (11)	7.5 (3.1)	9.1 (4.5)	18.8 (15.6)	3.3 (0- 19.3) [8]	1.8 (1.0-2.9)
P-value ⁱ		P=0.02	P=0.0125	P=0.0001	P=0.003	P=0.32	P=0.38
Mali							
All patients	61	39 (22)	9.6 (1.8)	8.2 (7.8)	14.0 (7.2) [60]	7.0 (0 - 17)	2.6 (1.8-3.5)
Uncomplicated	23	40 (22)	9.6 (1.5)	3.0 (1.7)	13.2 (7.4) [22]	2.0 (0- 12)	3.1 (2.3-4.4)
Severe	19	34 (22)	8.7 (2.4)	7.3 (4.3)	15.7 (8.3)	14.0 (9- 24)	2.8 (2.1-3.5)
Hyperparasitaemic	19	42 (24)	10.4 (1.1)	15.4 (9.4)	13.1 (5.9)	5.0 (2- 20)	1.8 (1.2-2.7)
P-value ⁱ		P=0.54	P=0.04	P<0.0001	P=0.48	P=0.003	P= 0.27 ^j P=0.03 ^k

^a Number studied.

^b Mean (Standard deviation).

^c Square brackets [] indicate n, where n differs due to missing data.

^d Pt: parasitaemia = percentage of infected RBC in 1000 RBC counted.

^e WBC: white blood cell count.

^f RF: rosette frequency (percentage of infected RBC binding 2 or more uninfected RBC in 200 infected RBC counted).

^g Median (Interquartile Range).

^h The mean (standard deviation) values for additional biochemical tests in the severe cases are pH 7.34 (0.15), bicarbonate 16.04 mmol/L (5.90), lactate 5.03 mg/dL (3.56). These tests were not available for uncomplicated cases in Kenya or for any Malian samples.

ⁱ Statistical analysis: All columns were analysed for the malaria severity groups by ANOVA or Student's t-test, except the RF and multiplication rates which were analysed by Mann-Whitney U test (Kenya) or Kruskal-Wallis test (Mali).

^j severe versus uncomplicated (Mann Whitney U test).

^k severe, uncomplicated and hyperparasitaemia groups (Kruskal Wallis test).

2.4.3 Field isolate multiplication rates are not associated with disease severity in Kenya and Mali

I measured the PMR in the first cycle of *in vitro* culture for 33 Kenyan and 61 Malian field isolates. A power calculation indicated that in both Kenya and Mali I had >99% power to detect a difference in PMR between severe and uncomplicated isolates as large as that seen in the previous study in Thailand (Chotivanich *et al.* 2000). With the sample size and variance shown here for Kenya (the smallest of the two studies), I had >90% power at a 5% error level to detect a difference in the PMR between severe and uncomplicated isolates as small as 1.8.

In both Kenya and Mali the multiplication rates were low (Kenya median 2.2, Mali median 2.6, Table 1). In Kenya there was no significant difference in the PMR of isolates from severe malaria patients compared to the PMR of isolates from uncomplicated malaria cases ($P=0.38$, Mann Whitney U, Figure 1A and Table 1). Similarly, in Mali there was no significant difference in the PMR of severe malaria isolates compared to uncomplicated malaria isolates ($P=0.27$, Mann Whitney U, Figure 1B and Table 1). Children with uncomplicated malaria had lower parasitaemia than children with severe malaria in both Kenya and Mali (Table 1), and parasitaemia was found to be significantly negatively correlated with PMR in both Kenya and Mali (Kenya: $\text{Rho}=-0.397$, $P=0.025$; Mali: $\text{Rho}=-0.312$, $P=0.02$; Figure 2). The data on the relationship between PMR and disease severity were therefore re-analysed after adjustment for host parasitaemia, and there was still no significant difference between the PMR of uncomplicated malaria isolates compared to severe malaria isolates ($F_{1,29}=0.343$, $P=0.56$ and $F_{1,38}=0.120$, $P=0.73$ for Kenya and Mali respectively).

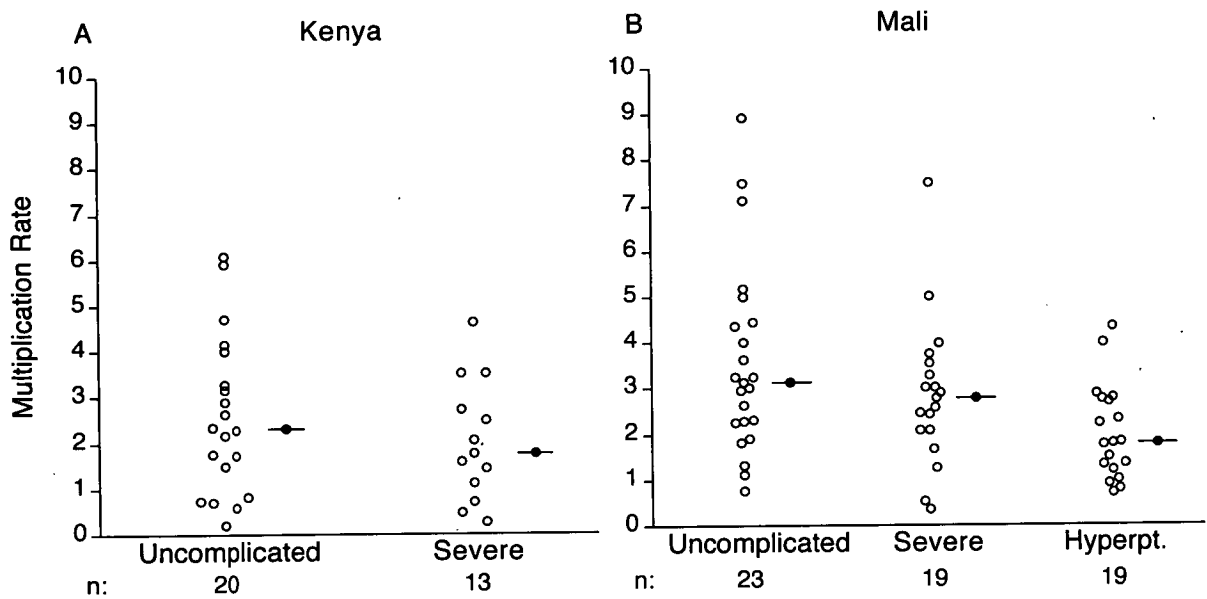
Figure 1:

Figure 1: Parasite multiplication rates (PMR) of Kenyan (A) and Malian (B) *P. falciparum* isolates from children diagnosed with uncomplicated or severe malaria. Isolates from Malian children diagnosed with hyperparasitaemia (Hyperpt.) are shown as a separate group. Equivalent children with hyperparasitaemia and no other symptoms or signs of severe malaria do occur in Kenya, but they were not recruited due to the study design. The median PMR for each group is indicated by the closed circle and line.

In Kenya the PMR of isolates from cerebral malaria cases ($n=3$, median 2.7) was not significantly different to the PMR of isolates from patients with other forms of severe disease ($n=10$, median 1.7, $P=0.74$, Mann Whitney U). Similarly, in Mali, the PMR of isolates from cerebral malaria cases ($n=5$, median 3.5) did not differ significantly from the PMR of isolates from other severe malaria cases ($n=14$, median 2.5, $P=0.17$, Mann Whitney U). The number of isolates in the sub-categories of severe disease is small, and a larger study would be required to determine conclusively whether there are any significant differences in PMR amongst isolates from patients with different clinical forms of severe malaria.

I also studied patients in Mali with hyperparasitaemia (>500,000 parasites per microlitre of blood) but no other symptoms or signs of severe malaria. These patients can be classified as “severe” using WHO criteria (World Health Organization 2000), however, they have a very low mortality rate and should probably be considered as an intermediate category between severe and mild uncomplicated malaria (Marsh *et al.* 1995; Lyke *et al.* 2003). Patients with hyperparasitaemia also occur in Kenya, but were not recruited in the Kenyan study because its aim was to compare the most severely ill children to those with mild uncomplicated disease, rather than considering the intermediate category. I found that isolates from patients with hyperparasitaemia had significantly lower PMR (median 1.8) than isolates from other Malian severe and uncomplicated malaria cases ($P=0.03$, Kruskal Wallis, Figure 1B and Table 1). The occurrence of lower PMR in isolates from patients with higher parasite burdens was also indicated by the negative correlation between parasitaemia and PMR (Figure 2).

Figure 2:

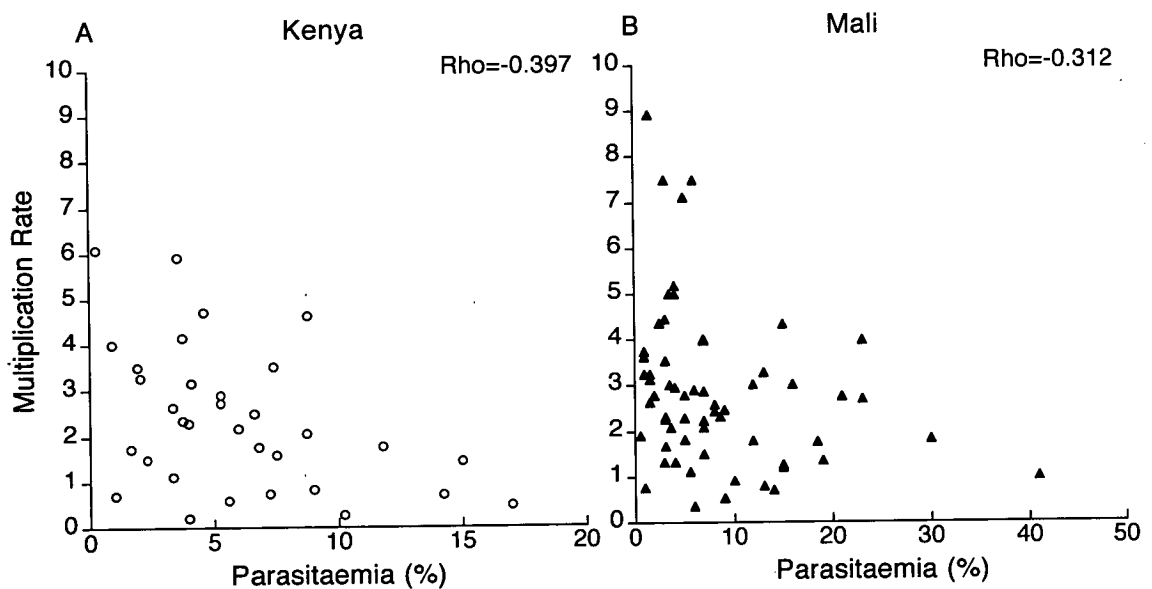


Figure 2: Scatter plots indicating the negative correlation between the PMR and host parasitaemia of *P. falciparum* isolates from Kenya (A, $n=33$) and Mali (B, $n=61$).

I also examined PMR in relation to three other variables: patient age as a possible indicator of host immunity, haemoglobin as an additional measure of disease severity and parasite rosette frequency (a known correlate of malaria virulence in African children) (Rowe *et al.* 1995; Rowe *et al.* 2002). The age and haemoglobin levels of the children and the parasite rosette frequency were not correlated with PMR in Mali ($P=0.98$, $P=0.72$ and $P=0.64$ respectively) or Kenya ($P=0.29$, $P=0.78$ and $P=0.70$ respectively, Spearman rank correlation).

The maximum PMR seen in the Kenyan isolates was 6.1, and in the Malian isolates was 8.9 (Figure 1). This is in contrast to the previous study from Thailand where more than half of the isolates from severe malaria patients showed between 8 and 25-fold multiplication (Chotivanich *et al.* 2000). I used identical methods to those described in the Thai study, however, my results are so clearly different to those reported earlier that I decided to examine the possibility of a technical problem in the PMR assay. I therefore studied PMR in a small, blinded set of Thai isolates (gift from Dr. K. Chotivanich and Professor N. White, Mahidol University, Thailand). The PMR assay was set up using blood from the same Caucasian donor who was used for the Mali samples. Despite the small numbers studied, the Thai severe malaria isolates had a higher PMR (median 7.7, ($n=4$: 1.4, 6.5, 9.0, 10.2)) than the Thai uncomplicated malaria isolates (median 2.2 ($n=4$: 0.4, 2.2, 2.2, 3.5)), as shown previously (Chotivanich *et al.* 2000). This difference in PMR between severe and uncomplicated Thai isolates was significantly greater than that found in the African samples (country*disease severity interaction: $F_{1,77}=5.20$, $P=0.008$). Thus, technical differences between studies are unlikely to explain the contrast between *P. falciparum* isolates from Thailand and Africa in the relationship between PMR and severe malaria.

2.4.4 Clinical and laboratory details of patients in the SI study

The laboratory data for the children in the SI study are shown in Table 2. The severe malaria patients had lower haemoglobin levels, higher parasitaemias and higher white cell counts than the children with uncomplicated malaria. The parasite isolates from the severe malaria patients had significantly higher rosette frequencies than the parasite isolates from the uncomplicated malaria and hyperparasitaemic patients (Table 2). Of the 46 Kenyan children with severe malaria, 20 had cerebral malaria, 11 had respiratory distress and prostration, 11 had prostration alone, 2 had respiratory distress alone and 2 were missing the clinical details on sub-category of severe disease. Of the 46 Malian children with severe malaria, 13 had cerebral malaria, 23 had other neurological impairment (prostration, obtundation or repeated seizures), 2 had respiratory distress, 5 had severe anaemia and 3 had jaundice. 6 Malian and 3 Kenyan children with severe disease died, and there were no deaths amongst the uncomplicated malaria or hyperparasitaemic children.

Table 2: Laboratory data and selectivity index for patients included in the SI analysis in Kenya and Mali

	N ^a	Age ^{bc} (months)	Hb ^{bc} (g/dL)	Pt ^{bd} (%)	WBC ^{bce} (x 10 ⁹ /L)	RF ^{cfg}	Selectivity Index (geo.mean (95%CI))
Kenya							
All patients	98	33 (26) [79]	8.1 (2.5) [74]	6.5 (6.1)	13.2 (13.1) [45]	4.25 (1-13.5) [62]	1.92 (1.65-2.19)
Uncomplicated	52	40 (27) [37]	9.4 (1.5) [32]	4.2 (2.9)	9.0 (3.9) [21]	1.5 (0-7) [31]	2.05 (1.55-2.55)
Severe ^h	46	27 (23) [42]	7.0 (2.6) [42]	9.0 (7.7)	16.9 (16.9) [24]	6.5 (2.5-17.5) [31]	1.79 (1.58-2.01)
P-value ⁱ		P=0.02 2	P<0.0001	P<0.0001	P=0.044	P=0.009	P=0.56
Mali							
All patients	104	39 (24)	9.0 (2.4)	7.6 (7.0)	13.9 (7.7) [101]	12 (2-22) [98]	1.61 (1.12-2.10)
Uncomplicated	33	38 (20)	9.4 (1.7)	3.3 (2.5)	12.9 (6.3) [32]	2 (0-14.5)	1.86 (0.60-3.11)
Severe	46	38 (25)	8.2 (2.9)	6.9 (5.7)	15.4 (9.3) [45]	18.5 (11.5-37) [40]	1.55 (0.86-2.25)
Hyperparasitaemic	25	44 (25)	10.2 (1.5)	14.8 (8.0)	12.3 (5.5) [24]	5 (2-19.5)	1.44 (1.21-1.67)
P-value ⁱ		P=0.58	P=0.003	P<0.0001	P=0.19	P<0.0001	P=0.62

^a Number studied.^b Mean (Standard deviation).^c Square brackets [] indicate n, where n differs due to missing data.^d Pt: parasitaemia (percentage of infected RBC in 1000 RBC counted).^e WBC: white blood cell count.^f RF: rosette frequency (percentage of infected RBC binding 2 or more uninfected RBC in 200 infected RBC counted).^g Median (Interquartile Range)^h The mean (standard deviation) values for additional biochemical tests in the severe cases are pH 7.26 (0.15) n=23, bicarbonate 13.97 mmol/L (5.85) n=23 and lactate 6.91 mg/dL (4.35) n=23. These tests were not available for uncomplicated cases in Kenya or for any Malian samples.ⁱ Statistical comparison: All columns were analysed for the malaria severity groups by ANOVA or Student's t-test, except the RF which was analysed by Mann-Whitney U test (Kenya) or Kruskal-Wallis test (Mali).

2.4.5 Selectivity index of field isolates is negatively correlated with parasitaemia

I examined thin blood smears from 98 Kenyan and 104 Malian children. The geometric mean SI (95% confidence interval, CI) was 1.92 (1.65–2.19) for Kenyan isolates and 1.61 (1.12–2.10) for Malian isolates, indicating non-random, selective invasion at both sites. The SI in both Kenya and Mali was negatively correlated with host parasitaemia, (Kenya $r = -0.523$, Mali $r = -0.526$, $P < 0.001$ in both cases, Figure 3). To allow direct comparison with previous data (Simpson *et al.* 1999), I grouped the SI into 4 categories (<2% parasitaemia; 2.1–5%; 5.1–10%; 10%–max, Figure 4). The <2% parasitaemia group had a significantly higher SI than the other parasitaemia groups in both Kenya and Mali ($P < 0.02$, Figure 4, Student's *t* test).

Figure 3:

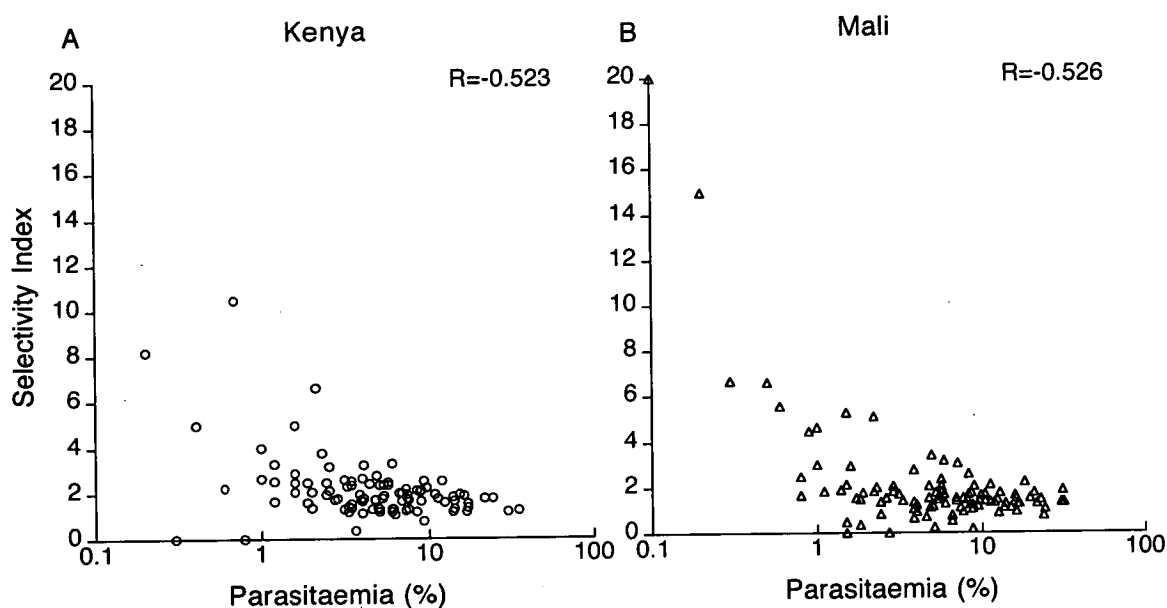


Figure 3: Scatter plots indicating the negative correlation between the selectivity index (SI) and host parasitaemia of *P. falciparum* isolates from Kenya (A, $n=98$) and Mali (B, $n=104$).

Figure 4:

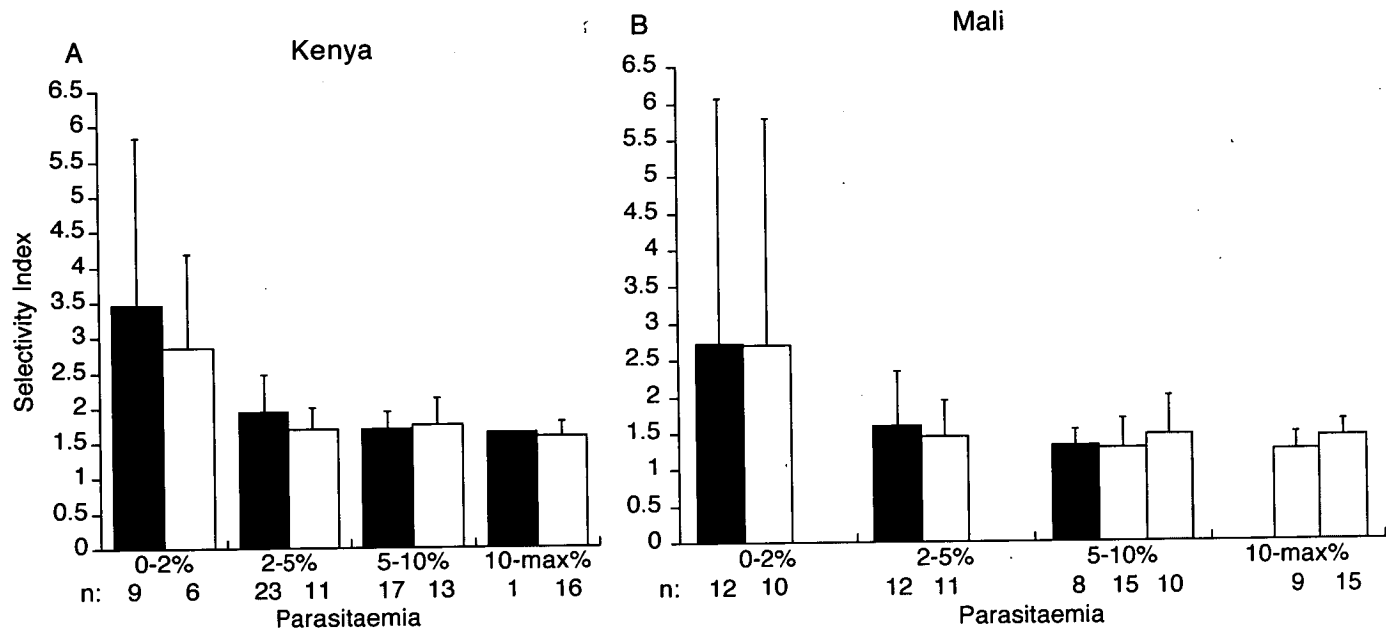


Figure 4: Geometric mean SI (95% CI) of *P. falciparum* isolates separated by patient malaria severity (uncomplicated, black bars; severe, white bars; hyperparasitaemia, grey bars) and host parasitaemia group. Four samples with a SI of 0 (i.e. no multiple-infected cells) were excluded (2 Kenyan and 2 Malian).

The SI was not correlated with age ($P=0.63$ and $P=0.76$), haemoglobin ($P=0.49$ and $P=0.68$) or parasite rosette frequency ($P=0.95$ and $P=0.24$ for Kenya and Mali respectively, simple regression). The latter result was of particular interest because I had hypothesised that rosetting might increase SI if it resulted in preferential invasion into the RBC forming the rosettes. The lack of correlation between rosetting and SI suggests that invasion is not targeted into the uninfected erythrocytes in rosettes, a finding that agrees with previous work (Clough *et al.* 1998).

2.4.6 Selectivity index of field isolates is not associated with disease severity

I examined whether the SI differed amongst isolates from children with uncomplicated or severe malaria or hyperparasitaemia (Figure 4 and Table 2). One-way analysis of variance with parasitaemia grouping included as a covariate showed no significant difference in the SI of isolates from the different disease severity groups ($F_{1,93}=0.14$, $P=0.71$ and $F_{1,73}=0.255$, $P=0.62$ for Kenya and Mali respectively). There was also no significant difference between uncomplicated and severe cases if only parasitaemias below 5% were analysed in accordance with previous data from Thailand (data not shown) (Simpson *et al.* 1999). With my sample sizes and variance I had >97% power to detect a difference between severe and uncomplicated isolates equivalent to that measured in Thailand (Simpson *et al.* 1999).

In Kenya the SI of isolates from children with cerebral malaria (geometric mean 1.72, 95% CI 1.29 to 2.15, $n=20$) did not differ significantly from the SI of isolates from children with other forms of severe disease (geometric mean 1.85, 95% CI 1.59 to 2.11, $n=26$, $F_{1,44}=0.53$, $P=0.47$, ANOVA). Similarly, in Mali, the SI of isolates from children with cerebral malaria (geometric mean 1.23, 95% CI 0.88 to 1.58, $n=13$) did not differ significantly from the SI of isolates from children with other forms of severe disease (Mali geometric mean 1.70, 95% CI 0.74 to 2.67, $n=33$, $P=0.18$, $F_{1,43}=1.894$, $P=0.18$, ANOVA).

2.4.7 Multiplication rate in relation to the selectivity index

I also looked at the relationship between SI and PMR. In Kenya there was a negative correlation between SI and PMR ($n=29$, $Rho=-0.410$, $P=0.03$, Spearman rank correlation, Figure 5A). However, in Mali there was no significant correlation between the SI and PMR ($n=60$, $Rho=0.015$, $P=0.91$, Spearman rank correlation, Figure 5B). It might be expected that isolates with high selectivity would have low

PMR giving a negative correlation between SI and PMR as seen in Kenya (figure 5A). It is unclear why this relationship was not seen in the Malian isolates (figure 5B).

Figure 5:

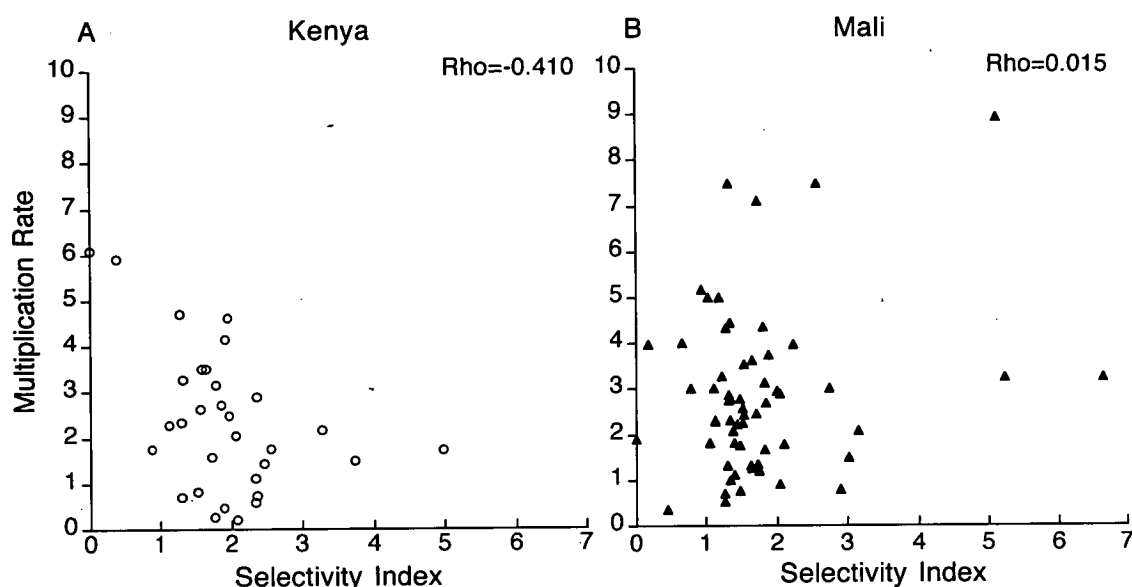


Figure 5: Scatter plots indicating the relationship between the PMR and SI of *P. falciparum* isolates from Kenya (A, n=29) and Mali (B, n=60). These samples are the subset from the SI data shown in Fig. 3 for which there is corresponding PMR data.

2.4.8 ABO blood group of the patient is not associated with PMR or SI of the parasite isolates

I looked at the PMR and the SI of the field isolates in relation to the ABO blood group of the patient they were isolated from. This is of interest as individuals with blood group A and B are at a higher risk of severe malaria (Pathirana 2005). I saw no association between the PMR and the patients' blood group in Mali ($P=0.21$, Kruskal Wallis). In Kenya there was a trend that isolates taken from patients with blood

group O (median PMR: 2.73, IQR: 2.02 to 3.76, n=17) had a higher PMR than isolates taken from patients with blood group A (median PMR: 1.75, IQR: 0.48 to 4.63, n=7) ($P=0.03$, Kruskal Wallis). This result was confounded though by the fact that the blood group A patients had higher parasitaemias (mean: 9.71, standard deviation 7.02, n=10) than blood group O patients (mean: 5.07, stdev 3.67, n=20) ($P=0.02$, Fisher's PLSD). Analysing them together in a parametric test looking at the effect of the blood group on the PMR (square-root transformed) and including parasitaemia, as a covariate, there is no significant effect of blood group ($P=0.12$, ANOVA). Furthermore I saw no association between the ABO blood type and the SI ($P=0.42$ and $P=0.33$, Kruskal Wallis, Kenya and Mali respectively).

I could confirm previous findings that the ABO blood group of RBC is associated with the rosetting frequency of field isolates. I found that there was a higher rosetting frequency of Kenyan field isolates in blood group A (median: 10.5, range: 1 – 70, n=6) versus blood group O RBC (1.5, 0 – 16, n=17) ($P=0.03$, Mann Whitney U). Although I did not see a high RF in blood group B RBC (0, 0-7, n=3). The association between RF and blood group A provides more evidence that rosetting of iRBC is linked to severe malaria, as this blood group has been associated with an increased risk of severe malaria (Pathirana 2005, Lell 1999).

2.5 Discussion

In this study I assessed two indicators of *P. falciparum* invasiveness (PMR and SI) and determined the relationship of these factors to malaria severity. The PMR measures parasite multiplication under standard conditions in the first cycle of *in vitro* culture, whereas the SI is determined from blood smears taken directly from patients. There was no significant difference in the PMR and SI of isolates from severe malaria patients compared to isolates from uncomplicated malaria patients in two diverse sites in Africa. This lack of association with severe malaria is in contrast to previous studies on PMR and SI from Thailand (Simpson *et al.* 1999; Chotivanich *et al.* 2000). I was able to exclude technical differences as the source of this discrepancy in results, because a small, blinded study of Thai isolates in my

laboratory confirmed the previously published data (Chotivanich *et al.* 2000). The PMR of the African isolates were mostly in the range of 1-6 fold (Figure 1), and are comparable to those of uncomplicated malaria isolates from Thailand (Chotivanich *et al.* 2000). However, isolates with high PMR in the 10-22 fold range characteristic of severe malaria cases in Thailand were absent from Africa. It seems extraordinary that many isolates in both Africa and Thailand should have such low PMR when every schizont produces approximately 24 merozoites. I only used healthy parasites that matured well for one cycle in culture for the PMR assays, however, I do not know how well the *in vitro* assay reflects PMR *in vivo*. The high PMR seen in Thai severe malaria isolates are in agreement with estimates of PMR *in vivo* from recent studies with the *P. falciparum* laboratory-adapted clone 3D7 infecting European volunteers (Cheng *et al.* 1997; Lawrence *et al.* 2000; Bejon *et al.* 2005), and from retrospective analysis of data collected during neurosyphilis treatment programmes (Gravenor *et al.* 1995; Simpson *et al.* 2002). To my knowledge, there are no comparable data available for *in vivo* PMR using fresh clinical isolates, or from any parasite clones in semi-immune individuals.

Why should there be a difference between Asia and Africa in the association of PMR and SI with parasite virulence? In Thailand (and most of Southeast Asia), malaria transmission levels tend to be low, there is little acquired immunity in the population, and severe malaria affects all age groups (Luxemburger *et al.* 1997). In contrast, in sub-Saharan Africa, malaria transmission tends to be moderate to high, older children and adults show partial immunity to malaria and severe disease mainly occurs in children under the age of 5 years. It is possible that the different levels of immunity to malaria in the two regions are responsible for the diverse results highlighted here. Partial immunity in African patients could delay the course of infection and lead to later presentation at hospital than Asian patients, by which time differences between isolates in PMR and SI may no longer be apparent. If such a scenario were true, one might expect African children experiencing their first malaria infections after maternally-acquired immunity has waned, to show higher PMR more typical of those seen in Thailand. However, there was no correlation between age and PMR in my study, and isolates from children between 6 and 12 months of age

who are unlikely to have had many previous malaria infections did not have higher PMR than isolates from older children (Kenya: infants, $n=5$, median PMR 1.5; older children, $n=25$, median PMR 2.3, $P=0.52$, Mann Whitney U. Mali: infants, $n=4$, median PMR 2.7; older children, $n=57$, median PMR 2.6, $P=0.79$, Mann Whitney U). There was also no significant correlation between age and SI, and isolates from children in the 6-12 month age group did not show significantly higher SI than isolates from older children (Kenya: infants, $n=13$, geometric mean SI 1.77 (1.38-2.16); older children $n=66$ geometric mean SI 1.86 (1.55-2.17), $F_{1,75}=0.15$, $P=0.70$, ANOVA. Mali: infants, $n=8$, geometric mean SI 1.70 (95% CI 0.06-3.34); older children, $n=96$, geometric mean SI 1.60 (1.08-2.12), $F_{1,100}=0.05$, $P=0.82$, ANOVA). The small number of children in the 6-12 month age group at both sites reflects the relative rarity of severe malaria in children under 1 year of age in these areas. A prospective study with active surveillance would be required to determine conclusively whether parasites from African children experiencing their first malaria infection show higher PMR and lower SI than parasites from older children.

An alternative explanation of my results is that parasites adapted to grow in semi-immune populations in sub-Saharan Africa have evolved different mechanisms of virulence compared to those adapted to grow in non-immune populations in Asia. The growth of parasites within the host during the asexual blood stage depends upon the multiplication potential of the parasite and the host's ability to remove infected red cells. It is plausible that in Asia, in the absence of significant levels of immunity, virulent parasites reach high parasite burdens within the host by having high PMR. In Africa, parasites with high PMR may not be effective in reaching high parasite burdens because of the presence of invasion-blocking antibodies in many hosts. In this case, parasite properties contributing to avoidance of the host's immune system clearance mechanisms may be more important in helping a parasite to achieve high parasite burdens and produce high levels of sexual stages and so maximise its transmission and fitness.

Whatever the explanation for the discrepancies between sub-Saharan Africa and Asia indicated by this study, my results do raise the possibility that different pathogenic

mechanisms of severe malaria may operate in parts of the world with different malaria transmission levels. This possibility is supported by studies of another potential virulence factor, rosetting, which is associated with severe disease in multiple sites in Africa (Carlson *et al.* 1990; Rowe *et al.* 1995; Kun *et al.* 1998), but not in Asia (Ho *et al.* 1991; Angkasekwinai *et al.* 1998). The hypothesis that Asian and African parasites differ in virulence properties could be tested in the absence of confounding effects of host immunity and host genetics by studying isolates infecting non-immune travelers.

One striking finding from this study was that isolates from Malian patients with hyperparasitaemia had significantly lower multiplication rates than other Malian isolates (Figure 1 and Table 1). Additionally, there was a negative correlation between *in vivo* parasitaemia and PMR across all isolates from both Kenya and Mali (Figure 2). These results seem counterintuitive, as one might expect that isolates reaching high parasitaemia *in vivo* would have the highest multiplication rates rather than the lowest. Density-dependent mechanisms are thought to occur *in vivo* to prevent excessive expansion of parasite populations, and can be determined by the availability of host red cells, the presence of host immunity or by a down-regulation of parasite invasiveness (Reuner *et al.* 1997; Bruce *et al.* 2000; Tyler *et al.* 2001; Bruce and Day 2003). My data are consistent with *P. falciparum* having the ability to down-regulate its multiplication rate in order to avoid overwhelming the host. I am not aware of any other experimental evidence to support such an idea, however, it is plausible that switching of invasion pathways (Dolan *et al.* 1990; Soubes *et al.* 1997; Cowman *et al.* 2000; Reed *et al.* 2000; Gaur *et al.* 2004) could potentially underlie such a process. It would be of great interest to examine the relationship between particular invasion pathways and parasite PMR and SI, and to determine whether the PMR and SI are stable properties of each isolate or can vary in response to external stimuli. An initial study found no association between invasion pathways and SI in Gambian field isolates (Baum *et al.* 2003). An alternative interpretation of the finding that isolates from hyperparasitaemic patients have very low PMR is that slowly multiplying parasites may do less damage to their hosts and therefore are able to reach high parasitaemias without causing the type of life-threatening disease

manifestations seen in the severe malaria group.

Could the relationship between PMR and *in vivo* parasitaemia explain the differences between African and Thai isolates? The interaction between parasitaemia and PMR was not commented upon in the Thai study, and the parasitaemias of the patients were not shown. However, high parasite burdens equivalent to those seen in Africa are not uncommon in Southeast Asia; therefore I think it unlikely that differences in parasitaemia levels explain the diverse results from the two areas. In the current study I have carried out additional analysis on the subset of samples with parasitaemias of less than 5%, and on all data after adjustment to take into account host parasitaemia effects. In both cases, there is no significant association between disease severity and PMR and SI in African children.

One similarity between Thai and African isolates is that red cell selectivity is highest at low parasitaemias, with isolates from patients with a parasitaemia of <2% having significantly higher SI than other groups (Figures 3 and 4). It may be that parasites with restricted invasion (high SI) are unable to reach high parasitaemias. A parallel to this occurs in *P. yoelii*, where virulent strains that invade normocytes reach higher parasitaemias than avirulent strains that have a strong reticulocyte preference (Walliker *et al.* 1976; Swardson-Olver *et al.* 2002). An alternative explanation for the high SI at low parasitaemia is that the presence of host anti-merozoite antibodies could lead to partial blocking of invasion and keep the parasitaemia low, while also agglutinating merozoites and increasing the number of multiple invasions (Miller *et al.* 1984).

In this study I have found no evidence to support a role for high parasite multiplication or lack of red cell selectivity as virulence factors in African children, a finding that contrasts sharply with previous work from Southeast Asia (Simpson *et al.* 1999; Chotivanich *et al.* 2000). It may be that other virulence factors such as rosetting (Carlson *et al.* 1990; Rowe *et al.* 1995; Kun *et al.* 1998) and platelet-mediated clumping (Pain *et al.* 2001) play a major role in parasite virulence in sub-Saharan Africa, whereas PMR and red cell selectivity are more important in Asia. If

distinct pathogenic mechanisms do underlie severe malaria in different parts of the world, this could have implications for the development of new strategies to treat and prevent severe disease. The differences in pathogenic mechanisms of severe malaria in regions of varying malaria transmission in both Africa and Asia are therefore worthy of further study.

CHAPTER 3: RBC selectivity is not associated with virulence in *P. chabaudi* infections

3.1 Abstract

Red cell selectivity was studied in *P. chabaudi* infections with three congenic *P. chabaudi* strains: CW0, N-lines and I-lines, which differ in their virulence to the host. The experimental design allowed me to look at the effects of parasitaemia, strain virulence, host immunity, course of infection and mosquito transmission on the selectivity index (SI). Invasion by *P. chabaudi* was found to be very unselective (geometric mean SI 1.17). There was no significant association of the SI with mosquito transmission ($P=0.41$), host immunity ($P=0.94$) or strain virulence ($P=0.65$, $P=0.25$ and $P=0.35$, pre-mosquito transmission, post-mosquito transmission naïve mice and post-mosquito transmission immunised mice respectively). The SI was overall negatively associated with parasitaemia ($P=0.01$), whereas this association was more strongly seen later during the infection. Independently of parasitaemia there was an increase in RBC selectivity from day 9 to day 14 of the infection ($P=0.02$, pre-mosquito transmission). The findings indicate that *P. chabaudi* virulence is not related to RBC selectivity. Furthermore, the interesting relationship between the time course of the infection and the SI reported here, is worth further investigation.

3.2 Introduction

The selectivity index (SI) is a measure of the red blood cell preference during merozoite invasion. It is defined as the observed number of multiple-infected RBC relative to that expected by chance. A SI of 1 implies RBC invasion to be completely random in terms of RBC selectivity, whereas deviations from 1 imply that the invasion process is selective, due to restriction of invasion into a subset of cells. RBC selectivity may be driven by both host factors and parasite factors ((Simpson *et al.*

1999), chapter 2). It has been mainly studied in *P. falciparum* isolates as described in the previous chapter (chapter 2) (Simpson *et al.* 1999; Chotivanich *et al.* 2000; Baum *et al.* 2003), but has been measured for a few other *Plasmodia* species (Simpson *et al.* 1999). The SI had only been studied in relation to virulence for *P. falciparum* isolates in Thailand (Simpson *et al.* 1999; Chotivanich *et al.* 2000), where it was found that parasites isolated from patients with severe malaria were less selective with lower SI than isolates from patients with uncomplicated malaria. The work presented in chapter 2 followed on from the Thai study to look at the SI of *P. falciparum* isolates from Mali and Kenya. In contrast to the previous findings from Thailand, there was no association between the SI and malaria severity of clinical isolates from African children. A full discussion of the possible reasons for the different findings in Africa compared to Asia are presented in the previous chapter. Whether these differences are driven by host factors, such as host immunity levels, the time of sampling the parasites, or intrinsic parasite variation, it is clear that more questions arose than were answered. Hence further work is needed to examine the complex relationship between SI and malaria virulence.

I decided to continue my work on the selectivity index of *Plasmodia* in relation to virulence in a different species – *P. chabaudi*. This species, a rodent malaria, is used frequently as a laboratory model to study *Plasmodia* virulence evolution – for example the effects of passaging, immune selection and strain competition (Mackinnon and Read 2003, 2004a; de Roode *et al.* 2005; Mackinnon *et al.* 2005a). *P. chabaudi* is a rodent model for *P. falciparum* malaria sharing some of the infection characteristics such as the cytoadherence properties of iRBC (discussed in (Mackinnon and Read 2004b)). The advantage of using this system here is the fact that the infection characteristics and virulence traits have been well studied and there are less confounding variables than there are when working with *P. falciparum* field isolates. An example of the course of infection of a *P. chabaudi* infection can be seen in figure 1, typically the peak parasitaemia occurs around day 7-9 after which the parasitaemia decreases until it is very low at day 14. The design of the study described here allowed me to look at differences in selectivity between unpassaged and passaged lines from the same ancestral strain with known differences in their

virulence to the host. The lines were derived from an avirulent ancestral line, CW0, and had been passaged through either naïve mice (N-lines) or immunised mice (I-lines). Furthermore, the same lines were transmitted through mosquitoes. It has been previously reported (Mackinnon and Read 2004a) that passaging both through naïve and immunised mice led to an increase in virulence of the lines, with the I-lines becoming even more virulent than the N-lines.

Figure 1:

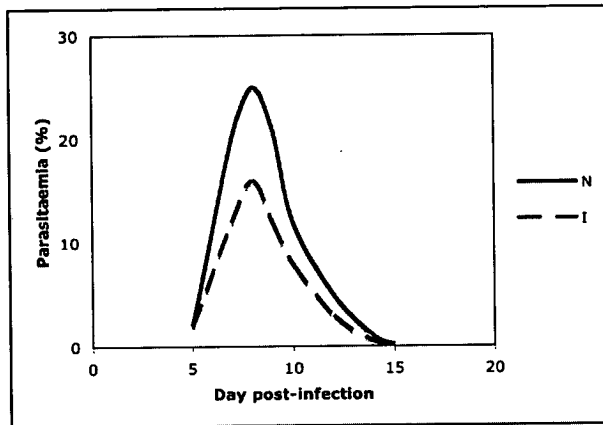


Figure 1: Schematic representation of the infection profile of naïve (solid line) or immunised mice (dashed line) infected with *P. chabaudi*. The parasitaemia (%) over the days 5 to 15 post-infection are shown. Adapted from (Mackinnon and Read 2003).

I hoped to gain insight into the possible factors driving RBC selectivity by looking at the SI of these congenic *P. chabaudi* lines: CW0, N-lines and I-lines. The first question I could address is whether in *P. chabaudi* infections there is a relationship between the SI and parasite virulence. One may expect that virulent clones are less selective as this has been reported previously for *P. yoelii*, where the virulent clones can invade normocytes (Walliker 1976), and for *P. falciparum* in Thailand (Simpson 1999). Similarly it is possible to address whether mosquito transmission may affect the SI. Mosquito transmission has been shown to decrease virulence of *P. chabaudi* lines and lead to an increase of another known virulence factor, rosetting (Mackinnon *et al.* 2005). If there were a relationship between the SI and virulence, one would expect an increase in SI after mosquito transmission as virulence

decreases. Another question is whether the SI changes over the time-course of an infection. During the infection, the host mounts an immune response to the asexual stages of *P. chabaudi* and immunity may have an effect on the SI. Finally with this experimental design I could also look at the SI in naïve versus immunised mice. Parasitaemia-limiting factors in the immunised mice may be expected to lead to an increase in SI of the parasite lines. The results of this study are presented below.

3.3 Materials and Methods

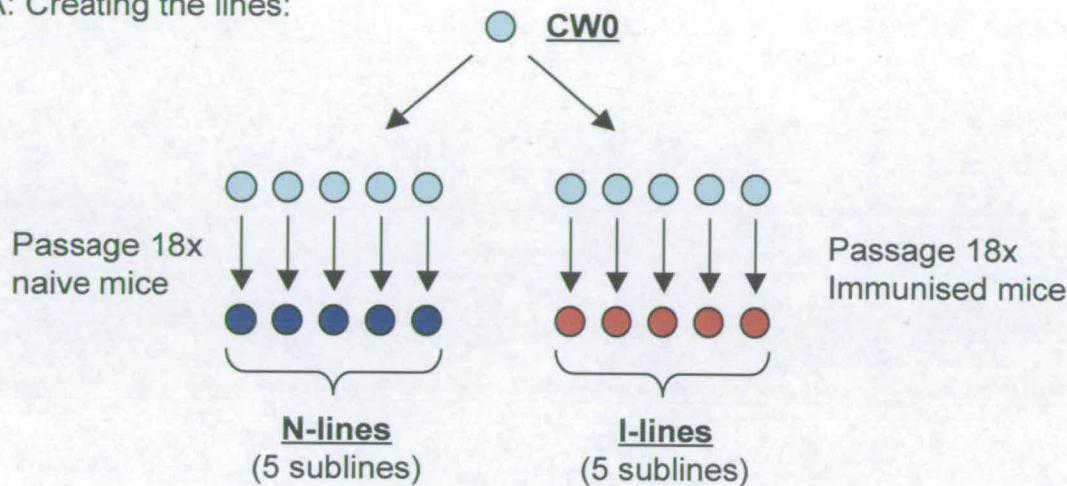
3.3.1 Experimental design

The experimental design for generating the lines has been described in detail elsewhere (Mackinnon and Read 2004a) and is shown in figure 2. In brief, two lines of *P. chabaudi* were selected from the ancestral avirulent line (CW-0, referred to here as CW0) by serial passaging through either naïve mice (N-lines) or immunised mice (I-lines) (mice: female C57Bl/6J, 7-10wk old). Immunised mice had been immunised with 10^4 parasites of a different clone, ER, on average 3 weeks prior to their use in the experiments detailed here (Mackinnon 2004). The N- and I-lines consisted of 5 independent sublines (figure 2A) and these had each been passaged once through mosquitoes. In the experiments reported here, for the pre-mosquito transmission experiments two mice were infected per subline giving a total of 10 mice for the N- or I-lines, and a further five mice were infected with the ancestral line (CW0) (figure 2B:1). For the post-mosquito transmission experiments the sublines were transmitted once through *Anopheles stephensi* mosquitoes. As for the pre-mosquito transmission experiments, two mice were infected per subline and five mice were infected with the ancestral line (figure 2B:2). 5 mice were also infected with the ancestral line, which had not been transmitted through the mosquito (not shown in figure). Furthermore, in the post-mosquito experiments both naïve and previously immunised mice were infected with the three *P. chabaudi* lines as described above (figure 2B:2). Within this dataset the effect of host immunity on the SI could be compared for day 7. The analysis was limited to day 7, as the

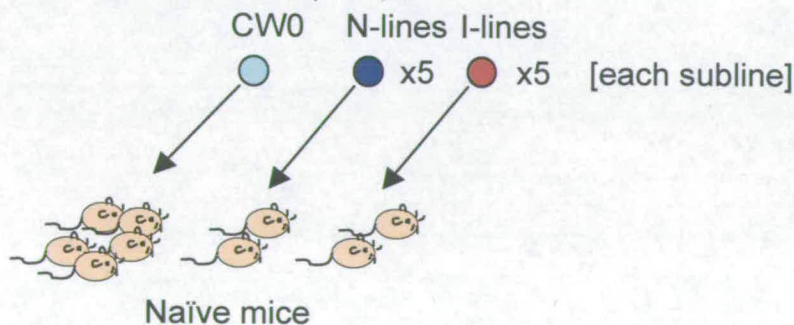
parasitaemia in the immunised mice was too low to accurately assess the SI later in the infection. For the pre-mosquito experiment the SI was counted from slides made on day 7, 9, 11 and 14 of the infection and for the post-mosquito in naïve mice experiment the SI was counted from slides made on day 7, 9 and 11 of the infection. The pre- and post-mosquito experiments were separate experiments done at different times and comparisons for a mosquito transmission effect are not strictly valid. Within the post-mosquito experiment it was possible to analyse the effect of mosquito transmission on the CW0 line, as 5 mice were infected with the ancestral line as described above. There were two repetitions of the pre- and post-mosquito experiments.

Figure 2: Schematic overview of the experimental design.

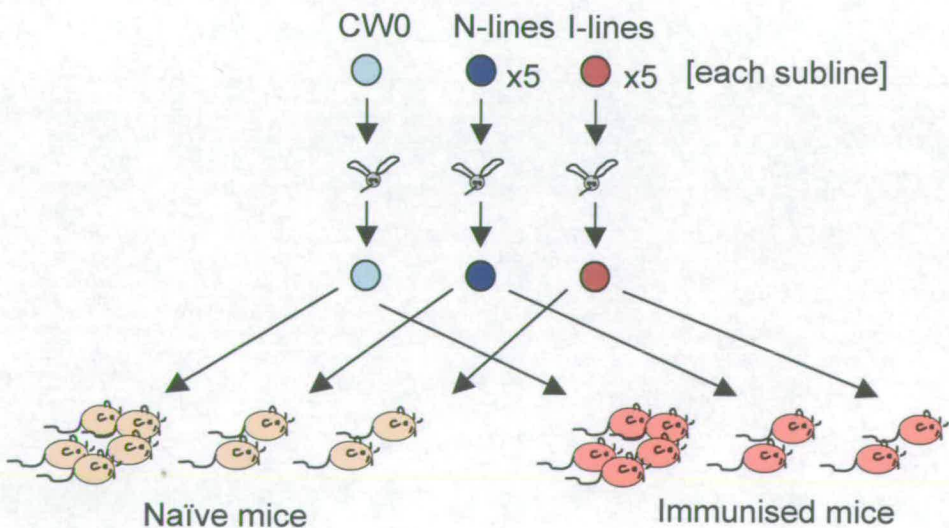
A: Creating the lines:



B: 1. Pre-mosquito transmission experiment:



2. Post-mosquito transmission experiment:



3.3.2 Selectivity index

The slides included in this study were prepared on days 7, 9, 11 and 14 of the study. They are thin smears stained with Giemsa. Generally 300 ring-infected RBC (iRBC) were counted per slide (Leica, 100x magnification, brightfield) and the number of rings in each RBC was recorded. In some cases due to lower parasitaemias less than 300 iRBC were counted, the minimum though being 100 iRBC counted. The SI was calculated as described previously (Simpson *et al.* 1999) Briefly, the number of observed multiple-infected RBC (i.e. 2 or more rings per RBC) was divided by the number of expected multiple-infected RBC. The latter was calculated according to a Poisson distribution, where lambda is $(-\ln(1-\text{parasitaemia}))$. The parasitaemia was counted as proportion of infected RBC per 500 RBC by Dr M. Mackinnon. The proportion of reticulocytes was counted on the Giemsa stained slide as reticulocytes are recognisably larger than RBC and stain a slightly darker shade of blue. This measure gave the proportion of reticulocytes out of all red blood cells. The red blood cell count was measured by flowcytometry (Coulter Electronics, Luton, UK).

3.3.3 Statistical analysis

All statistical analyses were done with Statview 5.0.1. The analyses were done with an ANOVA, using the log-transformed SI and log-transformed parasitaemia. The three experiments - pre-mosquito, post-mosquito in naïve mice and post-mosquito in immunised mice - were analysed independently for the main effects of line and day of infection. They were then pooled to look at the effects of treatment (naïve or immunised mice) or mosquito transmission (pre- or post-mosquito transmission). Here, the results from the minimal model are shown where non-significant effects and interactions were removed from the analyses. Significant interactions are described in the results. All analyses were carried out with and without including the $\log(\text{pt})$ as a covariate. Furthermore, all analyses were repeated excluding all parasitaemias that were $<1\%$ to allow for the bias of errors in parasitaemia counting at low densities (see below). Correlations between SI and the variables proportion of

reticulocytes and RBC count were made with a non-parametric Spearman rank correlation of raw data (SRC).

3.4 Results

3.4.1 The SI is associated with parasitaemia

There was a negative correlation of the SI with parasitaemia ($P=0.01$, $Rho=-0.173$, SRC, figure 3A) when all data points were analysed together. A correlation between the SI and parasitaemia was seen previously for *P. falciparum* (see chapter 2) and other studies (Simpson *et al.* 1999) and is mainly due to the high SI at the lower parasitaemias. Interestingly here, as is visible from the figure, the parasitaemia was not associated with the SI on all days – with day 7 being an exception – this is discussed in more detail below. To examine the effect of parasitaemia on the SI, all of the analyses described below were performed with and without adjustment for parasitaemia.

I considered the possibility that the high SI at the lower parasitaemias may be due to counting error in parasitaemia. The method of calculating the SI from a ratio means that the SI is particularly sensitive to deviations from the real parasitaemia at lower parasitaemias. This is because the expected number of multiple-infected RBC will be very small at low parasitaemias, and then even 1 observed multiple-infected RBC can create a large SI. This is illustrated in figure 3B. I calculated the number of multiple-infected RBC one would expect to see if the $SI=1.173$ over the range of parasitaemias that were included in this study. (The $SI=1.173$ is the mean SI from my data from samples above 5% parasitaemia, so excluding any bias in the SI at the lower parasitaemias.) I then increased and decreased the parasitaemia by a set amount (in this case by both 1% parasitaemia or 0.1% parasitaemia), keeping the number of multiple-infected RBC constant, and recalculated the SI. The new SI that one obtains, if the parasitaemia is over- or underestimated, are shown in the figure 3B in red lines (1% parasitaemia) and blue lines (0.1% parasitaemia). This highlights

the problem that the SI is liable to change more in an upward direction at the lower parasitaemias if there is an error in the parasitaemia, thereby introducing, on average, a positive bias in SI at low parasitaemias. Included in the graph are also the actual data points from my work with *P. chabaudi* (figure 3B). Although the possibility of a parasitaemia induced error was considered many data points lay beyond the error lines. Thus, there appears to be a real biological effect leading to the high SI at the lower parasitaemias.

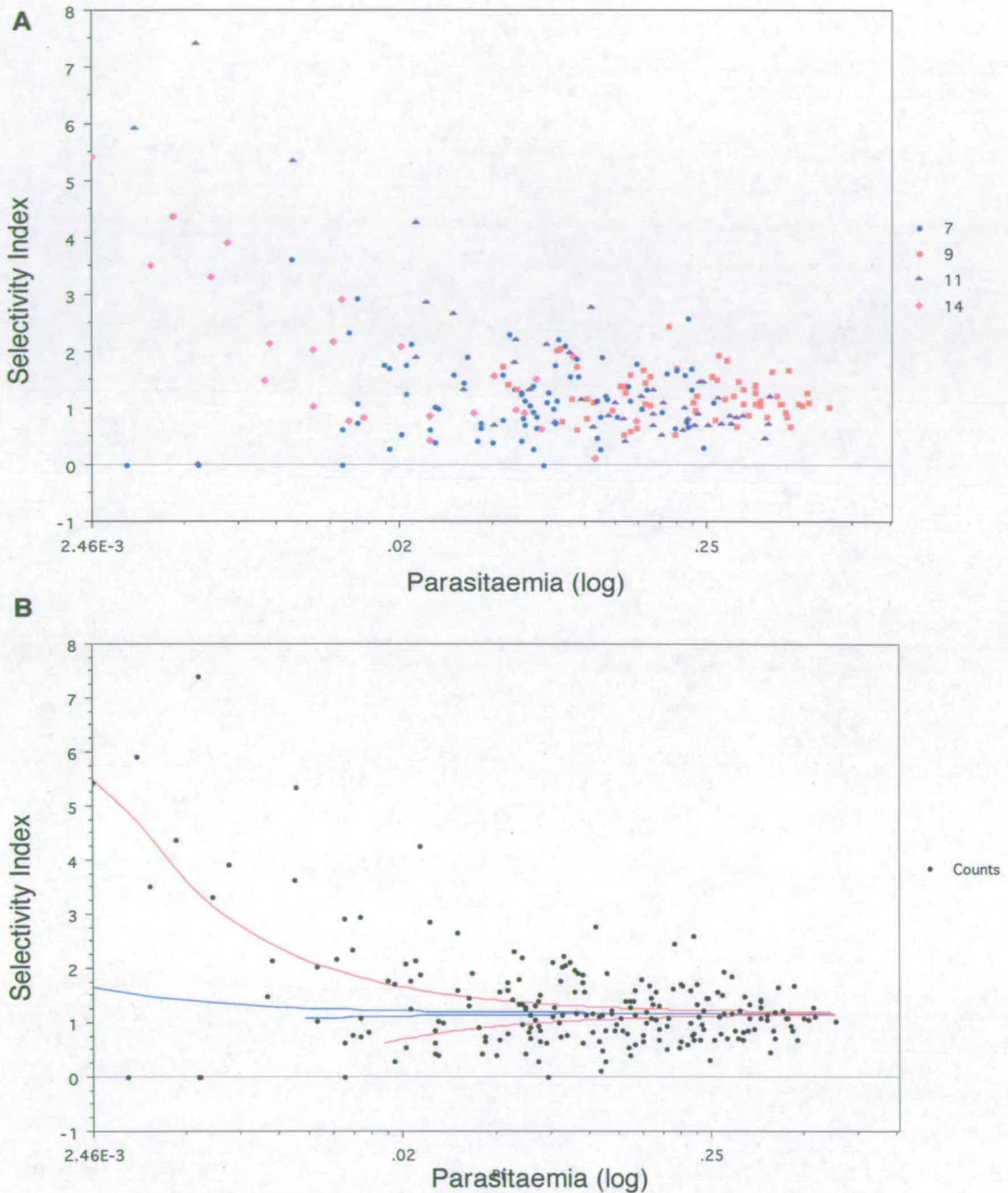
Figure 3:

Figure 3: Scatterplot of the SI against the parasitaemia, showing the data for all three experiments. In plot A the days of infection are indicated by separate symbols (day 7: purple circles, day 9: red squares, day 11: violet triangles, day 14: orange diamonds). In plot B besides the data (counts: black circles) the SI calculated for errors in parasitaemias (1%pt error red lines; 0.1% pt error blue lines) are indicated. See results for more details.

3.4.2 SI is not related to clone virulence

At all time points I had data for three parasite lines: CW0, N-lines and I-lines (figure 4A,B,C), although in the pre-mosquito dataset I only had data from 8 CW0 samples, compared to 66 and 68 slides for the I- and N-lines. The geometric mean (95% CI) for the three lines in the pre-mosquito data set was 1.411 (0.623-2.199), 1.169 (1.021-1.317) and 1.214 (0.966-1.462) for CW0, N-lines and I-lines respectively. There was no significant line difference between the three parasite lines when all days were analysed in either the pre-mosquito data set ($F_{2,139}=0.43$, $P=0.65$), the post-mosquito in naïve mice data set ($F_{2,49}=1.44$, $P=0.25$) or the post-mosquito in immunised mice data set ($F_{2,12}=1.23$, $P=0.33$). This was also the case if the parasitaemia or alternatively if only parasitaemias above 1% parasitaemia were included in the analyses (not shown).

I also looked at the effect of line on the SI on the individual days of infection (see figure 4 A,B,C). There was no difference on any of the days between the SI of the CW0, N-lines or I-lines in the pre-mosquito data set ($F_{2,37}=0.24$, $P=0.79$; $F_{2,37}=2.09$, $P=0.14$; $F_{2,34}=3.03$, $P=0.06$ and $F_{2,22}=0.69$, $P=0.52$ for day 7, 9, 11 and 14 respectively). The trend on day 11 is due to parasitaemia differences between the lines and is not maintained if parasitaemia is included in the analysis ($F_{2,31}=1.10$, $P=0.35$). Similarly, only on day 11 is there a significant difference in the SI of the three lines after mosquito transmission ($F_{2,18}=0.02$, $P=0.99$; $F_{2,20}=1.02$, $P=0.38$ and $F_{2,5}=15.11$, $P=0.003$ for day 7, 9 and 11 respectively). Again this can be explained by differences in the parasitaemias ($F_{1,3}=1.94$, $P=0.26$), although only the CW0 and I-lines were compared in this analysis as the N-lines had to be excluded as there was only one sample.

Figure 4:

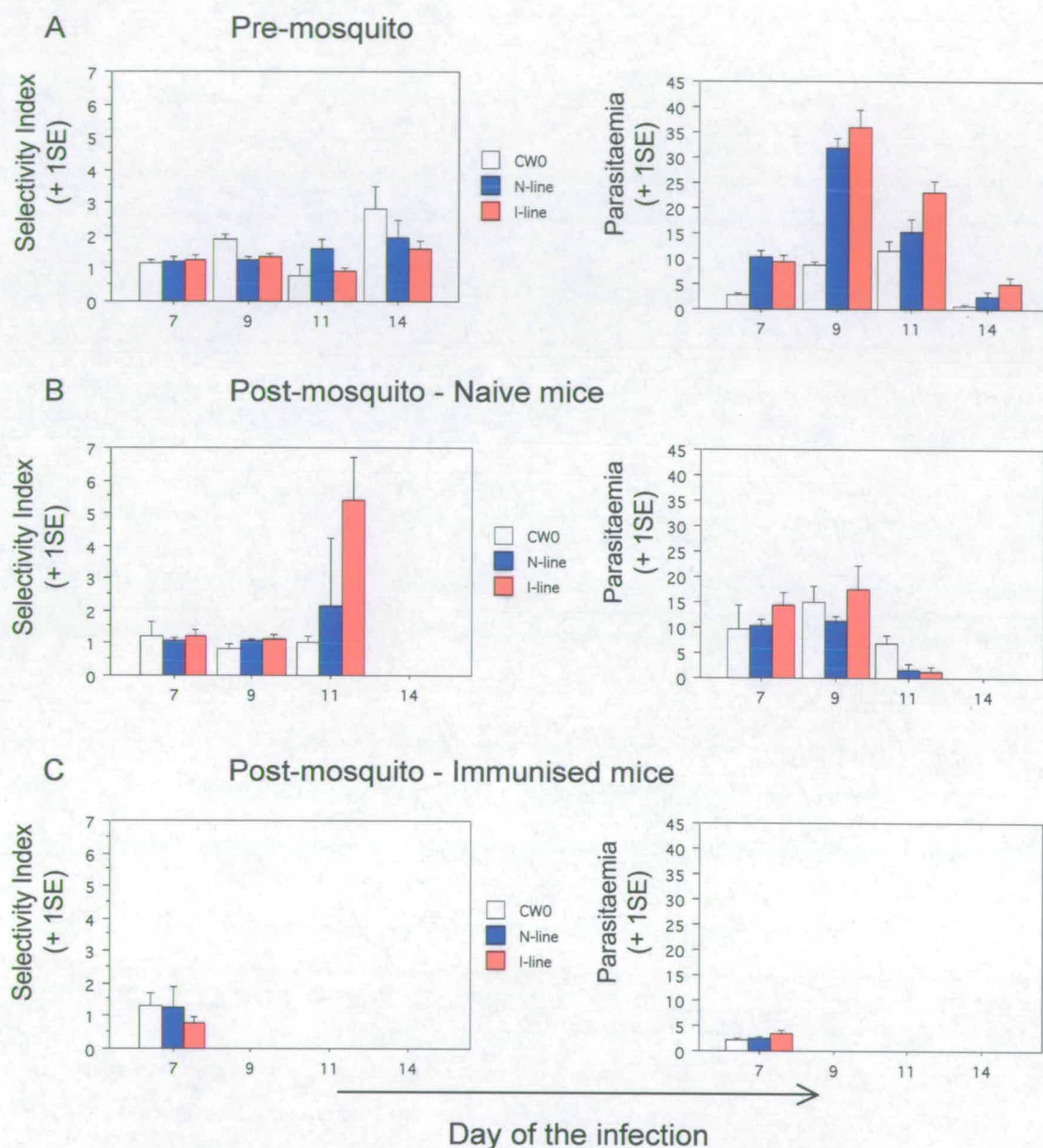


Figure 4: SI (left panel) and parasitaemia (right panel) of the three *P. chabaudi* lines: CW0 (white bars), N-line (blue bars) and I-line (red bars). The SI was counted for pre-mosquito transmission infections in naïve mice (A) and post-mosquito infections in both naïve (B) and immunised mice (C). The x-axis shows the day of the infection on which the slides were prepared. The mean and 1 standard error are shown.

Furthermore, I compared the non-passaged ancestral line (CW0) versus the passaged lines that had evolved to be more virulent (N- and I-lines, grouped as Non-CW0). There was no significant difference in either the pre-mosquito or post-mosquito data set ($F_{1,40}=0.71$, $P=0.40$ and $F_{1,50}=1.85$, $P=0.18$, respectively).

3.4.3 Mosquito transmission and the selectivity index in *P. chabaudi*

The experimental design allowed me to compare the selectivity index of the *P. chabaudi* isolates before and after mosquito transmission. I was interested in this as previously it has been shown that mosquito transmission can reduce virulence and increase the rosetting frequency of isolates (Mackinnon *et al.* 2005). The overall SI before mosquito transmission was 1.201 (geo. mean, 1.063 – 1.343, 95% confidence interval, $n=142$) and post-mosquito transmission in naïve mice was 1.115 (0.725 – 1.505, $n=52$). There was no significant effect of mosquito transmission on the selectivity index ($F_{1,192}=0.68$, $P=0.41$) (figure 4 A,B). This was significant if the parasitaemia was included in the analyses ($F_{1,190}=13.08$, $P=0.0004$), but there was also a strong interaction as the parasitaemia was considerably lower after mosquito transmission (parasitaemia x MT [pre/post mosquito] interaction: $F_{1,190}=11.11$, $P<0.001$), which makes the results hard to interpret. There was no effect of mosquito transmission on the RBC selectivity if the line was included in the analysis [i.e. CW0, I, N] ($F_{1,190}=0.40$, $P=0.53$). As the compared datasets were from experiments carried out at different times and therefore not strictly comparable, I also analysed an in-built pre- and post-mosquito transmission control for the CW0 line. In the experimental design the ancestral line was included in the same experiment as the mosquito transmitted CW0 line allowing a direct comparison of the effects of mosquito transmission. There was no significant difference between the SI before or after mosquito transmission of the ancestral line CW0 ($F_{1,29}=0.47$, $P=0.50$). Thus the decrease in virulence of *P. chabaudi* lines after mosquito transmission (Mackinnon *et al.* 2005) cannot be explained by an increase in RBC selectivity.

3.4.4 The SI over the course of the infection

The selectivity index was measured at multiple time-points during the infection as seen in figure 4 (left panel). The corresponding parasitaemias can be seen in the right hand panel (figure 4). I had data from day 7, 9, 11 and 14 for the pre-mosquito transmission infection. For post-mosquito transmission experiments, I had data for day 7, 9 and 11 in naïve mice, and only for day 7 of the infection in immunised mice because thereafter, parasitaemias were too low to obtain counts for SI calculations. The effect of the day of infection on SI varied between the pre- and post-mosquito groups and therefore is discussed separately below.

In the pre-mosquito data there was no day effect on the SI ($F_{3,134}=1.84$, $P=0.14$, figure 4A). If parasitaemia was included in the model, the day of infection was significantly associated with the SI ($F_{3,134}=6.87$, $P=0.0002$), but there was a significant interaction of the parasitaemia with the day of the infection (Day x parasitaemia interaction: $F_{3,134}=8.52$, $P<0.0001$) (figure 4A, right panel). Further investigation found that the interaction between the day of the infection and the parasitaemia was explained by a lack of a correlation between the SI and parasitaemia on day 7 in contrast to the negative correlation on the other days ($P=0.13$, $P=0.002$, $P=0.003$, $P=0.0003$, SRC, Day 7, 9, 11 and 14 respectively). Excluding day 7 from the model when looking at the effect of day of infection and parasitaemia on the SI removed the significant interaction of the two terms (Day x parasitaemia interaction: $F_{2,96}=0.820$, $P=0.44$). Nevertheless, the day of infection still had a significant effect on the SI ($F_{2,96}=3.868$, $P=0.02$), thus indicating that there is a day effect. As can be seen in figure 4A, the SI increases over time, with the exception of day 11.

In the post-mosquito data set there was a significant effect of the day of infection on the SI ($F_{2,49}=7.03$, $P=0.002$, figure 4B). If parasitaemia was included in the analysis there was no day effect on the SI ($F_{2,46}=2.47$, $P=0.1$) and there was no interaction between the parasitaemia and the day of infection. The high SI seen on day 11 of the post-mosquito naïve mice data set was due to very low parasitaemias on that day of

the infection (see above). When all parasitaemias below 1% were excluded from the analyses then there was no day effect on SI in either the pre-mosquito or post-mosquito data set ($F_{3,131}=1.53$, $P=0.21$ and $F_{2,47}=1.96$, $P=0.15$, respectively).

3.4.5 The SI in naïve compared to immunised mice

I looked at the SI of the selected N-lines and I-lines when grown in naïve versus immunised mice (figure 4 B,C). Only data for day 7 of the infection in immunised mice were available for this comparison because the parasitaemias were too low later in the infection. Although the parasitaemia was considerably lower in the immunised mice, there was no difference in the SI between the naïve and immunised mice ($F_{1,34}=0.01$, $P=0.94$) (figure 4B,C). The parasitaemia was not included in the analysis as there was no association between the SI and the parasitaemia on day 7 ($Rho=0.033$, $P=0.84$, SRC).

From the graphs (figure 4) one can see that the low parasitaemia on day 7 of the infection post-mosquito in immunised mice does not lead to the same larger mean and variance in SI as it does on both day 11 of the post-mosquito in naïve mice and day 14 in the pre-mosquito data set (figure 4). These may be considered to be roughly equivalent in terms of immunity, as supported by their similar parasitaemias, though different in the stage of the infection. This raises the question as to whether the high SI is more likely to occur at a later stage in the infection when possibly strain-specific immunity, anti-merozoite antibodies or some other parasitaemia-limiting factor is present. To address this, I repeated the above analysis but compared the day 7 post-mosquito data in immunised mice to the results from day 11 in naïve mice (post-mosquito). The parasitaemia between these two groups did not differ ($F_{1,24}=0.07$, $P=0.79$). However, there was a significant difference in the SI between the two groups ($F_{1,21}=5.62$, $P=0.03$). This was also the case if the parasitaemia was included in the analysis (Immune/naïve: $F_{1,20}=8.08$, $P=0.01$ and log(Pt): $F_{1,20}=12.53$, $P<0.01$). If parasitaemias below 1% were excluded from this analysis then there was a significant difference when parasitaemia was included in the analysis

(Immune/naïve: $F_{1,18}=4.766$, $P=0.04$ and $\log(\text{Pt})$: $F_{1,18}=4.21$, $P=0.06$), but there was no effect when parasitaemia was not included in the analysis ($F_{1,19}=1.654$, $P=0.22$).

3.4.6 The SI is not associated with RBC count or the proportion of reticulocytes

I found a weak correlation between the SI and the RBC count on the day the SI was counted ($\text{Rho}=0.188$, $P=0.03$, SRC, figure 5A), but the latter is strongly correlated with the parasitaemia ($\text{Rho}=-0.440$, $P<0.0001$, SRC). When parasitaemia was included in the analysis there was no correlation between the SI and the RBC count ($F_{1,139}=0.30$, $P=0.59$). Furthermore, looking at the effect of both the RBC count on the day of count and the day of the infection, there was no effect of the RBC count on the SI ($F_{1,137}=2.40$, $P=0.12$, ANOVA). There was no correlation between the SI and the proportion of reticulocytes present in the mouse blood ($\text{Rho}=0.170$, $P=0.19$, SRC, figure 5B). There was also no association if the day of infection was included in the analyses ($F_{1,55}=2.23$, $P=0.14$). This was expected, as *P. chabaudi* does not have a reticulocyte preference.

Figure 5:

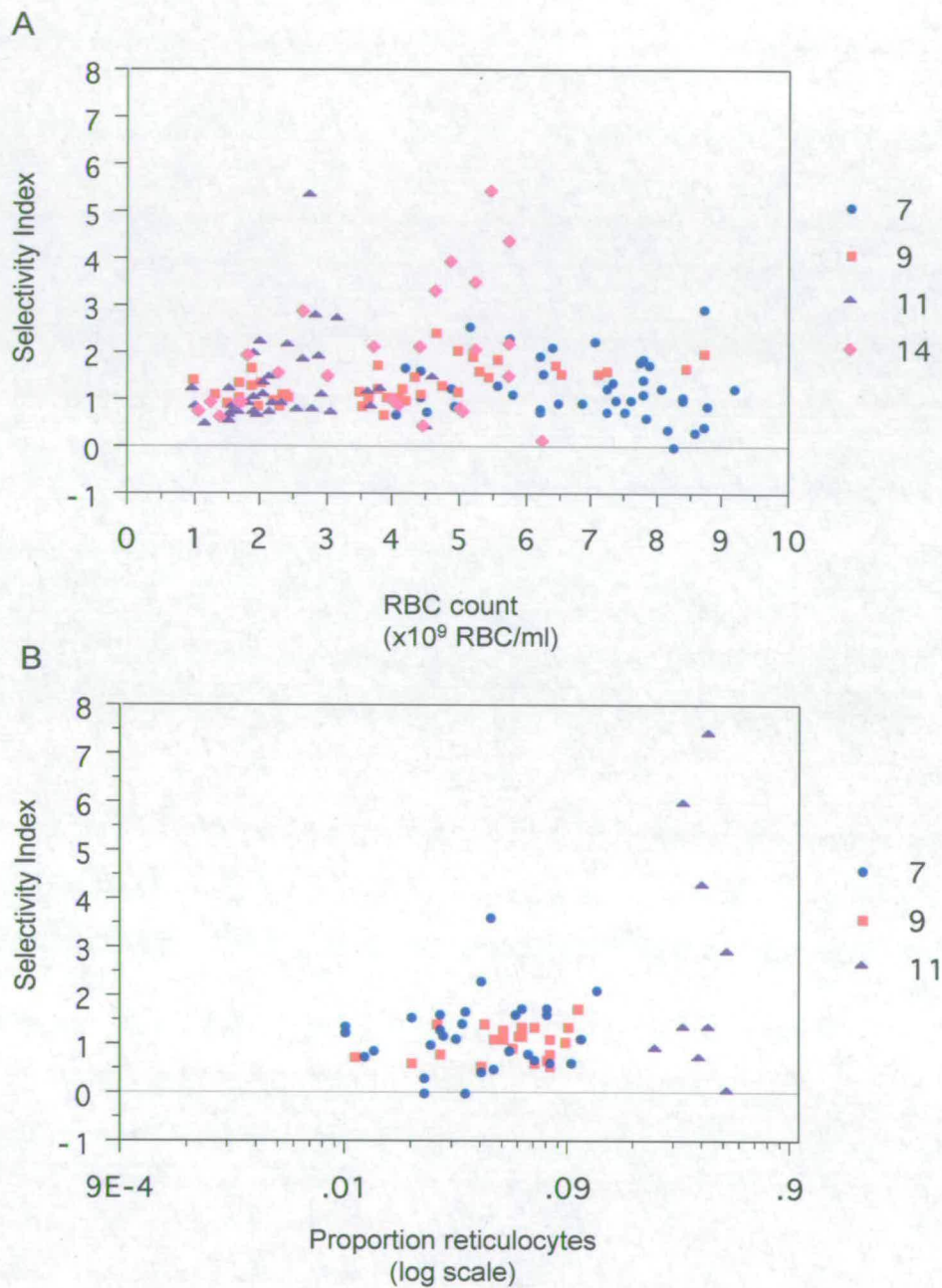


Figure 5: Scatterplots showing that there is no correlation between the SI and either RBC count (A) or the proportion of reticulocytes (B). The points are separately labeled for the day of the infection (day 7: purple circles, day 9: red squares, day 11: violet triangles, day 14: orange diamonds).

3.5 Discussion

I looked at the SI in congenic *P. chabaudi* lines that differed in virulence to address a number of questions as described earlier: i) Is the SI related to clone virulence? ii) Does mosquito transmission affect the SI of the three parasite lines? iii) Does the SI change over the time course of the infection? and iv) Does immunity affect the SI?. The following results were found. The three lines, CW0, I-lines and N-lines that were looked at differ in their virulence (Mackinnon and Read 2004a), but no significant difference in the SI in relation to virulence was seen. Similarly, there was no difference in the SI between the ancestral CW0 line and the passaged lines. In accordance with these findings mosquito transmission did not change the SI of the congenic parasite lines. There was an effect of the day of infection on the SI. In the post-mosquito data set, the change of the SI over the days of infection could be explained with parasitaemia. The lower parasitaemias in the later days of the infection (day 11) were negatively associated with high SI. In the pre-mosquito data set the relationship between the day of infection and parasitaemia with the SI is more complex. There is a significant effect of both parasitaemia and the day of infection on the SI. The pre-mosquito and the post-mosquito data set show the same negative relationship of the SI with parasitaemia and/or day respectively, the general result being an increase in SI over time. Furthermore I found that *P. chabaudi* growing in naïve or immunised mice did not differ in their selectivity. I saw no correlation between the SI and the proportion of reticulocytes, which is expected as *P. chabaudi* does not have a reticulocyte preference and shows a low overall selectivity. There was also no correlation between the SI and the RBC count when adjusted for RBC parasitaemia.

It is an unexpected finding that the SI of the three different lines is so similar, particularly on day 7 before the changes in parasitaemia occur, and this both before and after mosquito transmission. I had expected that CW0 would be the most selective line of the three as it is the least virulent of the three lines, and that passaging to create the N- and I-lines may lead to a decrease in selectivity along with the increase in virulence. Virulence of these lines was defined by minimum RBC

count, but there was also a positive relationship between maximum parasitaemia and virulence (Mackinnon and Read 2004a) and I was trying to identify whether that may be in part due to decreased RBC selectivity. The results though show no difference in SI between the lines and there is no evidence to support the hypothesis that *P. chabaudi* virulence is associated with RBC selectivity.

The underlying mechanisms of the virulence phenotypes of different *P. chabaudi* clones are not well understood. It is known that peak parasitaemia in an infection is positively correlated with the level of virulence caused to the host (Mackinnon 1999, Mackinnon 2003) and the early replication rates between N- and I-lines (M. Mackinnon, personal communication). There may be other factors, besides the asexual replication rate, which allow the more virulent strains to reach higher peak parasitaemias, persist for longer in the host and cause more damage to the host. Other mechanisms explaining how the different *P. chabaudi* clones cause more or less damage to their host are being elucidated. It is currently being studied whether the different clones trigger different levels of immunopathology, in terms of for example cytokine production (e.g. TNFalpha) in the host (Grainne Long, personal communication). This follows from previous work where hosts with different susceptibility to *P. chabaudi* infections show different immune responses (Cross and Langhorne 1998) and higher TNFalpha levels have been correlated with severe *P. falciparum* malaria (Allan *et al.* 1995).

How do my findings match the previous work looking at the association between field isolate SI and virulence in *P. falciparum*? In Thailand clinical isolates from severe malaria patients had lower RBC selectivity than isolates from uncomplicated malaria cases (Simpson *et al.* 1999). In my work, however, the SI of Malian and Kenyan field isolates was not associated with malaria severity in children (chapter 2). Similarly to the results with African *P. falciparum*, in the rodent model described in this chapter, there was no association between SI and the three congenic *P. chabaudi* lines, which have been shown to differ significantly in their virulence to the host. Thus, the studies with *P. falciparum* and *P. chabaudi* are inconsistent with

the Thai results, where it was found that the SI is lower in *P. falciparum* isolates that cause severe malaria and are hence more virulent.

There are of course limitations to the *P. chabaudi* model. The SI of this *Plasmodia* has, to my knowledge, not been studied before, although it is known that *P. chabaudi* does not have a reticulocyte preference (Jarra and Brown 1989). It is important to note that the SI of *P. chabaudi* is very low with a geometric mean of 1.167 (1.035 – 1.299 95% CI, n=209). The SI for *P. falciparum* is higher with a mean around 1.7 (see chapter 2). This may have important implications for the interpretation of the present results. As RBC selectivity is already very low in the ancestral line, CW0, then that may explain why no further reductions in selectivity in the passaged, more virulent N- and I-lines, is seen. It is possible that the adaptation of *P. chabaudi* from its natural host into mice has selected for isolates that can invade all RBC unselectively. It would be interesting to know what the SI of *P. chabaudi* is in its natural host the thicket rat, *Thamnomys rutilans*. Due to its low SI, *P. chabaudi* is not a good model to study RBC selectivity. There are other rodent models available; one for example is *P. berghei*, which has been shown to have a very high SI of 25.9 (Simpson *et al.* 1999). *P. berghei* is known to have a strong reticulocyte preference, which has also been shown to vary in different hosts (Ott 1968; Mons 1990). Similarly the selection for more virulent *P. yoelii* lines has shown that parasites become more virulent by becoming less selective and invading normocytes (Walliker *et al.* 1976), which would indicate a change in the SI. However, these models more accurately reflect *P. vivax*, which has a stronger reticulocyte preference than *P. falciparum* (Mons 1990).

In the *P. chabaudi* model the low parasitaemias mainly occur later in the infection and these are associated with higher selectivity. Interestingly though, it can be seen from the data in figure 4, that low parasitaemias in CW0 on day 7 (figure 4A) and in all lines on day 7 in immunised mice (figure 4C) do not seem to associate with higher SI. Thus this raises the possibility that low parasitaemias are less likely to be associated with higher SI earlier in the infection, than later in the infection, i.e. day 11 or 14. This suggests that during the infection, strain-specific immunity, anti-

merozoite antibodies or other parasitaemia-limiting factors generate the negative relationship between parasitaemia and higher SI. I investigated this by comparing the SI of *P. chabaudi* on day 7 of the infection in the immunised mice versus the SI of *P. chabaudi* on day 11 in the naïve mice (figure 4B,C). I found that these two groups did not differ statistically in their parasitaemia, but did differ significantly in their SI. This suggests that for a similar parasitaemia range, the SI is higher at a later stage in the infection. As the low parasitaemias are not associated with the higher SI on day 7 of the infection in immunised mice, one may conclude that the immunity generated by previous immunisation with a heterologous strain does not affect RBC selectivity. One may postulate that strain-specific immunity may affect RBC selectivity. Strain-specific immunity is known to play an important role in controlling *P. chabaudi* infections (Mota *et al.* 2001; Martinelli *et al.* 2005). Following from these findings it would be interesting to look in particular at low parasitaemia infections over the time-course of an infection to further investigate whether the association between low parasitaemias and higher SI generally occurs later in the infection.

Finally, a correlation between the SI and the parasitaemia was seen. As in my previous chapter (chapter 2) and in previous work (Simpson *et al.* 1999), this is mainly due to the higher SI at low parasitaemias (roughly below 2% parasitaemia). I considered whether the bias in the SI at low parasitaemias might partly be due to errors in counting and the method for calculating the SI, which is based on a ratio of the observed multiple-infected RBC divided by the expected multiple-infected RBC. I showed this with a theoretical calculation of SI for a set number of multiple-infected RBC and a change in parasitaemia by either 1% or 0.1%. The latter is very likely to occur at any parasitaemia. An error of 1% is less likely to occur, but may be possible. In this study the results fall beyond an error of 1% and therefore are likely to be explained by a biological effect rather than an error in counting the parasitaemia (figure 3B). Although this is only the first attempt to explain why the association between the SI and parasitaemias is seen, it will be important to try and sort out to what extent the SI at the lower parasitaemias is due to parasitaemia errors versus being a real effect. This could be analysed in a careful study both controlling for parasitaemias and accurately quantifying the low parasitaemias by other means

such as real-time PCR, which could also give a better understanding of the SI at low parasitaemias over the course of an infection as discussed above.

CHAPTER 4: Invasion profiles of Kenyan *P. falciparum* field isolates

4.1 Abstract

Invasion of RBC by *P. falciparum* occurs by multiple pathways, which can be studied by looking at invasion into enzyme treated RBC. Although most research to date has focused on laboratory strains, a small number of studies have looked at the invasion profile of field isolates from outpatients. As yet, there are no reports on the invasion pathways used by field isolates from patients with severe malaria. Here I looked at the invasion profiles of 31 Kenyan isolates from children with uncomplicated or severe malaria. There was considerable variation in regards to the invasion pathways used, the majority of isolates (23/31) invade by a neuraminidase resistant, trypsin sensitive pathway. The molecular basis of this pathway has not been clearly defined. There was an equal distribution of the isolates from children with uncomplicated or severe malaria across the different invasion profiles. Furthermore, the isolates from children with severe malaria did not significantly differ in their inhibition by the different enzyme treatments compared to isolates from children with uncomplicated malaria ($P>0.1$, all enzymes, Mann Whitney U). This work shows that there is no association between invasion profiles and malaria severity and that the majority of Kenyan isolates invade by a different pathway than described for Brazilian (Lobo 2004) or Gambian (Baum 2003) field isolates. This indication of geographical differences in the preferred invasion pathway used by *P. falciparum* isolates may have an important implication for vaccine design.

4.2 Introduction

Erythrocyte invasion by merozoites is a crucial step during the intra-erythrocytic asexual cycle of *P. falciparum*. This stage of the parasite infection causes an estimated 500 million clinical malaria cases per year (Snow *et al.* 2005) and understanding the parasite's biology is important to identify new drugs and vaccines.

Four key stages of invasion have been described: merozoite attachment to the erythrocyte, apical reorientation, tight-junction formation and finally erythrocyte invasion. The molecular basis of these processes is not well understood. It is now clear that in contrast to *P. vivax*, which requires the Duffy antigen for invasion, multiple pathways mediate erythrocyte invasion by *P. falciparum* merozoites (Gaur *et al.* 2004). Invasion pathways have been identified by looking at the invasion of merozoites into mutant erythrocytes (Pasvol and Wilson 1982; Hadley *et al.* 1987) and by removing RBC surface receptors with enzyme treatments (Mitchell *et al.* 1986; Perkins and Holt 1988; Dolan *et al.* 1994; Binks and Conway 1999), the commonly used enzymes being neuraminidase, trypsin and chymotrypsin. Neuraminidase removes sialic acids from the RBC surface. Invasion into these RBC defines whether a parasite isolate is sialic-acid dependent or independent. Trypsin cleaves protein at lysine or arginine residues (Stryer 1995) and removes surface glycoproteins such as glycophorin A (GPA) and glycophorin C (GPC). Chymotrypsin has a different specificity to trypsin cleaving at tyrosine, tryptophan, phenylalanine and methionine residues (Stryer 1995) and removes for example glycophorin B (GPB).

Invasion profiles of various laboratory *P. falciparum* strains have been defined by studying invasion into enzyme treated RBC. Isolates are termed dependent or sensitive if invasion is decreased after a specific enzyme treatment, e.g. an isolate that does not invade a neuraminidase treated RBC invades via a sialic-acid dependent or neuraminidase sensitive pathway. Similarly if invasion of an isolate is not decreased by a specific enzyme treatment they are termed resistant or independent. A number of invasion profiles have been described as shown in table 1 and they are briefly discussed below.

Table 1: Invasion profiles described for *P. falciparum* laboratory strains.

Invasion Profile type ^a	Parasite Ligand	RBC receptor	Reference
Nm _S T _S CT _R	EBA-175 BAEBL	GPA GPC	(Pasvol 1982)(Camus 1985) (Sim 1994) (Maier 2003)(Mayer 2003)
Nm _S T _R CT _S	? JESEBL	GPB Receptor E	(Dolan 1994) (Gilberger 2003)
Nm _S T _R CT _R	PfRh1	Receptor Y	(Rayner 2001)(Triglia 2005)
Nm _R T _S CT _?	?	Receptor X	(Dolan 1994)
Nm _R T _R CT _S	PfRh2b MSP1 AMA1	Receptor Z Band 3 Kx ^b	(Duraisingh 2003) (Goel 2003) (Kato 2005)
Nm _R T _? CT _?	PfRh4	?	(Stubbs 2005) ^c

a Invasion profile type abbreviations: Nm = neuraminidase, T = trypsin, CT = chymotrypsin, S = sensitive and R = resistant

b Erythrocyte membrane protein, Kx (Kato 2005)

c PfRh4 has so far only been associated with the sialic-acid independent pathway in some but not all sialic-acid independent laboratory *P. falciparum* isolates (Stubbs 2005)

The best-defined and characterised profile is a neuraminidase and trypsin sensitive, yet chymotrypsin resistant invasion phenotype. This invasion pathway is mediated by GPA as the erythrocyte receptor and EBA-175 as the parasite ligand (Camus and Hadley 1985; Sim *et al.* 1994). Another receptor-ligand interaction defined by this enzyme profile is mediated by the ligand BAEBL binding to GPC (Mayer *et al.* 2002; Maier *et al.* 2003). The next invasion profile is a neuraminidase sensitive, trypsin resistant and chymotrypsin sensitive invasion phenotype. This invasion profile has been described for the receptor GPB, which is trypsin resistant, but the parasite ligand remains unknown (Dolan *et al.* 1994). The same invasion profile could point to an invasion pathway via the unknown receptor E and the parasite ligand, JESEBL (Gilberger *et al.* 2003). A neuraminidase sensitive and trypsin resistant, yet chymotrypsin resistant invasion profile is mediated by interactions between the unknown RBC receptor Y and the *P. falciparum* protein PfRh1 (Rayner *et al.* 2001; Triglia *et al.* 2005). A neuraminidase resistant and trypsin sensitive profile has been described for the unknown receptor X, which so far has no known ligands (Dolan *et al.* 1994). Finally another described invasion profile shows

resistance to both neuraminidase and trypsin, yet sensitivity to chymotrypsin treatment of the RBC. There are two described pathways that fit this invasion profile. The first describes an undefined parasite ligand interacting with an unknown receptor Z in a sialic-acid and trypsin independent manner (Duraisingh *et al.* 2003). Another speculative pathway is an interaction between the RBC protein band 3 and MSP1 on the merozoite surface (Goel *et al.* 2003). Furthermore a possible interaction between the *P. falciparum* protein AMA1 with the erythrocyte membrane protein, Kx, has been described (Kato *et al.* 2005), although in this case the proposed interaction only occurs after trypsin treatment indicating that prior modification of the protein is necessary. Most recently it has been described that PfRh4 activation may be involved in a pathway switch to sialic acid-independent pathways (Stubbs *et al.* 2005). In this study, W2mef parasites either selected to grow on neuraminidase treated RBC or where EBA-175 was disrupted had increased expression levels of PfRh4 and the pseudogene PEBL, which are located in a head-to-head orientation on chromosome 4 (Stubbs *et al.* 2005). Switching of *P. falciparum* laboratory isolates when selected on neuraminidase treated RBC had been described previously (Mitchell *et al.* 1987, Dolan *et al.* 1990) indicating how parasites can adapt to employ different invasion pathways under selective pressure. There are also multiple allelic types of two *P. falciparum* proteins, BAEBL and JESEBL. The different allelic types mediate different erythrocyte-binding specificities to enzyme treated erythrocytes when the recombinant regions containing the different polymorphisms are expressed on transfected cells (Mayer *et al.* 2002; Mayer *et al.* 2004). However, although the presence of BAEBL and JESEBL allelic types has been confirmed in field isolates (Lobo *et al.* 2004), they may not be functionally important as the allelic types have not been associated with different invasion profiles in field isolates (Lobo *et al.* 2004).

Blood group antigens may also be associated with invasion, as a recent paper described higher invasion rates for laboratory *P. falciparum* strains into A1 type RBC (Cheng *et al.* 2005). Blood group antigens are surface immunodominant sugars on the outside of the RBC membrane. Although there are a large number of blood group antigens (see Reid and Lomas-Francis 1997) only a few of them are of

particular interest here: the ABO blood group system and the MNS blood group system. The ABO blood group system contains the A and B antigens. These two antigens are defined by specific sugar residues added to the H antigen precursor, more specifically the addition of a N acetyl galactosamine to form the A antigen and a galactose residue to form the B antigen (Reid and Lomas-Francis 1997). These antigens are carried on glycoprotein molecules, such as band 3. The ABO blood group antigens are highly abundant. The MNS blood group system are found on the above described glycoproteins GPA and GPB (Reid and Lomas-Francis 1997). These proteins have been directly associated with *P. falciparum* invasion pathways as detailed above.

The description of the above invasion pathways is based on work with laboratory-adapted *P. falciparum* strains. The results for each strain are consistent across laboratories indicating that the invasion phenotype is stable (Dolan *et al.* 1994; Binks and Conway 1999), with the exception of a report showing different invasion profiles for two 3D7 lines (Cortes *et al.* 2004). Following from the work on laboratory isolates there has been recent interest to study the invasion phenotypes of field isolates to understand the RBC invasion process in human *P. falciparum* infections. This is of particular relevance for vaccine design to blood-stage antigens, which may target specific invasion pathways. To date there are three studies looking at the invasion profiles of field isolates collected in India, The Gambia and Brazil respectively (Okoyeh *et al.* 1999; Baum *et al.* 2003; Lobo *et al.* 2004). These studies have reported on the invasion profiles of field isolates invading neuraminidase or trypsin treated RBC (Okoyeh *et al.* 1999; Baum *et al.* 2003) or neuraminidase, trypsin or chymotrypsin treated RBC (Lobo *et al.* 2004). Overall large variation between the invasion pathways used was found for all sites, although there generally seems to be one preferred invasion profile employed by the majority of isolates from each study site. 12 out of 15 Indian isolates used an alternative pathway to the EBA-175/GPA pathway (Okoyeh *et al.* 1999). Whereas, in both The Gambia and Brazil the neuraminidase and trypsin sensitive invasion profile was the predominant one (30/38 isolates in The Gambia and 7/14 isolates in Brazil) (Baum *et al.* 2003) (Lobo *et al.* 2004). Furthermore, in contrast to the conclusions from the study in The

Gambia that the majority of isolates invade through the EBA-175/GPA pathway, the Brazilian study challenges the notion that the neuraminidase and trypsin sensitive invasion profile indicates invasion by the EBA-175/GPA pathway, as they show that 5 of the 7 Brazilian isolates with this phenotype can invade RBC, which lack GPA (Lobo *et al.* 2004). 5 out of 14 Brazilian isolates were trypsin resistant, which is an unusual phenotype (Lobo *et al.* 2004).

All of the above studies were based on field isolates collected from patients with uncomplicated malaria (Okoyeh *et al.* 1999; Baum *et al.* 2003; Lobo *et al.* 2004). I was interested in the invasion profiles of isolates collected from severe malaria patients. Parasite isolates from severe malaria patients have been associated with high multiplication rates and a decreased RBC selectivity in Thailand (Simpson *et al.* 1999; Chotivanich *et al.* 2000) and it is unknown whether isolates from patients with severe malaria may use a specific invasion pathway or possibly a larger range of invasion pathways than isolates from patients with mild malaria. In this chapter the invasion profile of Kenyan field isolates collected from children diagnosed with either uncomplicated or severe malaria is reported and the association between invasion profile and disease severity is examined.

4.3 Materials and Methods

4.3.1 Parasite isolates

Parasite isolates were collected from Kilifi, Kenya. At this site, malaria transmission is seasonal (June to August and December to February), with infective bites ranging from 10-30 per year (Kinyanjui *et al.* 2004). Children with cerebral malaria (unrousable coma with a Blantyre score ≤ 2), prostration (inability to sit or in babies, to breast feed) or respiratory distress (abnormally deep breathing) were considered to have severe malaria. The clinical definition identifies approximately the same group of children at risk of life-threatening malaria as those identified by the more comprehensive WHO criteria (Marsh *et al.* 1995). Uncomplicated cases were

children with malaria presenting at hospital with no signs of severe disease, who were treated as outpatients with oral therapy. Blood samples were collected after obtaining informed consent from the children's parents or guardians, and protocols were approved by ethical review boards in the UK and Kenya. The isolates were collected into heparinised tubes and processed within 12 hours of collection. Lymphocytes and the buffy coat were removed, the RBC washed three times in incomplete RPMI and resuspended in complete RPMI. The parasites were cultured in 3%CO₂, 1%O₂, and 96%N₂ at 37°C. Cultures were monitored by Giemsa smears for 18-36 hours, and only those with normal morphology that matured to the schizont stage were included in the study.

Thin smears were prepared from blood collected from the patient at arrival at hospital and were stained with Giemsa.

4.3.2 Selectivity Index

300 ring-infected RBC were counted per slide and the number of rings in each RBC was recorded. The SI was calculated as described in detail by Simpson *et al.* (Simpson *et al.* 1999) Briefly, the number of observed multiple-infected RBC (i.e. 2 or more rings per RBC) was divided by the number of expected multiple-infected RBC. The latter was calculated according to a Poisson distribution, where lambda is $(-\ln(1-\text{parasitaemia}))$. The parasitaemia was counted as proportion of infected RBC per 1000 RBC.

4.3.3 Parasite culture

The parasites studied were *P. falciparum* clone 7G8 (Hadley *et al.* 1987) and strain Dd2. Parasites were cultured in complete RPMI (cRPMI: RPMI 1640 (BioWhittaker) plus 25mM HEPES (BioWhittaker), 2mM glutamine (BioWhittaker), 25mM glucose (BioWhittaker), 25µg/ml gentamicin (Gibco) and 10% pooled human AB serum) at 1-2% haematocrit in O+ RBC at 37°C with 3%CO₂, 1%O₂, and 96%N₂ at 37°C. Laboratory strains were synchronised by sorbitol (Sigma) lysis (Lambros and Vanderberg 1979). Incomplete RPMI (incRPMI) contains the following: RPMI 1640 plus 25mM HEPES, 2mM glutamine, 25mM glucose and 25µg/ml gentamicin.

4.3.4 Donor erythrocytes

Donor RBC for the work with Kenyan field isolates were collected from a Caucasian donor (O+). 10ml whole blood were collected into 2ml acid citrate dextrose (ACD, Gibco), mixed to prevent coagulation and stored at 4°C for a maximum of two weeks. Before use the white blood cells were removed by layering 6ml of whole blood over 5ml lymphoprep (Axis Shield) and centrifuging for 20minutes at 400g. This results in a clean RBC pellet with all white blood cells in the layers above. The packed RBC were washed twice with 10ml incRPMI, resuspended at 50% haematocrit and used within one week. O+ RBC for experiments with laboratory strains were from the Blood Transfusion Unit. They were treated as described above.

4.3.5 Enzyme preparation

Frozen aliquots of trypsin (Sigma), chymotrypsin (Sigma) and the soybean trypsin inhibitor (Sigma) were prepared. For these the enzymes were dissolved in incRPMI to the required concentrations: trypsin (T, 1mg/ml), chymotrypsin (1mg/ml) and soybean trypsin inhibitor (STI, 0.5mg/ml). They were then frozen in 1ml aliquots at -20°C. The neuraminidase (*Vibrio cholerae*, Calbiochem) was reconstituted in dH₂O

and stored at 4°C following manufacturers instructions. Another supply of neuraminidase (*Vibrio cholerae*, Roche) was stored at 4°C.

4.3.6 Enzyme treatment

The enzyme treatment is based on previous protocols as described (Baum *et al.* 2003). An aliquot of each of the required enzymes was thawed. A tube with 50mU neuraminidase in 1ml incRPMI was prepared and one control with 1ml incRPMI. To each tube (control, neuraminidase, chymotrypsin, trypsin) 100µl packed cell volume of donor RBC were added. The tubes were incubated on a rotating wheel (ThermoDenley Cell Mixer) for 1hour at 37°C. After that time all samples were washed once with 1ml incRPMI and then soybean trypsin inhibitor was added to the trypsin and chymotrypsin samples to inhibit the enzyme. IncRPMI was added to the control and neuraminidase sample. The samples were incubated on a rotating wheel for 10 minutes at room temperature. The samples were washed three times before resuspending the RBC at 50% haematocrit in incRPMI. The enzyme treated RBC were stored at 4°C and used for a maximum of one week after which the samples were freshly prepared.

4.3.7 Agglutination of enzyme treated RBC

A 20 µl sample of each of the different RBC samples was diluted in 200µl 0.9% NaCl (Sigma). 40µl of the 5% haematocrit sample of each was added to the same volume of anti-M antibody (Biotest Uk Ltd), anti-S antibody (Biotest Uk Ltd) or Peanut (*Arachis hypogaea*) lectin (Sigma, reconstituted in PBS and stored in aliquots at -20°C) in Pyrex tubes. The samples were mixed and the tubes covered with parafilm. The samples were incubated for 5minutes (anti-S antibody) or 30 minutes at room temperature (anti-M antibody and PNA lectin). The anti-S antibody samples were centrifuged for 1 minute at 150g before the agglutination was assessed. To check for agglutination the pellets were gently dislodged, the agglutination assessed

by eye and the following scores given: one large agglutinate (++), small agglutinates (+), no agglutinates (-).

4.3.8 Invasion pathway assay

The assay to determine PMR in the first cycle of *in vitro* culture was set up as described (Chotivanich *et al.* 2000). All parasites were cultured as described above until the mature schizont stage. The schizonts were enriched by centrifugation through 55% Percoll (Sigma). The enriched schizonts were washed twice and adjusted to around 1% parasitaemia with normal group O+ red blood cells and enzyme-treated red blood cells, from one single healthy Caucasian donor. They were incubated in 96-well plates (Corning) at a volume of 50µl at 2% haematocrit with duplicate wells for each isolate. The 96-well plate was placed within a gas modulator chamber (Billups-Rothenberg) to allow culturing in 3%CO₂, 1%O₂, and 96%N₂ for 24hrs at 37°C. Thin blood films of the pre-invasion sample and the ring-invaded RBC after 24hrs were made and stained with Giemsa to determine the parasitaemia. At least 1000 RBC from each slide of the duplicate wells per sample were counted. The PMR is the number of ring-infected RBC after invasion divided by the pre-invasion parasitaemia. Invasion inhibition was measured as $(100 - ((\text{Enzyme-treated PMR} / \text{control PMR}) \times 100))$. In a few cases no ring-iRBC were seen and the invasion inhibition was measured as 100%. Where the PMR into enzyme-treated RBC was higher than into control RBC the invasion inhibition is given as a negative value, due to an increase in invasion.

4.3.9 Statistical analysis

All statistical tests on the Kenyan field isolate data were non-parametric tests as the data were non-normally distributed. To calculate the median and interquartile range and for statistical analyses any negative invasion inhibition results were counted as 0. The Mann-Whitney U test or Kruskal Wallis test were used to look for differences

between two or more groups respectively. Correlations between the selectivity index (SI) and the invasion inhibition data were carried out with a Spearman Rank Correlation.

4.4 Results

4.4.1 Controlling for enzyme treatment of RBC by agglutination

I used two different methods to control for the enzyme treatment of the RBC for my study of the invasion pathways of Kenyan field isolates. Firstly, removal of epitopes from the RBC surface by the enzymes can be checked with two monoclonal antibodies and a lectin. The two monoclonal antibodies, anti-M and anti-S, recognise epitopes that are trypsin and chymotrypsin sensitive respectively. The peanut lectin will only agglutinate neuraminidase treated RBC. Table 2 shows the agglutination profile. After trypsin treatment the anti-M antibody does not agglutinate the RBC, whereas RBC treated with chymotrypsin are not agglutinated by the anti-S antibody. This shows the specificity of the two enzymes used. Furthermore agglutination of the RBC by the peanut lectin, PNA, was only observed after neuraminidase treatment. It is worth noting that upon neuraminidase treatment the anti-M and anti-S antibodies do not agglutinate the RBC as the epitopes recognised carry sialic acid residues (Sim *et al.* 1994).

Table 2: Agglutination of RBC after enzyme treatment

Enzyme Treatment	<i>Anti-M antibody</i>	<i>Anti-S antibody</i>	<i>PNA^a</i>
Control	++	++	-
Neuraminidase	-	-	++
Chymotrypsin	++	-	-
Trypsin (1 mg/ml)	-	++	-

a PNA, peanut (*Arachis hypogaea*) lectin

4.4.2 Controlling for the enzyme treatment of RBC with *P. falciparum* laboratory strains of a known invasion phenotype

Invasion pathways have been studied mainly with laboratory isolates to date. It is known that most laboratory strains have a stable invasion phenotype, which has been demonstrated in different laboratories. A good overview of this is given in Binks et al. (Binks and Conway 1999). For studies with field isolates a number of laboratory isolates are included in the study for comparative purposes (Baum et al. 2003; Lobo et al. 2004). I looked at the invasion phenotype of two laboratory isolates, 7G8 and Dd2. These two strains were selected as they show different profiles (Binks and Conway 1999). 7G8 is sialic-acid independent – invasion into RBC by this isolate is highly inhibited by trypsin (80 – 95% inhibition compared to the control) and to a much lesser degree by neuraminidase (30 – 40%). Dd2 on the other hand is highly inhibited by neuraminidase (90 – 100%), but less so by trypsin (50%). I did three independent experiments with these two isolates the results of which can be seen in figure 1.

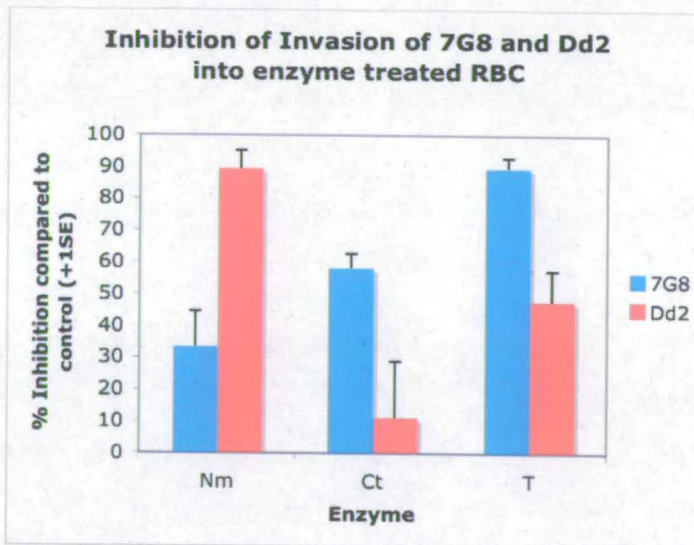


Figure 1: Invasion inhibition (mean + 1SE) of two laboratory strains, 7G8 (blue) and Dd2 (red), following enzyme treatment of RBC. The enzyme treatments are listed on the X-axis and concentrations were as follows: Nm=neuraminidase 50mU/ml, Ct=chymotrypsin 1mg/ml and T=trypsin 1mg/ml.

The invasion profiles seen in regards to neuraminidase and trypsin treated RBC matched those predicted, which confirmed that both the enzyme treatment and the invasion assay were giving results comparable with previous research. Unfortunately little is known about the chymotrypsin invasion phenotype of parasites, as there has only recently been an interest in this pathway.

4.4.3 Invasion profiles of Kenyan *P. falciparum* isolates

The invasion profiles of 31 Kenyan isolates were examined (table 3). These isolates were a subset of those studied in chapter 2. Invasion inhibition by three different enzyme-treatments of RBC was measured: treatment with neuraminidase (Nm, 50mU/ml), chymotrypsin (CT, 1mg/ml) and trypsin (T, 1mg/ml).

The invasion inhibition of Kenyan field isolates into Nm treated RBC varied from -32.4 (indicating an increase in invasion) to 100% inhibition (i.e. no ring iRBC were counted), with a median inhibition of 12.8%. The inhibition into CT treated RBC ranged from -66.7 to 100%, with a median inhibition of 62.8%. Invasion into trypsin treated RBC varied from 28 to 100%, with a median inhibition of 89.1%.

The invasion profiles of the field isolates were defined by their Nm, CT and T invasion inhibition. Inhibition above 50% compared to the control was defined as sensitive and below 50% was defined as resistant according to the common definition (Baum *et al.* 2003; Lobo *et al.* 2004). The Kenyan isolates showed 6 out of a possible 8 phenotype combinations. Eighteen of the Kenyan isolates showed a neuraminidase resistant and chymotrypsin and trypsin sensitive profile (table 3). This profile matches the profile of 7G8. Five of the Kenyan isolates were neuraminidase and chymotrypsin resistant and trypsin sensitive. Three of the Kenyan isolates were sensitive to all enzymes: neuraminidase, chymotrypsin and trypsin. Three of the isolates showed no sensitivity to chymotrypsin and were neuraminidase and trypsin sensitive – this being the predominant invasion profile in a sample of Brazilian isolates and Gambian isolates (although the chymotrypsin sensitivity is unknown for the Gambian isolates) (Lobo *et al.* 2004) (Baum 2003). One isolate showed the same

Table 3: Invasion profile of Kenyan field isolates

Invasion Profile type	Isolate ^a	% Inhibition compared to control in RBC treated with:		
		Nm (50mU/ml)	CT (1mg/ml)	T (1mg/ml)
Nm_RT_SCT_S	<i>7G8</i>	<i>33.3 (19.2)</i>	<i>58.2 (7.5)</i>	<i>80.7 (5.5)</i>
	6 U	9.3	62.8	86.1
	7 U	12.8	76.6	83.0
	8 S	7.1	78.6	78.6
	9 S	-32.4	90.1	97.2
	10 S	0	73.2	87.8
	11 U	49.1	59.7	75.4
	12 S	-22.8	62.9	85.7
	13 U	8.5	56.3	95.8
	14 U	1.1	56.5	89.1
	15 U	12.5	58.3	79.2
	16 S	0	92.9	92.9
	17 U	7.5	64.2	79.1
	18 U	42	94	88
	19 U	17.0	59.6	85.1
	20 U	10.3	92.3	92.3
	21 S	23.5	70.6	94.1
	22 S	33.3	55.6	92.6
	23 U	29.6	77.8	77.8
Nm_RT_SCT_R	1 U	13.8	43.1	100
	2 U	8.9	48.2	91.1
	3 S	-13.2	42.1	89.5
	4 S	6.3	-66.7	100
	5 U	-23.6	28.3	94.4
Nm_ST_SCT_S	29 S	100	90.2	82.5
	30 U	56.0	100	100
	31 U	56.1	63.4	100
Nm_ST_SCT_R	26 S	75.2	30.2	96.0
	27 U	100	31.5	100
	28 U	58.0	38.6	81.3
Nm_ST_RCT_R	<i>Dd2</i>	<i>89.2 (10.2)</i>	<i>11.1 (30.5)</i>	<i>47.6 (16.9)</i>
	25 U	58.5	8.3	28.6
Nm_RT_RCT_S	24 S	16	100	28

^a The disease severity of the host, from whom the parasite was isolated, is indicated beside the isolate number as S=severe malaria and U=uncomplicated malaria. The laboratory strains 7G8 and Dd2 are indicated in italics, for these two strains the mean of three experiments and the standard deviation are shown.

profile as the laboratory strain Dd2 and was neuraminidase sensitive and chymotrypsin and trypsin resistant. Finally, one isolate was resistant to both neuraminidase and trypsin treatment, but was sensitive to chymotrypsin treatment. There are two more profiles, which are theoretically possible, but were not seen in this study: no isolates were resistant to neuraminidase, chymotrypsin and trypsin treatment and no isolates had a neuraminidase sensitive, chymotrypsin sensitive and trypsin resistant profile.

The invasion inhibition following neuraminidase treatment of RBC was lower than in previous studies (Okoyeh 1999, Baum 2003, Lobo 2004). This was investigated further to consider any possible problems with the neuraminidase used for the assay. 15 frozen isolates of samples used in the original study were received from Kenya. Of these 5 grew after thawing in Edinburgh and could be used in an invasion assay using a different neuraminidase supply to control for enzyme problems. Comparing the invasion inhibition by neuraminidase of these 5 isolates in the experiments carried out in Kenya (10.3% median, 0% to 32.8% interquartile range) I found that although when repeating the work the inhibition was slightly higher (33% median, 16.4% to 44.7% IQR) there was no significant difference ($P=0.18$, Mann Whitney U) and even in the repeat experiments the inhibition is still strikingly lower than from previous reports (The Gambia 62% median invasion inhibition, Brazil 70% median invasion inhibition (Baum *et al.* 2003; Lobo *et al.* 2004).

4.4.4 Invasion profiles and malaria severity in Kenyan isolates

Previous studies looking at invasion pathways used by field isolates have been carried out with isolates taken from uncomplicated malaria patients that were outpatients. It is unknown whether isolates from severe malaria patients may invade by a specific invasion pathway. I looked at field isolates taken from children diagnosed with either uncomplicated or severe malaria, to compare the invasion inhibition into enzyme treated RBC of the two groups. 19 of the isolates were from children with uncomplicated malaria. The mean age (months) was 46.3 (34.9, standard deviation) and the mean haemoglobin level was 9.8 (g/dL, 1.3 standard

deviation). 12 of the isolates were from children with severe malaria. The mean age of the severe malaria children was 19.6 (11.2) and their mean haemoglobin level was 7.7 (3.1). The severe malaria patients were diagnosed with the following symptoms: 3 cerebral malaria, 4 prostration and respiratory distress, 1 respiratory distress, 1 prostration and severe anaemia and 3 prostration only. As seen in table 3 the isolates from either group showed a similar distribution amongst the recorded invasion profiles (table 3). The median inhibition compared to the non-enzyme treated control of isolates from patients with uncomplicated or severe malaria for each of the enzyme-treatments is shown in figure 2. The inhibition did not differ between the uncomplicated and severe malaria isolates for any of the enzyme treatments ($P>0.1$, for all enzyme treatments, Mann Whitney U test).

Figure 2:

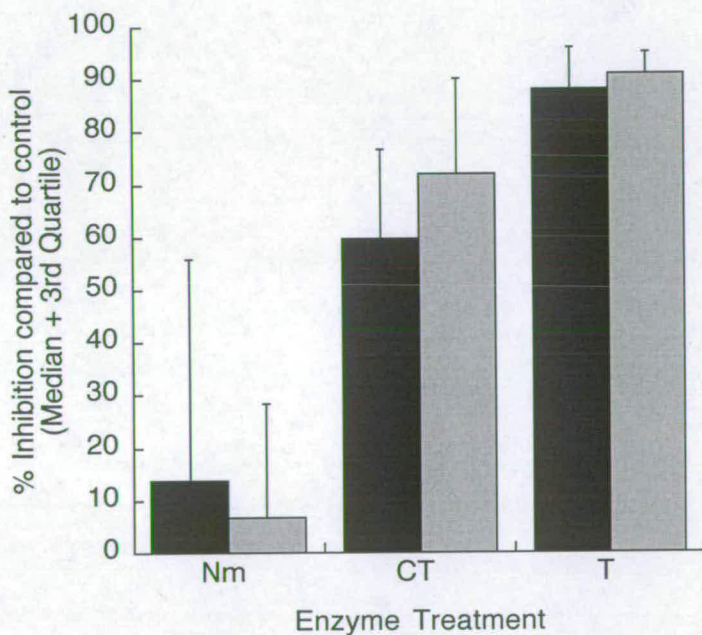


Figure 2: Invasion inhibition (%) of Kenyan field isolates from children with mild (dark grey bars) or severe malaria (light grey bars). The RBC were treated with neuraminidase (Nm, 50mU/ml), chymotrypsin (CT, 1mg/ml) and trypsin concentration (T, 1mg/ml). The median plus the 3rd Quartile is shown.

4.4.5 Invasion profiles and ABO blood group type in Kenyan isolates

Independently from malaria severity, I found there was a significant difference in neuraminidase sensitivity between isolates taken from patients of different ABO blood group types (figure 3). 17 isolates had been isolated from patients with blood group O, 6 from patients with blood group B and 5 from patients with blood group A. The single sample from a patient with blood group AB was excluded from this analysis and for two samples the blood group data was missing. It was found that isolates from patients with blood group B were more highly inhibited by neuraminidase treatment of RBC with a median inhibition of 44.7% (23.5 to 58.0 IQR, n=6) compared to 7.1% (3.1 to 34.4 IQR, n=5) and 9.3% (0 to 23.3 IQR, n=17) for blood group A and O respectively ($P=0.02$, Kruskal Wallis). This would indicate that isolates growing in the B blood group type are more dependent on sialic acid for invasion of O type RBC (the donor used for all experiments was group O). No other patient variable (age, parasitaemia, haemoglobin levels or white blood cell count) was associated with the level of inhibition into enzyme-treated RBC.

Figure 3:

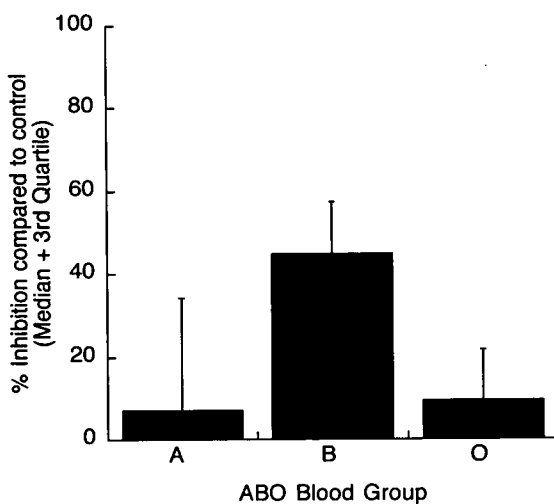


Figure 3: Invasion inhibition (%) into neuraminidase treated RBC of Kenyan field isolates from children with the ABO blood group A (n=5), B (n=6) or O (n=17). The median inhibition (+ 3rd Quartile) is shown.

4.4.6 Invasion profiles and the selectivity index.

I determined the selectivity index (SI) for the isolates included in this study for a larger study described elsewhere (Deans et al., chapter 2). The geometric mean SI for the samples included in this study was 1.78 (1.43 – 2.13, 95% confidence interval) and there was no significant difference between the SI for isolates from uncomplicated and severe malaria patients ($P=0.97$, Mann Whitney U). SI was not correlated with the level of inhibition for any of the enzyme treatments ($P>0.1$, Spearman Rank Correlation). No other parasite variable (parasite multiplication rate or rosetting frequency) was found to be associated with the level of invasion inhibition by enzyme treatment of the RBC.

4.5 Discussion

In this chapter the invasion of Kenyan field isolates into erythrocytes treated with three different enzymes was examined in order to assess the invasion pathways used in natural malaria infections. 6 out of 8 possible invasion profiles were seen, indicating that *P. falciparum* field isolates in Kenya use a variety of invasion pathways. In contrast to previous studies from The Gambia and Brazil (Baum *et al.* 2003; Lobo *et al.* 2004), but in accordance with a study from India (Okoyeh *et al.* 1999), it was found that the neuraminidase- and trypsin-sensitive, chymotrypsin-resistant pathway characteristic of EBA-175/GPA-mediated invasion was rare in Kenyan field isolates, where only 3/31 showed this profile (Table 3).

Because the Kenyan isolates showed less neuraminidase sensitivity than expected from previous reports (Baum *et al.* 2003; Lobo *et al.* 2004), I repeated the neuraminidase inhibition assay with a smaller number of samples using a neuraminidase enzyme from a different supplier. The low level of inhibition of invasion into neuraminidase treated RBC was confirmed using the new enzyme, showing that this finding is not due to technical problems with the assay. In addition the work from Dr Susana Nery (a collaborator from Dr David Conway's group who

is examining gene expression profiles in the same isolates as I have studied), has shown that the neuraminidase inhibition levels of the original isolates correlates with EBA-175 expression levels determined by quantitative RT-PCR (Susana Nery, personal communication). The EBA175 levels are negatively correlated with PfRh4 in Kilifi field isolates (Susana Nery, personal communication) and as PfRh4 expression is associated with the switch to sialic-acid independent invasion (Stubbs *et al.* 2005) this may be a predominant invasion pathway used by Kenyan isolates. However, the full role of Rh4 in invasion of these isolates is not clear, and the invasion profile of PfRh4-mediated invasion has not yet been described.

There may be selective pressure leading to the high sialic-acid independence and lack of use of the EBA-175/GPA pathway by field isolates collected in Kilifi. One may hypothesize that across geographical regions there are different preferences for which invasion pathway is mainly used. Another example is the fact that the Brazilian isolates showed a significantly reduced sensitivity to trypsin than expected from previous results, particularly as a relatively high trypsin concentration was used (Lobo *et al.* 2004). In Brazil 5 out of 14 field isolates are trypsin resistant compared to 2 out of 31 Kenyan field isolates. Similarly the measured sensitivity to chymotrypsin varied between our study and the previous report from Brazil, with only one 1 of 14 Brazilian isolate reported as chymotrypsin sensitive (Lobo *et al.* 2004), whereas 22 out of 31 Kenyan isolates were sensitive to chymotrypsin treatment. To further study the variability of invasion pathways from different regions it would be ideal to carry out a large study looking at the invasion profiles of field isolates collected from multiple geographical locations, but analysed together using one blood donor. This way one would be able to more accurately address to what extent variability in the invasion profiles exists across different study sites. As blood polymorphisms are common and glycophorin A and B are thought to be amongst the fastest evolving human proteins (Baum *et al.* 2002; Wang *et al.* 2003), one can imagine how blood polymorphism effects may shape the invasion pathway preference of field isolates from different geographical regions.

Regional differences in the invasion profiles that the majority of isolates use may have important implications for bloodstage vaccines, which target antigens involved in invasion pathways. For example a vaccine to EBA-175, which may block the EBA-175/GPA pathway, may not be as effective in Kenya as it would be in The Gambia, as the majority of Kenyan isolates were found to invade by a different pathway. Furthermore, the large redundancy of invasion pathways used by field isolates indicates that a bloodstage vaccine may not inhibit *P. falciparum* invasion and growth, but may instead select for parasites, which invade by different mechanisms.

The majority of the Kenyan isolates (23/31) fell within two invasion profiles defined as neuraminidase resistant and trypsin sensitive, which is a not very well defined pathway described solely by the involvement of a potential receptor X (Hadley *et al.* 1987; Dolan *et al.* 1994). Furthermore, specific allelic types of the merozoite antigens BAEBL and JESEBL have been described to have a neuraminidase resistant and trypsin sensitive erythrocyte binding profile (Mayer *et al.* 2002; Mayer *et al.* 2004) in addition to their main invasion pathway as shown in table 1, although this association between specific allelic types and invasion pathways has not been supported by field isolate data (Lobo *et al.* 2004). The finding that the putative pathway involving receptor X occurs commonly in natural infection argues that further research to delineate this pathway should be considered a priority.

Three other Kenyan isolates showed neuraminidase, trypsin and chymotrypsin sensitivity. Such an invasion profile has not been described previously, partly because chymotrypsin has not been used frequently in the past to describe invasion phenotypes. Possibly a pathway exists where the receptor is sensitive to both enzymes (trypsin and chymotrypsin) and is sialic acid dependent. Alternatively, these three isolates may only invade efficiently through a number of pathways and removing any of the different pathways by enzyme treatment reduces invasion by more than 50%.

Two further invasion profiles were seen. A neuraminidase sensitive, trypsin and chymotrypsin resistant profile was seen, the same invasion profile seen for the laboratory strain Dd2. This sialic-acid dependent profile has been described for the PfRh1 – Receptor Y interaction (Rayner *et al.* 2001; Triglia *et al.* 2005). And finally a neuraminidase and trypsin resistant, yet chymotrypsin sensitive profile was seen, indicating a possible invasion preference through the PfRh2b ligand binding to receptor Z (Duraisingh *et al.* 2003) and/or the suggested MSP-1 binding to band 3 pathway (Goel *et al.* 2003). As expected, no isolates exhibited independence from known invasion pathways and were all sensitive to at least one of the enzyme treatments. And no isolates invaded by the neuraminidase and chymotrypsin sensitive, yet trypsin resistant pathway, as described for the parasite ligand JESEBL and its potential receptor E (Gilberger *et al.* 2003).

Whether growth in a certain blood group affects the invasion profiles of a parasite is unknown, despite some evidence for differences in ABO types in regards to some membrane surface characteristics. It has been found that specific sialic acids were associated with different ABO blood groups, although these only made up a small part of the more commonly shared surface sialic acids (Bulai *et al.* 2003). Similarly, it has been shown that there are more high affinity EGF binding sites on blood group A RBC compared to the other ABO blood groups (Engelmann *et al.* 1992). For the Kenyan isolates I saw a significant difference in the level of inhibition by neuraminidase treatment depending on the ABO blood group of the host that the parasites were isolated from ($P=0.02$, Kruskal Wallis), with isolates from patients with blood group B type being most highly inhibited by neuraminidase than isolates from patients with blood group A or O. In addition, the three isolates that were sensitive to all three enzyme treatments (described above) were all isolated from hosts with a non-O blood group type (two from a blood group B and one from a blood group A type) and may have been less sensitive to enzyme treatment if they had invaded enzyme treated blood group A or B erythrocytes. Evidence for potential differences in invasion into different ABO blood group types was shown in a recent publication, where it was found that *P. falciparum* laboratory strains can invade A1-type RBC at higher invasion rates than other blood groups (Chung *et al.* 2005). It has

also been shown that both higher levels of rosetting and severe malaria are associated with blood group A (Rowe *et al.* 1995; Lell *et al.* 1999; Pathirana *et al.* 2005). It remains unclear whether the results seen here may only have occurred by chance as the sample size is small and the study was not designed to look at the invasion inhibition in regards to host ABO blood group type, but these findings together with the above described studies indicate that this deserves further investigation. It would be interesting to look at invasion profiles of laboratory or field isolates when invading different ABO blood group types, and also after selecting parasites to grow in different ABO blood group types and then studying their invasion into the same and different ABO blood group types.

The work described in this chapter is the first study looking at the invasion profiles of field isolates collected both from children with uncomplicated malaria and with severe malaria. I found that the isolates from both groups showed the same invasion profiles and there was no significant association between invasion profile and disease severity. In this study, the sample size was relatively small with only 12 isolates from children with severe malaria. So one cannot rule out that a difference may have been seen in a larger study and further work is required. Yet, the lack of association between invasion profile and disease severity is consistent with the finding that with the same isolates, there was no significant difference in the multiplication rates between isolates from children with uncomplicated or severe malaria contrasting with previous results from Thailand (chapter 2, (Chotivanich *et al.* 2000)). Due to the strong association between parasite multiplication rate and disease severity in Thailand, a study looking at the invasion profiles of Thai field isolates, to examine whether the high multiplication rates displayed by isolates from patients with severe malaria can be associated with overall less sensitivity to enzyme treatment of erythrocytes or a specific preferred invasion profile, may provide further insight.

CHAPTER 5: *Plasmodium falciparum*: rosettes do not protect merozoites from invasion-inhibitory antibodies

A version of this chapter has been submitted as: Deans, AM & Rowe, JA. *Plasmodium falciparum*: rosettes do not protect merozoites from invasion-inhibitory antibodies. *Journal of Experimental Parasitology*. In press 2005.

5.1 Abstract

Rosetting is a parasite adhesion phenotype associated with severe malaria in African children. Why parasites form rosettes is unknown, although enhanced invasion or immune evasion have been suggested as possible functions. Previous work showed that rosetting does not enhance parasite invasion under standard *in vitro* conditions. I hypothesised that rosetting might promote invasion in the presence of host invasion-inhibitory antibodies, by allowing merozoites direct entry into the erythrocytes in the rosette and so minimising exposure to plasma antibodies. I therefore investigated whether rosetting influences invasion in the presence of invasion-inhibitory antibodies to MSP-1. I found no difference in invasion rates between isogenic rosetting and non-rosetting lines from two parasite strains, R29 and TM284, in the presence of MSP-1 antibodies ($P=0.62$ and $P=0.63$, Students t-test, TM284 and R29 respectively). These results do not support the hypothesis that rosettes protect merozoites from inhibitory antibodies during invasion. The biological function of rosetting remains unknown.

5.2 Introduction

The binding of infected red blood cells (iRBC) to uninfected red blood cells (RBC) is a *Plasmodium falciparum* adhesion phenotype known as rosetting. *P. falciparum* field isolates vary in the extent to which they form rosettes, and high levels of rosette formation have been strongly associated with severe malaria in African children in

numerous studies (e.g., (Carlson and Wahlgren 1992; Ringwald *et al.* 1993; Rowe *et al.* 1995). A human polymorphism associated with deficiency of the RBC rosetting receptor Complement Receptor One (CR1) that reduces rosetting (Rowe *et al.* 1997), has been shown to confer protection from severe malaria in a malaria endemic region (Cockburn *et al.* 2004), thus providing more evidence for an association between rosetting and life-threatening malaria.

How rosetting may contribute to the development of severe malaria is not understood. Other *Plasmodium* species that infect humans form rosettes (Udomsanpetch *et al.* 1995; Angus *et al.* 1996; Lowe *et al.* 1998) but do not typically cause the same severe malaria symptoms as *P. falciparum*. Obstruction of the microvasculature and impairment of blood flow by rosettes has been shown in an *ex vivo* model (Kaul *et al.* 1991), and it is likely that rosettes aggravate the pathologies caused by cytoadherence of iRBC by further blocking the microvasculature (Nash *et al.* 1992). Cytoadherence, where iRBC bind to the endothelium lining the blood vessels (reviewed in (Newbold *et al.* 1999), is a parasite property unique to *P. falciparum* among the human *Plasmodium* species, which may explain why rosetting is not associated with severe malaria in *P. ovale*, *P. malariae* or *P. vivax*. Rosetting also occurs in several rodent and primate plasmodia such as *P. chabaudi* (Mackinnon *et al.* 2002), *P. fragile* (David *et al.* 1988), *P. coatneyi* (Kawai *et al.* 1995) and *P. cynomolgi* (Handunnetti *et al.* 1989). The widespread occurrence of rosetting in a variety of plasmodium species suggests that it may have an important biological function, however this function is currently unknown.

Rosetting in *P. falciparum* field isolates has been positively associated with host parasitaemia in African children (Rowe *et al.* 2002). It had been postulated that the function of rosetting could be to facilitate merozoite invasion (Wahlgren *et al.* 1989), which would promote high asexual blood stage parasitaemias in the host. However, experiments with a culture-adapted laboratory strain PA1 showed conclusively that there

was no difference in invasion rates between the isogenic rosetting and non-rosetting parasites in this strain (Clough *et al.* 1998).

I hypothesised that increased invasion of rosetting parasites may only occur in the presence of host immunity, when rosettes could potentially act as an immune evasion mechanism by shielding the merozoites from host invasion-inhibitory antibodies. It is plausible that merozoites released from a rosetting iRBC may be able to reinvade the neighbouring RBC without prolonged exposure to host antibodies in the plasma, as indicated in Figure 1A. Merozoites from non-rosetting parasites would be exposed to plasma antibodies that could inhibit their invasion (Figure 1B). If this were the case, one would expect rosetting parasites to have higher invasion rates than isogenic non-rosetting parasites in the presence of invasion inhibiting antibodies. In order to test this hypothesis I compared the invasion rates of isogenic rosetting and non-rosetting parasite lines of two culture-adapted *P. falciparum* strains in the presence of invasion-inhibitory antibodies to MSP1 (merozoite surface protein one) (Singh *et al.* 2003).

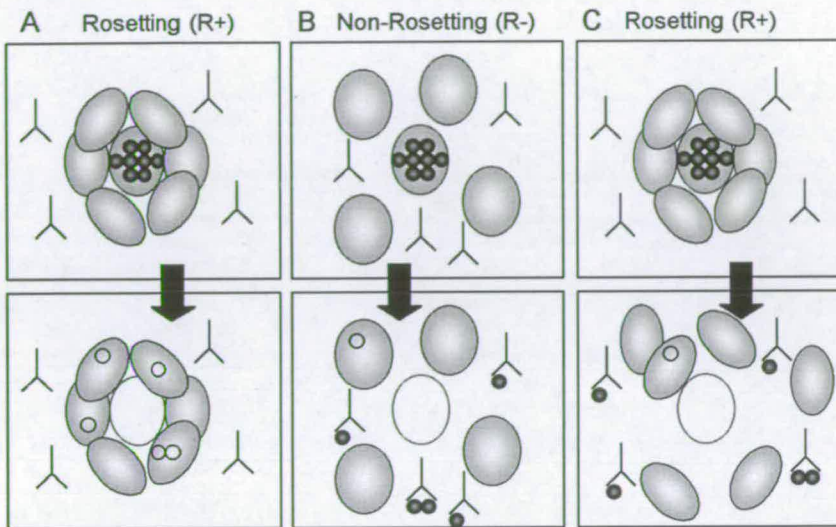
Figure 1:

Figure 1: Diagram showing how merozoites released from rosetting and non-rosetting *P. falciparum* iRBC could be differentially inhibited by invasion-inhibitory antibodies. A) Rosetting might reduce the effect of invasion-inhibitory antibodies if merozoites invade directly into the RBC in rosettes, thereby avoiding prolonged exposure to antibodies in the host's plasma. B) Merozoites derived from non-rosetting parasites would be exposed to antibodies in the plasma and hence would show lower invasion rates than merozoites from rosetting parasites. C) If merozoites from rosetting parasites do not preferentially invade the RBC in the rosette but are exposed to plasma antibodies in a similar way to merozoites from non-rosetting parasites, they would be expected to show reduced invasion rates in the presence of host antibodies.

5.3 Materials and Methods

5.3.1 Parasite culture

The parasites studied were *P. falciparum* clone R29 (derived from the IT strain)(Roberts *et al.* 1992) and strain TM284 (Scholander *et al.* 1996). R29 and TM284 are genotypically distinct and express different PfEMP1 variants (Rowe *et al.* 1997)(J.A.Rowe unpublished data). Parasites were cultured in complete RPMI (cRPMI: RPMI 1640 plus 25mM HEPES, 2mM glutamine, 25mM glucose, 25µg/ml gentamicin and 10% pooled human serum) at 1-2% haematocrit in O+ RBC at 37°C with 3%CO₂, 1%O₂, 96%N₂. Parasites were synchronised by sorbitol lysis (Lambros and Vanderberg 1979). To select for isogenic rosetting and non-rosetting parasite lines the cultures were selected using plasmagel flotation (Pasvol *et al.* 1978; Rowe *et al.* 1997). Following suspension in plasmagel solution, the rosetting trophozoite stage parasites settle to the bottom with the RBC pellet and the non-rosetting trophozoite stage parasites float in the upper layer above the RBC pellet. The non-rosetting upper layer was carefully removed and both layers were washed and then returned to culture. Repeated plasmagel selections over 1-2 weeks results in rosetting lines with >65% rosette frequency and non-rosetting lines with <6% rosette frequency.

5.3.2 Invasion assays

Synchronous trophozoite stage parasite cultures were diluted to approximately 1% parasitaemia with RBC and resuspended in cRPMI at 2% haematocrit. 50µl samples were set up in quadruplicate or triplicate wells in a 96-well flat-bottom plate (Corning). The wells immediately surrounding the samples had been prepared to contain 100µl incomplete RPMI to prevent dehydration during the assay. The plate was placed in a gas modulator chamber and gassed with 3%CO₂, 1%O₂, 96%N₂ for 3minutes before being placed at 37°C for 24 hours. From the remaining sample, pre-invasion smears were

made to count the exact pre-invasion parasitaemia on a Giemsa-stained thin smear. After 24 hours, Giemsa-stained thin smears were made from all wells and the parasitaemia was assessed by counting 1000 RBC. The invasion rate is the post-invasion parasitaemia divided by the pre-invasion parasitaemia.

Invasion inhibition assays were carried out using rabbit polyclonal IgG to MSP1-42 (Singh *et al.* 2003). In initial experiments a monoclonal antibody to MSP1 (McBride and Heidrich 1987; Blackman *et al.* 1990), two other rabbit polyclonal antibodies to MSP1 (gift from Dr Mike Blackman) and a rabbit polyclonal IgG to AMA-1 (gift from Dr Allan Saul) were tested, however, none of these reagents gave significant inhibition of invasion with R29 or TM284. For the assays with the anti-MSP1-42 antibody, the starting cultures were diluted to 0.9-1.3% parasitaemia with RBC and resuspended to 2% haematocrit with no added antibody (control) or a final concentration of 1.5 mg/ml of anti-MSP1 IgG. Assays were set up and invasion rates assessed as described above, with triplicate wells for each antibody and control. For shaking invasion assays the 96-well plate in the modulator chamber was rotated at 150-160rpm during the 24 hour incubation period (Orbital shaker, Brunswick Scientific). To ensure that the invasion-inhibitory antibody did not disrupt rosettes, samples of the rosetting parasites were incubated with the MSP1-42 antibodies at the above concentrations for 30 minutes at 37°C and then the RF was assessed.

5.3.3 Assessment of rosette frequency

A 200µl aliquot of culture suspension containing mature pigmented trophozoite stage parasites was stained with 25µg/ml ethidium bromide for 5 minutes at 37°C. 15µl of culture suspension was placed on a microscope slide and covered with a coverslip. The stained sample was viewed with a 40x objective using a Leica fluorescence microscope with both UV and brightfield light to allow visualisation of both iRBC and RBC simultaneously. 200 iRBC were counted and scored for rosetting, with a rosette being

defined as an iRBC binding two or more RBC. The percentage of the 200 iRBC in rosettes is termed the rosette frequency (RF). For the invasion assays, rosetting cultures had a RF of above 65% and non-rosetting cultures had a RF of less than 6%.

5.3.4 Statistical analysis

Statistical analysis was done with Statview 5.0.1 software. The invasion rates of isogenic rosetting and non-rosetting parasites were analysed with a paired Students t-test, paired by experiment to control for variations in invasion due to RBC donor, human sera and other factors. The percentage inhibition of invasion by the MSP1-42 antibody of rosetting and non-rosetting parasites was analysed with a Students t-test.

5.4 Results

5.4.1 Invasion rates of isogenic rosetting and non-rosetting parasites

I firstly set out to investigate whether isogenic rosetting and non-rosetting parasite lines derived from R29 and TM284 differed in their invasion rates under standard *in vitro* culture conditions. Over seven (TM284) or eight (R29) experiments, there was no significant difference in the invasion rates of the isogenic rosetting and non-rosetting lines (Table 1, $P=0.86$ and $P=0.21$, Paired Students t-test, TM284 and R29 respectively). I did find that TM284 has a higher invasion rate (6.6 mean (2.0 standard deviation)) than the parasite strain R29 (4.4 (1.6) ($P=0.03$, Students t-test (only R-)).

Table 1: Parasite rosette frequency and invasion rate for two isogenic rosetting and non-rosetting parasite strains

Strain	R ^a	N	RF ^b	Invasion Rate ^c	Paired t-test ^d
TM284	R+	7	74 (4.8)	6.7 (1.6)	$P=0.86$
	R-	7	3 (2.4)	6.6 (2.0)	
R29	R+	8	77 (8.3)	5.3 (1.8)	$P=0.21$
	R-	8	2 (1.6)	4.4 (1.6)	

^a R represents the rosetting phenotype: R+ is selected for rosetting and R- is selected to not form rosettes in culture

^b Rosette Frequency counted as % iRBC binding two or more uninfected RBC; Mean (standard deviation)

^c Invasion rate is the number of ring-iRBC from triplicate or quadruplicate wells from each experiment after 24 hr invasion divided by the starting schizont parasitaemia; Mean (standard deviation)

^d Paired t-test for the invasion rate of the isogenic R+ and R- cultures paired by experiment to account for variations due to RBC age, RBC donor, human sera and other factors.

5.4.2 Identification of an invasion-inhibitory antibody

To examine the hypothesis that rosetting may support invasion in the presence of host invasion-inhibitory antibodies (figure 1A and B), I had to identify antibodies that would inhibit the invasion of non-rosetting parasites. Invasion-inhibitory antibodies are often described in the literature and are used as a tool to identify *P. falciparum* proteins, which may be involved in the RBC invasion process. As generating antibodies was beyond the scope of this PhD project, I obtained several antibodies as gifts (see methods) and tried a range of antibody concentrations to check for invasion inhibition. The maximum inhibition seen with the different antibodies is shown in figure 2. I did not get a significant inhibition of invasion for four antibodies. Two rabbit polyclonal antibodies to MSP1 did not inhibit invasion of both R29 and TM294 at a 1/100 or 1/400 dilution of the rabbit sera in one experiment (data not shown). In three experiments with TM284 a 1/10 dilution of the same polyclonal antibodies increased invasion by roughly 10% and 13% (Rb-01 and Rb-03, Figure 2). An AMA1 polyclonal antibody did not inhibit invasion of R29 at three different concentrations (0.3, 0.6 and 1.25mg/ml) and at the highest concentration (1.25mg/ml) inhibited invasion by only 16% in one experiment (figure 2). The inhibition was not significant compared to the control ($P=0.57$, Students t-test). The murine monoclonal antibody mAb12.8, which has been shown to be an invasion-inhibitory antibody (Blackman *et al.* 1990; Guevara Patino *et al.* 1997), was tested in 5 experiments at a concentration of 0.5mg/ml on R29 and TM284 respectively. mAb 12.8 inhibited invasion by 16% and 18.4% in R29 and TM284 respectively (figure 2), but this was not significant compared to the control with no added antibody ($P=0.74$ and $P=0.20$, R29 R- and TM284 R- respectively, Students t-test).

The only antibodies to significantly inhibit invasion by more than 20% was a rabbit polyclonal antibody to MSP1-42 (Singh *et al.* 2003) ($P=0.02$ and $P<0.001$, R29 R- and TM284 R- respectively, Students t-test, figure 2). This antibody was therefore used to

study invasion inhibition in isogenic rosetting and non-rosetting parasites as described below.

Figure 2:

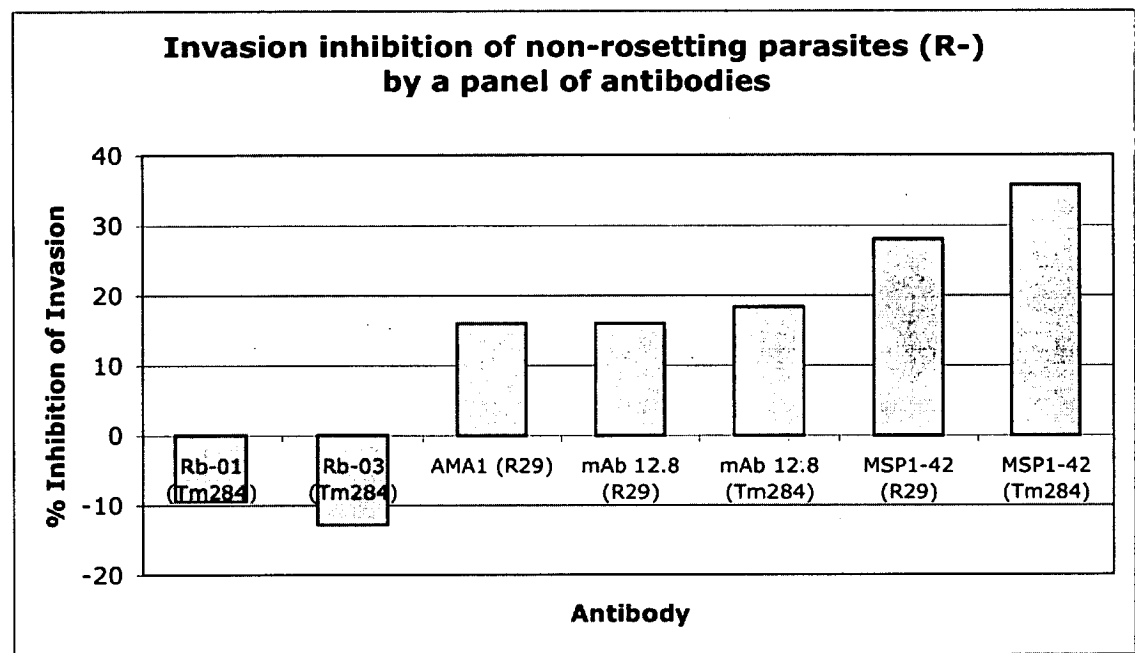


Figure 2: Invasion inhibition by a panel antibodies tried on either R29 or Tm284 as indicated in the brackets. Invasion inhibition was measured on non-rosetting parasites in an invasion assay as described in the methods. % Mean inhibition of invasion is relative to the control invasion rate. Details for the individual antibodies: Rb01 and Rb03 were used at a 1/10 dilution in 3 experiments with Tm284. AMA1 was used at 1.25mg/ml in 1 experiment on R29. mAb12.8 was used at 0.5mg/ml in 5 experiments with both R29 and Tm284. MSP1-42 was used at 1.5mg/ml in two experiments with both R29 and Tm284.

5.4.3 Invasion rates of isogenic rosetting and non-rosetting parasites in the presence of antibodies to MSP-1

To examine the hypothesis that rosetting may support invasion in the presence of host invasion-inhibitory antibodies (Figure 1A and B), I studied the invasion rates of isogenic rosetting and non-rosetting parasites in the presence of antibodies to MSP1-42 (Singh *et al.* 2003). In two experiments with both TM284 and R29, I found that the percentage inhibition of invasion in the presence of the MSP1-42 antibodies did not differ between the isogenic rosetting and non-rosetting parasites (Table 2, $P=0.62$ and $P=0.63$, Students t-test, TM284 and R29 respectively). The mean inhibition of invasion for rosetting parasites was 33.0% (95% confidence interval 25.5% to 40.5%) and for non-rosetting parasites was 31.8% (95% confidence interval 26.3% to 37.3%).

Table 2: Invasion inhibition of isogenic rosetting and non-rosetting *P. falciparum* parasites from two strains

Strain	Exp.	R ^a	RF ^b	Invasion Rate ^c		Inhibition ^d
				Control	MSP-1 Ab	
TM284	1	R+	70	7.4 (1.2)	4.9 (0.1)	33.3
		R-	2	6.8 (1.4)	4.7 (0.3)	30.2
	2	R+	68	9.0 (1.2)	6.2 (0.9)	31.2
		R-	0	8.7 (0.2)	5.2 (0.7)	40.2
R29	1	R+	76	5.1 (1.2)	3.8 (0.3)	24.4
		R-	0	2.5 (0.3)	1.8 (0.2)	28
	2	R+	76	5.9 (0.3)	3.4 (0.2)	42.9
		R-	0	4.3 (1.2)	3.1 (0.3)	28.9

^a R represents the rosetting phenotype: R+ is selected for rosetting and R- is selected to not form rosettes in culture

^b Rosette Frequency counted as % iRBC binding two or more uninfected RBC

^c Invasion rate is the mean (standard deviation) number of ring-iRBC from triplicate wells after 24 hr invasion divided by the starting schizont parasitaemia

^d Percent inhibition of invasion of the isogenic R+ and R- parasites in the presence of the MSP-1 antibody compared to the control invasion rate. There was no significant difference in the inhibition between the R+ and R- parasites (P=0.62 and P=0.63, Students t-test, TM284 and R29 respectively).

To ensure that my results were not due to rosettes being disrupted by the invasion-inhibitory antibodies, I assessed the effect of the MSP1-42 antibodies on rosetting. For both R29 and TM284 I found that the rosette frequency did not change in the presence of the MSP1-42 antibodies (R29: control with no antibody 48% RF, 1.5mg/ml MSP1-42 52% RF; TM284: control with no antibody 83% RF, 1.5mg/ml MSP1-42 84% RF).

In the invasion inhibition experiments (Table 2), the data show a trend towards rosetting R29 parasites having higher overall invasion rates than R29 non-rosetting parasites in the control cultures without added antibodies. However, as shown in Table 1, an analysis of a larger number of experiments does not support a significant difference in invasion rates between these isogenic lines.

5.4.4 Invasion inhibition of shaking cultures

In addition, I attempted to examine the inhibitory effect of the MSP1-42 antibodies on R29 and TM284 cultures under shaking conditions. However, in two experiments with TM284 no inhibition of invasion was seen when the cultures were shaken rather than static ($P \geq 0.50$, both R+ and R-, Student's t-test, figure 3). I therefore could not repeat the above experiments under shaking conditions.

Figure 3:

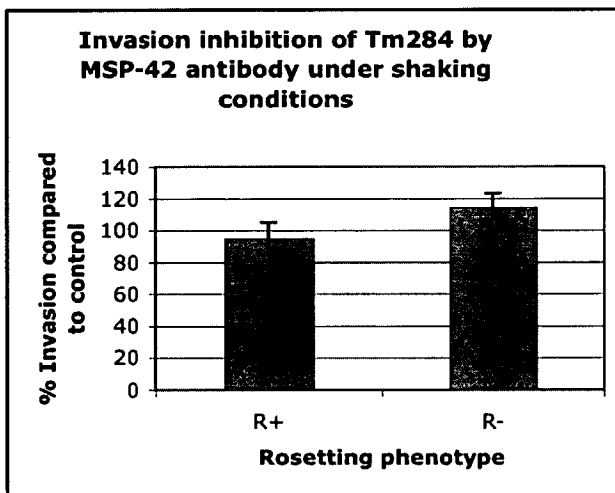


Figure 3: Lack of invasion inhibition of isogenic rosetting (R+) and non-rosetting (R-) Tm284 parasites by the antibody MSP-42 (1.5mg/ml) under shaking conditions. The mean (+ standard deviation) of two experiments, each with triplicate wells, is shown.

5.5 Discussion

In this chapter I show that rosettes do not protect from invasion-inhibitory antibodies. I also confirmed the previous findings of Clough *et al.* that rosetting does not enhance invasion (Clough *et al.* 1998). This had been shown for one parasite line, PA1, and I repeated the findings with R29 and TM284.

The experiments were complicated at first by the fact that it was necessary to identify an antibody that inhibited the invasion of the two *P. falciparum* strains under investigation. Antibodies to the merozoite surface antigens MSP1 and AMA1 are known to inhibit invasion and have been associated with protection from clinical malaria (John *et al.* 2004; Polley *et al.* 2004). I tested a range of antibodies to MSP-1 and AMA-1 to find a suitable antibody for these experiments, but found it difficult to identify a reagent giving significant invasion inhibition of R29 or TM284 non-rosetting parasites as shown in figure 2. There may be two reasons for this. First of all the merozoite surface antigens MSP1 and AMA1, to which the antibodies were generated, are polymorphic and the invasion-inhibitory effects of these antibodies may therefore be strain-specific. The MSP1 monoclonal antibody mAb12.10, which was generated by the same group as the mAb12.8 used here, showed 72% inhibition of the T9-94 strain and only 50% inhibition of invasion of the T9-96 strain at a concentration of 0.5mg/ml (Blackman *et al.* 1990). In addition human antibodies isolated from a malaria endemic region, which recognise the EGF1 domain in MSP1-19 similar to the mAb12.8, did not show any invasion-inhibitory activity (Chappel *et al.* 1994). In regards to AMA1, antibodies to either 3D7 or W2mef both inhibited the homologous strain by around 60-80% but the heterologous strain by only around 20% similar to my findings (Healer *et al.* 2004). Another study also reported less inhibition of invasion of heterologous *P. falciparum* strains by AMA1 antibodies (Kennedy *et al.* 2002) and antibodies elicited by AMA1 in a Phase I vaccine

trial rarely inhibited invasion by more than 20% (Malkin *et al.* 2005). From my findings and the above reports, it is apparent that one would need to generate strain specific antibodies against R29 or TM284 antigens to see a maximum inhibition of invasion, but that lay beyond the scope of this PhD project.

Although I believe the above explains the lack of invasion inhibition, as a second reason I cannot rule out problems with a decrease in activity of the antibodies due to storage or the shipping since they were generated. Reproducing invasion inhibition results may be a problem as a decrease in invasion-inhibitory activity by an antibody used by the same research group on the same homogenous strain has been described, which provides further evidence for problems with reproducibility of invasion-inhibitory effects (Healer *et al.* 2004).

Despite those initial problems, it was possible to identify a rabbit polyclonal antibody to MSP1-42 (Singh *et al.* 2003) that gave a significant inhibition of invasion. The inhibition with MSP1-42 was not as high as it had been shown with this antibody on the 3D7 strain, for which 69% inhibition was reported (S.Mahanty, personal communication) and was not maintained under shaking conditions (see results 3.4). However, in the static invasion assays the inhibition was significant compared to the control sample for both R29 and TM284 (figure 2, Table 2). Therefore this antibody was suitable for experiments to determine if rosettes protect from invasion-inhibitory antibodies.

I found that rosetting and non-rosetting parasites were inhibited to the same degree by the MSP1-42 antibody for two parasite strains, R29 and TM284 ($P=0.63$ and $P=0.62$, respectively, Table 2). I showed that this was not due to the disruption of rosettes, as the rosettes were stable in the presence of MSP1-42. This indicates that rosettes do not protect merozoites from invasion-inhibitory antibodies as hypothesised in Figure 1A, but rather that rosetting parasites are inhibited by invasion-inhibitory antibodies to the same

extent as non-rosetting parasites (Figure 1B and 1C). It is unclear whether rosettes are stable during schizogony or whether they break apart before the schizonts rupture. My results point towards the latter being the most likely possibility. This suggestion is consistent with previous findings showing that the RBC forming the rosette are not preferentially targeted during invasion (Clough *et al.* 1998). This result is important as the biological function of the rosetting phenotype is so far unknown. In addition it has potential implications for vaccine research as my findings do not support any evidence that rosettes may protect parasites from invasion-inhibitory antibodies generated by blood-stage vaccines.

Although I found no evidence to support the hypothesis that rosettes protect merozoites from invasion-inhibitory antibodies, it is yet to be determined whether rosettes may affect the interactions of iRBC with other components of the host's immune system. Contact-dependent activation of natural killer cells – an important first line in innate immunity against *P. falciparum* - has been reported (Artavanis-Tsakonas *et al.* 2003), and this activation may not occur if uninfected RBC surround the iRBC. Contrary to this, binding of iRBC to dendritic cells has been shown to modulate the function of antigen-presenting cells to possibly delay an immune reaction (Urban *et al.* 1999), so rosetting may prevent the binding of iRBC to receptors on the surface of dendritic cells and may promote dendritic cell activity. Furthermore, rosettes may shield the iRBC from phagocytosis by macrophages and neutrophils. It will be interesting to compare natural killer cell activation, dendritic cell modulation and phagocytosis of rosetting and non-rosetting parasites in an attempt to understand the biological function of rosetting.

CHAPTER 6: Discussion

6.1 Summary

The focus of my study was to investigate potential *P. falciparum* virulence factors that may contribute to the pathogenesis of severe malaria. The work involved looking at virulence factors in field isolates from two distinct regions in Africa, looking at RBC selectivity in the *P. chabaudi* model and furthermore studying the rosetting phenotype in laboratory strains. I made the following findings:

1. Parasite multiplication rate and selectivity index are not associated with malaria severity in Africa.
2. Selectivity index is not associated with parasite virulence in the *P. chabaudi* model. There does seem to be a day effect on SI.
3. There is no significant difference between invasion pathways of Kenyan field isolates from patients with uncomplicated or severe malaria.
4. Rosetting does not protect iRBC from invasion-inhibitory antibodies.

A full discussion of my findings can be found at the end of each chapter. Here I will discuss how my findings link together and where this leaves future research.

6.2 Severe malaria – Africa and Asia

Understanding what causes severe malaria is crucial to develop better drugs and vaccines in the future. Multiple factors may predispose an individual to severe malaria (Miller *et al.* 2002; Greenwood *et al.* 2005), yet there is little understanding of the precise contribution of different factors in the pathogenesis of severe malaria.

This study looked at a number of parasite virulence factors in relation to malaria severity in Africa and the following conclusions can be drawn: There is no evidence for differences between *P. falciparum* parasites that are isolated from children with uncomplicated or severe malaria in regards to their multiplication rates or their RBC selectivity, contrasting previous results from Thailand (Simpson *et al.* 1999; Chotivanich *et al.* 2000). This is not the first report where there is a discrepancy between findings from Africa and Asia in relation to a *P. falciparum* phenotype, as rosetting has been strongly associated with severe malaria in Africa (Carlson *et al.* 1990; Rowe *et al.* 1995; Kun *et al.* 1998), however the same is not the case in Asia (Ho *et al.* 1991; Angkasekwinai *et al.* 1998). Although further studies are needed to investigate why the results from Asia and Africa differed, one may conclude that in Africa parasite virulence factors other than the parasite multiplication rate or RBC selectivity may be associated with the development of severe malaria. Results from the work described here further support rosetting as a virulence factor associated with severe malaria (chapter 2 and A. Rowe, personal communication), but there are also other virulence factors such as autoagglutination and cytoadherence, which may play an important role.

I found no association between the SI and malaria severity of *P. falciparum* infections in Kenya and Mali. Several reasons for this were discussed (chapter 2), two of which were first that it is due to host immunity, as in Africa the population rapidly builds up a level of semi-immunity to the parasite and children above 5 years rarely develop severe malaria. Second, I postulate that a low level of immunity may delay the onset of clinical symptoms and, hence, the time until presentation at the hospital. Intriguingly, when studying the SI in the *P. chabaudi* model I did not see an association between virulence and the SI, but there is an indication that the SI may change over the time-course of the infection. This may be driven by strain-specific immunity or other host responses to the infection over time. Although tentative, this may explain differences seen between Thailand and Africa. One may hypothesise that Thai patients with severe malaria have no acquired immunity to *P. falciparum* and are at an early stage in the infection, comparable to day 7 in the *P. chabaudi* model, and parasites from these patients show low RBC selectivity (Simpson *et al.*

1999). Whereas, the Thai mild malaria patients are controlling the parasite growth and increasing RBC selectivity, comparable to a later day in the *P. chabaudi* model and hence a higher SI than isolates from Thai patients with severe malaria. In African children, where some immunity is present, possibly delaying the time until presentation at the hospital, in children that both develop uncomplicated or severe malaria the selectivity index is higher, comparable to a later day in the *P. chabaudi* model, similar to the Thai uncomplicated patients. It would be very interesting to look at a birth cohort in Africa to examine the SI in the first infection when host immunity should be very low. As these cohorts are sometimes followed for many years, one could look at the SI in later infections as well. Although there is the complication that due to maternal antibodies the infants experiencing their first infection may not be regarded as naïve in terms of immunity to malaria.

Due to the above mentioned difficulties, a good way to compare Asian and African parasite isolates in regards to their PMR and SI would be by studying parasites isolated from non-immune Caucasian travellers who return to the UK and develop malaria. Such a study design could eliminate variables such as differences in host genetics, immunity levels, previous drug treatment, previous malaria infections, and time of presentation to the hospital. Furthermore, as the assays for parasites from both Africa and Asia could be carried out by the same laboratory this would remove any across laboratory variation. There are of course limitations and one problem may be the smaller number of travellers returning from Asia who develop severe *P. falciparum* malaria. The question is of course what may one expect to see? If there are genuine differences in PMR between Asian and African parasites, then one may expect to see a significant difference in PMR between the two groups. If the low PMR seen in Africa is because of host immunity, then one may expect to see high PMR of isolates from travellers returning from African malaria endemic locations, that are similar to the high PMR of isolates from patients with severe malaria in Africa (Chotivanich *et al.* 2000). At the same time, it would be possible to look at other parasite virulence factors such as rosetting and autoagglutination.

A very different approach to study differences in PMR is to identify genes associated with high PMR. One method used to address this topic is linkage group selection (Martinelli *et al.* 2005) and there is currently an ongoing project using this method to identify genes linked with a higher growth rate phenotype in *P. chabaudi* (Naomi Gadsby, personal communication). Any identified genes, could be used to find *P. falciparum* homologues. It would then be possible to look at the expression of these genes in both Thai and African isolates to see if there is a genetic difference between the high PMR Thai isolates and the low PMR Thai and African isolates.

An unexpected result described in chapter 2 was the finding that parasites from children diagnosed with hyperparasitaemia had the lowest PMR. This group of patients are themselves very interesting as they are able to harbour such high parasite infections, yet do not show any signs of severe malaria. This group may be invaluable for identifying which parasite factors one should target for a drug design or vaccine that will prevent severe malaria. One may of course argue that these are children, who are on their way to develop severe malaria, but there are a number of strong reasons to believe they truly form a separate category. First of all, they are relatively old and have high haemoglobin levels (chapter 2). The mortality rate of children with hyperparasitaemia is very low at only 0.01% compared to an overall mortality rate of 8.7% of children with severe malaria in the same population (Lyke *et al.* 2003). They do not have as high rosette frequencies as parasites from children with severe malaria (chapter 2 and A. Rowe, personal communication). Also, when looking at the blood group information of the parasites collected in Mali, severe malaria is associated with blood group A (A. Rowe, personal communication), yet the hyperparasitaemia children show a similar distribution of the blood groups as the uncomplicated malaria patients. The blood group distributions of both the uncomplicated and the hyperparasitaemia children differ from the distribution of the children, which have severe malaria (A. Rowe, personal communication). An ongoing study looking at the *var* genes, which are expressed in the Malian children, has also found that the *var* genes expressed by children with uncomplicated malaria are distinct from the *var* genes expressed by parasites from children with severe malaria (Helen Kyriacou, personal communication). One may speculate that

parasites, which can develop high parasitaemias in the host without severe pathology symptoms, express an 'avirulent' PfEMP1 type. This 'avirulent' PfEMP1 type may be associated with binding to certain receptors, possibly CD36 rather than receptors such as ICAM1, which may facilitate the development of severe malaria. Functional studies looking at the binding specificities of this 'avirulent' PfEMP1 type compared to for example the variant surface antigens associated with severe malaria could address this question. It may also be associated with decreased PMR as shown in this study. As far as I am aware, there has not been any association between any PfEMP1 types and PMR described to date. Unfortunately, in this study the invasion pathways of the Malian isolates were not investigated, but it may be worth following up which invasion pathways parasites isolated from patients with hyperparasitaemia use.

The work described here looking at the invasion pathways of parasites from children with uncomplicated and severe malaria in Kenya showed no significant difference between the two groups. This may not be so surprising as there was no significant difference between the PMR of these two groups. Although independently of PMR, there could have been a difference in invasion pathways used by isolates from children with uncomplicated or severe malaria. Invasion pathways may not link to parasite virulence, but it still remains to study the invasion pathways used by parasites with high PMR in Thailand, where such clear differences in PMR between uncomplicated and severe malaria patients were seen (Chotivanich *et al.* 2000).

6.3 *Plasmodium chabaudi* as a model to study RBC selectivity

In the *P. chabaudi* model it was found that there is no association between the SI and parasite virulence. This supports my finding from Africa and as described in more detail above may indicate that the differences in SI found for Thai isolates may have been due to other factors, such as time in the infection, rather than being an intrinsic parasite virulence factor. This is the first report of a SI for *P. chabaudi*. Overall, I found RBC selectivity to be low, as expected for a parasite that does not have a preference for reticulocytes or young RBC. *P. chabaudi* may provide a good model for further study of the SI over the course of the infection and for the identification of

host factors that may lead to an increase in SI later in infection, as it is possible to monitor the infections over time. Such a study could provide evidence to test the hypothesis outlined above that the differences in results from Southeast Asia and Africa in regards to the SI and malaria severity, are due to the timing or patients presentation at hospital.

6.4 Severe malaria – rosetting as a virulence factor

In this study I found rosettes do not protect merozoites from invasion inhibitory antibodies. A relationship between rosetting and malaria severity has been demonstrated in Africa, yet how rosettes contribute to the development of severe malaria is unknown. A previous study (Clough *et al.* 1998) and work presented in this thesis, where rosetting of neither laboratory strains (chapter 5) nor field isolates (chapter 2) were associated with higher PMR, show that rosetting does not enhance parasite growth per se. Rosetting is though positively correlated with parasitaemia *in vivo* indicating that rosettes may have some effect on parasite growth or survival in the host (Rowe *et al.* 2002). I tested the hypothesis that rosettes may protect merozoite invasion from invasion-inhibitory antibodies and so maintain a higher growth rate in the presence of host immunity. I could not find evidence to support this hypothesis (chapter 5). It now remains to be investigated whether rosettes may protect from other forms of host immunity – such as phagocytosis, activation of natural killer cells or other contact-dependent immune suppression mechanisms.

6.5 Conclusions

The results described in this thesis emphasise that there is still a lot to be learnt about *P. falciparum* virulence factors. I showed that results found in Africa are not the same as Thai infections and that a virulence factor other than parasite multiplication rate or RBC selectivity may play an important role in the development of severe malaria in Africa. Other virulence factors, such as rosetting or autoagglutination, are known, but how they may be associated with severe malaria is not well understood.

Possibly they maintain parasite growth in the host, where some degree of acquired immunity may be present. I investigated this hypothesis for rosetting, but found that rosettes do not protect merozoite invasion from invasion-inhibitory antibodies. So the function of rosetting remains to be investigated further. The invasion pathways used by parasite isolates were found to be diverse, but there was no indication that the invasion profile is a virulence factor as there was no significant difference in invasion profiles between isolates from uncomplicated and severe malaria patients. Finally, these findings have serious implications for future research and malaria intervention strategies. Will a vaccine disrupting rosettes protect children in Africa from severe malaria, yet not affect the severe malaria pathologies in Asia? And vice versa, antibodies that inhibit the rapid growth of parasites may be protective in Asia, however still leave the individual at risk in Africa? Future work will be required to address these questions.

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Appendix

Publications arising from this thesis:

1. Arising from the work presented in chapter 5:

Title: *Plasmodium falciparum*: rosettes do not protect merozoites from invasion-inhibitory antibodies.

Authors: Anne-Marie Deans and J. Alexandra Rowe

Journal: Experimental Parasitology

Status: Accepted. In press 2005.

2. Arising from the work presented in chapter 2:

Title: Low multiplication rates of African *Plasmodium falciparum* isolates and lack of association of multiplication rate and red cell selectivity with malaria virulence

Authors: Anne-Marie Deans, Kirsten E. Lyke, Mahamadou A. Thera, Christopher V. Plowe, Abdoulaye Kone, Ogobara K. Doumbo, Oscar Kai, Kevin Marsh, Margaret J. Mackinnon, Ahmed Raza and J. Alexandra Rowe

Journal: American Journal of Tropical Medicine and Hygiene

Status: Accepted. In press 2005.

3. Arising from the work presented in chapter 3:

Title: Red cell selectivity is not associated with *P. chabaudi* strain virulence.

Authors: Anne-Marie Deans, J. Alexandra Rowe and Margaret J. Mackinnon

Journal: To be submitted to the International Journal of Parasitology

Status: In preparation.

4. Arising from the work presented in chapter 4:

4.1 Title: Invasion pathways and *P. falciparum* disease severity.

Authors: Anne-Marie Deans, Susanna Nery, Oscar Kai, Kevin Marsh, Margaret J. Mackinnon, Ahmed Raza, Dave Conway and J. Alexandra Rowe

Journal: To be submitted to Infection and Immunity.

Status: In preparation. This manuscript will be submitted back to back with work from Dr. Susana Nery and Dr. David Conway in regards to the expression profiles of invasion proteins from the same samples (see below).

4.2 Title: Expression of *Plasmodium falciparum* genes involved in erythrocyte invasion varies among isolates cultured directly from patients.

Authors: Susanna Nery, Anne-Marie Deans, Moses Mosobo, Kevin Marsh, Alexandra Rowe and Dave Conway

Journal: Molecular and Biochemical Parasitology.

Status: Submitted February 2006.



Research brief

Plasmodium falciparum: Rosettes do not protect merozoites from invasion-inhibitory antibodies

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Abstract

Rosetting is a parasite adhesion phenotype associated with severe malaria in African children. Why parasites form rosettes is unknown, although enhanced invasion or immune evasion have been suggested as possible functions. Previous work showed that rosetting does not enhance parasite invasion under standard in vitro conditions. We hypothesised that rosetting might promote invasion in the presence of host invasion-inhibitory antibodies, by allowing merozoites direct entry into the erythrocytes in the rosette and so minimising exposure to plasma antibodies. We therefore investigated whether rosetting influences invasion in the presence of invasion-inhibitory antibodies to MSP-1. We found no difference in invasion rates between isogenic rosetting and non-rosetting lines from two parasite strains, R29 and TM284, in the presence of MSP-1 antibodies ($P=0.62$ and $P=0.63$, Student's t test, TM284 and R29, respectively). These results do not support the hypothesis that rosettes protect merozoites from inhibitory antibodies during invasion. The biological function of rosetting remains unknown.

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Index Descriptors and Abbreviations: *Plasmodium falciparum*; Rosetting; Invasion inhibition; Immune evasion; Rosette formation; Erythrocyte invasion; RBC, red blood cells; iRBC, infected red blood cells; cRPMI, complete RPMI; RF, rosetting frequency; MSP-1, merozoite surface protein 1; AMA1, apical membrane antigen 1

1. Introduction

The binding of infected red blood cells (iRBC) to uninfected red blood cells (RBC) is a *Plasmodium falciparum* adhesion phenotype known as rosetting. *P. falciparum* field isolates vary in the extent to which they form rosettes, and high levels of rosette formation have been strongly associated with severe malaria in African children in numerous studies (e.g., Carlson and Wahlgren, 1992; Ringwald et al., 1993; Rowe et al., 1995, 2002b). A human polymorphism associated with deficiency of the RBC rosetting receptor Complement Receptor 1 that reduces rosetting (Rowe et al., 1997), has been shown to confer protection from severe malaria in a malaria endemic region (Cockburn

et al., 2004), thus providing more evidence for an association between rosetting and life-threatening malaria.

How rosetting may contribute to the development of severe malaria is not understood. Other *Plasmodium* species that infect humans form rosettes (Angus et al., 1996; Lowe et al., 1998; Udomsanpetch et al., 1995) but do not typically cause the same severe malaria symptoms as *P. falciparum*. Obstruction of the microvasculature and impairment of blood flow by rosettes has been shown in an ex vivo model (Kaul et al., 1991), and it is likely that rosettes aggravate the pathologies caused by cytoadherence of iRBC by further blocking the microvasculature (Nash et al., 1992). Cytoadherence, where iRBC bind to the endothelium lining the blood vessels (reviewed in Newbold et al., 1999), is a parasite property unique to *P. falciparum* among the human *Plasmodium* species, which may explain why rosetting is not associated with severe malaria in *P. ovale*, *P. malariae* or *P. vivax*. Rosetting also occurs in several rodent and primate plasmodia

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such as *Plasmodium chabaudi* (Mackinnon et al., 2002), *Plasmodium fragile* (David et al., 1988), *Plasmodium coatneyi* (Kawai et al., 1995), and *Plasmodium cynomolgi* (Handunnetti et al., 1989). The widespread occurrence of rosetting in a variety of plasmodium species suggests that rosetting may have an important biological function, however this function is currently unknown.

Rosetting in *P. falciparum* field isolates has been positively associated with host parasitaemia in African children (Rowe et al., 2002a). It had been postulated that the function of rosetting could be to facilitate merozoite invasion (Wahlgren et al., 1989), which would promote high asexual blood stage parasitaemias in the host. However, experiments with a culture-adapted laboratory strain PA1 showed conclusively that there was no difference in invasion rates between the isogenic rosetting and non-rosetting parasites in this strain (Clough et al., 1998).

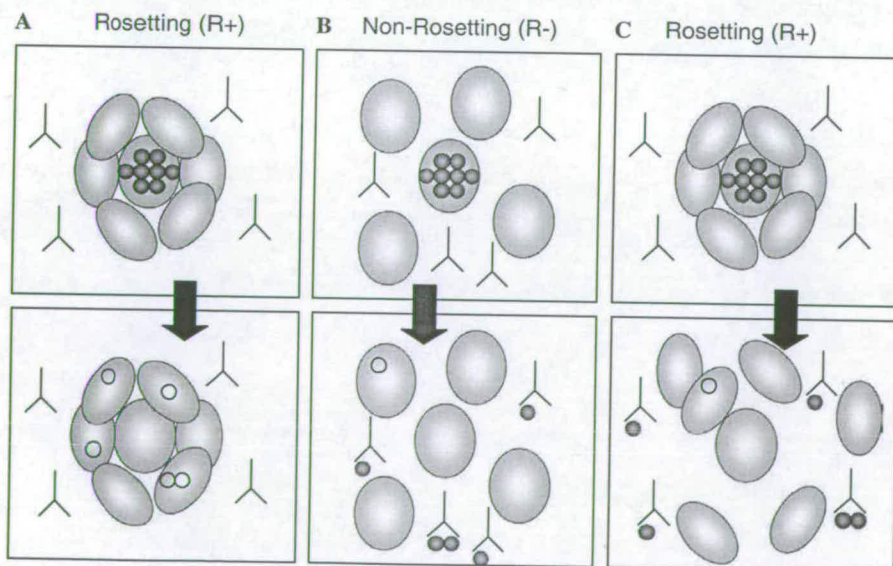
We hypothesised that increased invasion of rosetting parasites may only occur in the presence of host immunity, when rosettes could potentially act as an immune evasion mechanism by shielding the merozoites from host invasion-inhibitory antibodies. It is plausible that merozoites released from a rosetting iRBC may be able to reinvade the neighbouring RBC without prolonged exposure to host antibodies in the plasma, as indicated in Fig. 1A. Merozoites from non-rosetting parasites would be exposed to plasma antibodies that could inhibit their invasion (Fig. 1B). If this were the case, one would expect rosetting parasites to have higher invasion rates than isogenic non-rosetting parasites in the presence of invasion inhibiting

antibodies. To test this hypothesis, we compared the invasion rates of isogenic rosetting and non-rosetting parasite lines of two culture-adapted *P. falciparum* strains in the presence of invasion-inhibitory antibodies to MSP-1 (merozoite surface protein one) (Singh et al., 2003).

2. Methods

2.1. Parasite culture

The parasites studied were *P. falciparum* clone R29 (derived from the IT strain) and strain TM284 (Rowe et al., 1997). R29 and TM284 are genotypically distinct and express different PfEMP1 variants (Rowe et al., 1997 and unpublished data). Parasites were cultured in complete RPMI (cRPMI: RPMI 1640 plus 25 mM Hepes, 2 mM glutamine, 25 mM glucose, 25 µg/ml gentamicin, and 10% pooled human serum) at 1–2% haematocrit in O⁺ RBC at 37 °C with 3% CO₂, 1% O₂, and 96% N₂. Parasites were synchronised by sorbitol lysis (Lambros and Vanderberg, 1979). To select for isogenic rosetting and non-rosetting parasite lines, the cultures were selected using plasmagel flotation (Pasvol et al., 1978; Rowe et al., 1997). Following suspension in plasmagel solution, the rosetting trophozoite-stage parasites settle to the bottom with the RBC pellet and the non-rosetting trophozoite-stage parasites float in the upper layer above the RBC pellet. The non-rosetting upper layer was carefully removed and both layers were washed and then returned to culture. Repeated plasmagel selections over 1–2 weeks result in rosetting lines with >65% rosette



1. Diagram showing how merozoites released from rosetting and non-rosetting *P. falciparum* iRBC could be differentially inhibited by invasion-inhibitory antibodies. (A) Rosetting might reduce the effect of invasion-inhibitory antibodies if merozoites invade directly into the RBC in rosettes, thereby avoiding prolonged exposure to antibodies in the host's plasma. (B) Merozoites derived from non-rosetting parasites would be exposed to antibodies in the plasma and hence would show lower invasion rates than merozoites from rosetting parasites. (C) If merozoites from rosetting parasites do not preferentially invade the RBC in the rosette but are exposed to plasma antibodies in a similar way to merozoites from non-rosetting parasites, they would be expected to show reduced invasion rates in the presence of host antibodies. Our results indicate that merozoites released from rosetting parasites are inhibited to the same extent as merozoites released from non-rosetting parasites (B and C).

frequency and non-rosetting lines with <6% rosette frequency.

2.2. Invasion assays

Synchronous trophozoite-stage parasite cultures were diluted to approximately 1% parasitaemia with RBC and resuspended in cRPMI at 2% haematocrit. Fifty microlitre samples were set up in quadruplicate or triplicate wells in a 96-well flat-bottomed plate. The wells immediately surrounding the samples had been prepared to contain 100 µl incomplete RPMI to prevent significant evaporation from test wells during the assay. The plate was placed in a gas modulator chamber and gassed with 3% CO₂, 1% O₂, and 96% N₂ for 3 min before being placed at 37°C for 24 h. From the remaining sample, pre-invasion smears were made to count the exact pre-invasion parasitaemia on a Giemsa-stained thin smear. After 24 h, Giemsa-stained thin smears were made from all wells and the parasitaemia was assessed by counting 1000 RBC. The invasion rate is the post-invasion parasitaemia divided by the pre-invasion parasitaemia.

Invasion inhibition assays were carried out using rabbit polyclonal IgG to MSP-1-42 (Singh et al., 2003). We also tested a monoclonal antibody to MSP-1 (Blackman et al., 1990; McBride and Heidrich, 1987), two other rabbit polyclonal antibodies to MSP-1 (gift from Dr. Mike Blackman) and a rabbit polyclonal IgG to AMA-1 (gift from Dr. Allan Saul), however, none of these reagents gave significant inhibition of invasion with R29 or TM284. For the assays with the anti-MSP-1-42 antibody, the starting cultures were diluted to 0.9–1.3% parasitaemia with RBC and resuspended to 2% haematocrit with no added antibody (control) or a final concentration of 1.5 mg/ml of anti-MSP-1 IgG. Assays were set up and invasion rates were assessed as described above, with triplicate wells for each antibody and control. To ensure that the invasion-inhibitory antibody did not disrupt rosettes, samples of the rosetting parasites were incubated with the MSP-1-42 antibodies at the above concentration for 30 min at 37°C and then the rosette frequency was assessed.

2.3. Assessment of rosette frequency

A 200 µl aliquot of culture suspension containing mature pigmented trophozoite-stage parasites was stained with 25 µg/ml ethidium bromide for 5 min at 37°C. Ten microlitres of culture suspension was placed on a microscope slide and covered with a coverslip. The stained sample was viewed with a 40× objective using a Leica fluorescence microscope with both UV and brightfield light to allow visualisation of both iRBC and RBC simultaneously. Two hundred iRBC were counted and scored for rosetting, with a rosette being defined as an iRBC binding two or more RBC. The percentage of the 200 iRBC in rosettes is termed the rosette frequency (RF). For the invasion assays, rosetting cultures had a RF of above 65% and non-rosetting cultures had a RF of less than 6%.

2.4. Statistics

Statistical analysis was done with Statview 5.0.1 software. The invasion rates of isogenic rosetting and non-rosetting parasites were analysed with a paired Student's *t* test, paired by experiment to control for variations in invasion due to RBC donor, RBC age, and human sera. The percentage inhibition of invasion by the MSP-1-42 antibody of rosetting and non-rosetting parasites was analysed with a Student's *t* test.

3. Results and discussion

3.1. Invasion rates of isogenic rosetting and non-rosetting parasites

We first set out to investigate whether isogenic rosetting and non-rosetting parasite lines derived from R29 and TM284 differed in their invasion rates under standard in vitro culture conditions. Over seven (TM284) or eight (R29) experiments, there was no significant difference in the invasion rates of the isogenic rosetting and non-rosetting lines (Table 1, *P* = 0.86 and *P* = 0.21, paired Student's *t* test, TM284 and R29, respectively). We therefore confirmed the previous findings of Clough et al. that rosetting does not enhance invasion (Clough et al., 1998).

3.2. Invasion rates of isogenic rosetting and non-rosetting parasites in the presence of antibodies to MSP-1

To examine the hypothesis that rosetting may enhance invasion in the presence of host invasion-inhibitory antibodies (Figs. 1A and B), we studied the invasion rates of isogenic rosetting and non-rosetting parasites in the presence of antibodies to MSP-1-42 (Singh et al., 2003). In two experiments with both TM284 and R29, we found that the isogenic rosetting and non-rosetting parasites did not differ significantly in the percentage inhibition of invasion in the presence of the MSP-1-42 antibodies (Table 2, *P* = 0.62 and

Table 1

Rosetting frequency and invasion rate for two pairs of isogenic *P. falciparum* rosetting and non-rosetting parasites

Strain	R ^a	N	RF ^b	Invasion rate ^c	Paired <i>t</i> test ^d
TM284	R+	7	74 (4.8)	6.7 (1.6)	<i>P</i> = 0.86
	R–	7	3 (2.4)	6.6 (2.0)	
R29	R+	8	77 (8.3)	5.3 (1.8)	<i>P</i> = 0.21
	R–	8	2 (1.6)	4.4 (1.6)	

^a R, rosetting phenotype; R+, rosetting; and R–, non-rosetting.

^b RF, rosette frequency counted as percent iRBC binding two or more uninfected RBC; mean (standard deviation).

^c Invasion rate is the number of ring-iRBC from triplicate or quadruplicate wells from each experiment after 24 h invasion divided by the starting schizont parasitaemia; mean (standard deviation).

^d Paired *t* test for the invasion rate of the isogenic R+ and R– cultures paired by experiment to account for variations due to RBC age, RBC donor, and human sera.

Table 2
Invasion inhibition of two pairs of isogenic rosetting and non-rosetting *P. falciparum* parasites

Strain	Experiment	R ^a	RF ^b	Invasion rate ^c		Inhibition ^d
				Control	MSP-1 Ab	
TM284	1	R+	70	7.4 (1.2)	4.9 (0.1)	33.3
		R–	2	6.8 (1.4)	4.7 (0.3)	30.2
	2	R+	68	9.0 (1.2)	6.2 (0.9)	31.2
		R–	0	8.7 (0.2)	5.2 (0.7)	40.2
R29	1	R+	76	5.1 (1.2)	3.8 (0.3)	24.4
		R–	0	2.5 (0.3)	1.8 (0.2)	28.0
	2	R+	76	5.9 (0.3)	3.4 (0.2)	42.9
		R–	0	4.3 (1.2)	3.1 (0.3)	28.9

^a R, rosetting phenotype; R+, rosetting; and R–, non-rosetting.

^b RF, rosette frequency counted as percent iRBC binding two or more uninfected RBC.

^c Invasion rate is the mean (standard deviation) number of ring-iRBC from triplicate wells after 24 h invasion divided by the starting schizont parasitaemia.

^d Percent inhibition of invasion of the isogenic R+ and R– parasites in the presence of the MSP-1 antibody compared to the control invasion rate. There was no significant difference in the inhibition between the R+ and R– parasites ($P=0.62$ and $P=0.63$, Student's *t* test, TM284 and R29, respectively).

$P=0.63$, Student's *t* test, TM284 and R29, respectively). The mean inhibition of invasion for rosetting parasites was 33.0% (95% confidence interval 25.5–40.5%) and for non-rosetting parasites was 31.8% (95% confidence interval 26.3–37.3%). Therefore, we found no evidence to support the hypothesis that rosettes protect merozoites from invasion-inhibitory antibodies as hypothesised in Fig. 1A. Instead, the data suggest that rosetting parasites are inhibited by invasion-inhibitory antibodies to the same extent as non-rosetting parasites (Figs. 1B and C).

Although we have found no evidence to support the hypothesis that rosettes protect merozoites from invasion-inhibitory antibodies, because of the variation in the experiments shown in Table 2, we cannot completely exclude the possibility of a minor difference in invasion inhibition between rosetting and non-rosetting parasites. To increase the sensitivity of these experiments, we attempted to identify a reagent that would provide greater inhibition of rosetting for repeated assays, however, none of the other reagents to MSP-1 and AMA-1 that were tested (see Section 2) showed any significant invasion inhibition in either R29 or TM284. We also attempted to examine the inhibitory effect of the MSP-1-42 antibodies on R29 and TM284 cultures under shaking conditions, however, no inhibition of invasion was seen when the cultures were shaken rather than static (data not shown).

To ensure that our results were not due to rosettes being disrupted by the invasion-inhibitory antibodies, we assessed the effect of the MSP-1-42 antibodies on rosetting. For both R29 and TM284, we found that the RF did not change in the presence of the MSP-1-42 antibodies (R29—control with no antibody 48% RF, 1.5 mg/ml MSP-1-42 52% RF; TM284—control with no antibody 83% RF, 1.5 mg/ml MSP-1-42 84% RF).

It is unclear whether rosettes are stable during schizogony or whether they break apart before the schizonts rupture. Our results point towards the latter being the most likely possibility. This suggestion is consistent with previous findings showing that the RBC forming the rosette are not preferentially targeted during invasion (Clough et al., 1998).

In the invasion inhibition experiments (Table 2), the data show a trend towards rosetting R29 parasites having higher overall invasion rates than R29 non-rosetting parasites in the control cultures without added antibodies. However, as shown in Table 1, an analysis of a larger number of experiments does not support a significant difference in invasion rates between these isogenic lines.

Although we have found no evidence to support the hypothesis that rosettes protect merozoites from invasion-inhibitory antibodies, it remains possible that rosettes may affect the interactions of iRBC with other components of the host's immune system. Contact-dependent activation of natural killer cells—an important first line in innate immunity against *P. falciparum*—has been reported (Artavanis-Tsakonas et al., 2003), and this activation may not occur if uninfected RBC surround the iRBC. Contrary to this, binding of iRBC to dendritic cells has been shown to modulate the function of antigen-presenting cells to possibly delay an immune reaction (Urban et al., 1999), so rosetting may prevent the binding of iRBC to receptors on the surface of dendritic cells and may promote dendritic cell activity. Furthermore, rosettes may shield the iRBC from phagocytosis by macrophages and neutrophils. Future experiments will compare natural killer cell activation, dendritic cell modulation, and phagocytosis of rosetting and non-rosetting parasites in an attempt to understand the biological function of rosetting.

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