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SEQUESTRATION, VIRULENCE AND FUTURE INTERVENTIONS IN *PLASMODIUM FALCIPARUM* MALARIA

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Stockholm 2005
To Malin & Rebecka – my family
ABSTRACT

The *Plasmodium falciparum* infected erythrocyte (iRBC) withdraws from the peripheral circulation to hide in the deep microvasculature during maturation. This phenomenon, referred to as sequestration, is believed to cause extensive clogging of the vessels and to contribute to the development of severe malaria. Adhesion of the iRBC to the endothelium (cytoadhesion) as well as to uninfected and infected erythrocytes (rosetting and autoagglutination respectively) is thought essential in this process. This interaction with host cell receptors is mediated by parasite derived proteins of which *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is the most studied.

The investigations performed within this thesis aim to further elucidate the correlation between PfEMP1-mediated adhesive events and clinical manifestations of severe malaria. This is accomplished by analysis of fresh clinical parasite isolates and *in vivo* studies in a new animal model. Furthermore, immunization and adjuvant drug treatment, both targeting the adhesive properties here shown associated with severe disease, have been evaluated in our new model.

Examination of the binding properties of iRBC from children with different forms of severe- or mild malaria, revealed the total adhesive capacity and the capability to simultaneously adhere to several receptors to be associated with severe disease. It also demonstrated that the levels of binding to heparin and blood group A as well as the rosetting rate are significantly higher with iRBC from children with severe malaria as compared to mild disease. These findings implicate the adhesive features of a specific binding domain, the DBL-1α domain of the PfEMP1 to be important in bringing about of severe malaria.

By the injection of human iRBC into non-manipulated rats it was shown that parasites sequester in the lungs of the animals and induce pathological changes in a strain specific manner. Additional *in vitro* experiments confirmed the adhesion of iRBC to the rat endothelium and demonstrated the involved receptors to be the same as those of the human endothelium. The approach of injecting human iRBC was further explored in non-human primates.

As adhesion of iRBC to heparin was found associated with severe disease, we generated a new heparin-like glycan devoid of anticoagulant activity to study anti sequestering effects. A substantial decrease in sequestration *in vivo* was achieved with this substance in both rats and primates, suggesting drugs based on glycans as promising candidates for adjuvant treatment of severe malaria.

The FCR3S1.2 parasite is well recognized by sera from children in a malaria endemic region and exhibit all the adhesive features here associated with severe disease. It further sequesters and induces specific pathology in our rat model and is therefore suggested as a severe malaria model parasite. Immunization of animals with the DBL-1α domain of the PfEMP1 of this parasite is here demonstrated to generate antibodies recognizing iRBC and capable of inhibiting sequestration *in vivo* in both rats and primates. This indicates this domain to be a promising component in a future vaccine.

In conclusion we have established a role for the DBL-1α domain of PfEMP1 in bringing about severe malaria and demonstrated how immunization with this domain, or targeting of its adhesive properties with a new glycan, can inhibit sequestration *in vivo*. We have also established a new animal model in which sequestration and possibly additional pathological events can be studied.

Keywords: severe malaria, *Plasmodium falciparum*, sequestration, endothelial adhesion, erythrocyte rosetting, PfEMP1, DBL1α, animal model, vaccine, adjuvant treatment
LIST OF PUBLICATIONS

This thesis is based on the following papers. They will be referred to by their roman number in the text.

I. Fresh Isolates from Children with Severe *Plasmodium falciparum* Malaria bind to Multiple Receptors.
   INFECTION AND IMMUNITY, SEPT 2001, P. 5849-5856

II. Whole Body Imaging of Sequestration of *Plasmodium falciparum* in the Rat.
   INFECTION AND IMMUNITY, NOV 2005, P. 7736-7746

III. Release of Sequestered Malaria Parasites upon Injection of a Depolymerised Glycosaminoglycan.
   SUBMITTED MANUSCRIPT

IV. Immunization with PfEMP1-DBL1α Generates Antibodies that Disrupt Rosettes and Protect against Sequestration of *Plasmodium falciparum*-infected Erythrocytes.
   VACCINE 22 (2004) P. 2701-2712

V. Generation of Cross Protective Antibodies against *Plasmodium falciparum* Sequestration by Immunization with a PfEMP1-DBL1α domain.
   MANUSCRIPT
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# 1 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>iRBC</td>
<td><em>Plasmodium falciparum</em> infected erythrocyte</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin sulphate A</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>PfEMP1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 1</td>
</tr>
<tr>
<td>PfEMP3</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein 3</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>NUD</td>
<td><em>Non ultra descriptive</em> (without further description)</td>
</tr>
<tr>
<td>CIDR</td>
<td>Cysteine-rich inter domain region</td>
</tr>
<tr>
<td>DBL</td>
<td>Duffy binding-like domain</td>
</tr>
<tr>
<td>ATS</td>
<td>Acidic terminal segment</td>
</tr>
<tr>
<td>NTS</td>
<td>N-terminal segment</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>dGAG</td>
<td>Depolymerized glycosaminoglycan</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>KAHARP</td>
<td>Knob associated histidine rich protein</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite surface protein</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombospondin-related adhesive protein</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>AMA-1</td>
<td>Apical membrane antigen 1</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnet assisted cell sorting</td>
</tr>
<tr>
<td>CSP</td>
<td>Circumsporozoite protein</td>
</tr>
<tr>
<td>VSA</td>
<td>Variant surface antigen</td>
</tr>
<tr>
<td>PfS</td>
<td><em>Plasmodium falciparum</em> surface protein</td>
</tr>
<tr>
<td>RESA</td>
<td>Ring-infected erythrocyte surface antigen</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest Virus</td>
</tr>
<tr>
<td>Tc</td>
<td>Technetium</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
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</tbody>
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2 INTRODUCTION

2.1 Background

While the malaria parasite can be described as nothing but successful, combating malaria disease has indeed not been. Human malaria remains a persistent plague in large parts of the world and kills more people, with the exception of TB and possibly HIV, than any other communicable disease. 107 countries in Africa, Asia and America were reported as malaria endemic between 1990 and 2002 (WHO, 2005). Having this said, talking about malaria in reality to some extent equals talking about Africa south of Sahara; while representing less than 50% of the malaria affected countries, the majority of the clinical episodes and the absolute bulk of all malaria related deaths occur in this region. The reasons for this are manifold: The dominance of *Plasmodium falciparum* (the causative of the most severe form of malaria), the abundance of the most efficient malaria vector (*Anopheles gambiae*) and the lack of well funded healthcare and infrastructures capable of mounting sustainable campaigns against malaria. Add to this the emerging resistance against insecticides and anti-malarial drugs and the result is nothing short of catastrophic (Snow et al., 2005).

Malaria was early on eliminated from parts of the world less suitable for disease transmission as a result of socio-economic changes, such as better housing and draining of marshes, often a consequence of agricultural and demographic expansion rather than specific anti-malarial efforts. The first and only major attempt to address the global malaria situation was initiated in the mid 1950’s when WHO, encouraged by the effectivity of DDT in the eradication of malaria in the USA a few years earlier, launched the Global Eradication of Malaria program. The new wonder drug chloroquine was not initially included in the program, as it was judged too expensive, but mass usage was thrown in as a desperate action in 1966 when the program already was in an irreversible state of failure. The program was officially declared dead in 1972 and WHO shifted its policy from eradication to malaria control in the remaining areas. Although striking technical reasons, such as DDT and chloroquine resistance, were evident parts of the global failure, one can not help wondering why this effort, after succeeding in Europe and other places, was halted at the border of the poorest part of the world. There are indications that malaria eradication in Africa in practice never was attempted (Liston, S, 1998).

With the WHO initiative Roll Back Malaria, launched by Gro Harlem Brundtland in 1988, a new optimistic era in malaria control has been entered. The initiative is fuelled by recent advances within malaria research, best exemplified by the recent mapping of both the genome of the *Anopheles* mosquito and the *P. falciparum* parasite, but possibly even more by a previously unseen political and economical commitment by actors spanning from governments and international organizations to private foundations (Gardner et al., 2002; Holt et al., 2002). Although there is always reason for caution regarding the patience of this type of international efforts, and history tells us that patience will indeed prove to be crucial in this battle, there are positive signs to be noted: In contrast to the eradication program of the 50’s that solely depended on DDT and was badly equipped to tackle the wide range of different political, economical
and cultural environments were it was to be implicated, the new initiative has a wider approach, spanning from promotion of existing anti-malarial tools such as insecticide treated bed nets and new drug combinations to research on infrastructures and vaccines.

2.2 History

There is reason to believe that malaria, an ancient companion of humanity, has killed more people than any other disease and thereby have had an enormous impact on our history. Malaria probably emanated from tropical Africa and was introduced in Europe via the Nile valley and the close contacts between ancient Egypt and Greece. The intermittent nature of the malaria fever and the association between the disease and mosquito bites are described as early as in the Chinese medical writing Nei Ching (2700 B.C.) and in the writings of Vedic (1600 B.C.) (Desowitz, 1991; Hoeppli, 1956; Hoeppli, 1959). Further evidence of early malaria prevalence can be found in the Ebers papyrus (1550 B.C.), and spleen enlargement, possibly as a result of malaria, has been noted in 3000 year old mummies (Breasted, 1930; Bruce-Chwatt, 1988; Leake, 1952). With the rise of the Greek empire 1600 B.C. the information gets more coherent and malaria symptoms are mentioned frequently from the late 5th century and forward by Aristotle, Plato and Sophocles. Despite the existence of older sources, the Greek Hippocrates (4th century B.C.) is traditionally credited with the first adequate medical description of malaria, including the different febrile intervals of malaria and the characteristic enlargement of the spleen (Garnham, 1966). Hippocrates also associated the disease with the unhealthy surroundings of the marshes and considered the causative agent of malaria to be the Miasma (harmful or corrupting atmosphere or influence; from Greek *miasma*, ”pollution”) that put the four “humours” of the body in imbalance. The Miasma theory is evident even today as Malaria literally means “bad air” (mal’aria).

The impact of malaria on human history is as obvious as it is hard to estimate. Malaria is believed to have struck and killed Alexander the great as he, after conquering what was then all of the “known world”, was gathering his giant army to turn east. Had he succeeded in joining Greece and Asia, West and East, history would most certainly look very different. To what extent malaria influenced the ultimate destiny of the ancient Greek and Roman cultures remains speculative, but it is not hard to imagine malaria as a contributing factor in the decline of these empires. Neither is it hard to imagine that malaria had a profound impact on the process of colonization of America. As there is no mentioning in the medical records of Mayas, Olmecs or Aztecs, it can be concluded that malaria was brought to America by Columbus and the settlers by the end of the 15th century (a statement supported by the lack of immunity in the South American monkeys as well as of human hemoglobinopathies conferring protection). The African continent would most likely have played a very different role on the global arena if it was not for the substantial contribution by malaria to its chronic poverty. In short, malaria has and continues to affect the course of human history by bringing economic, social and military ruin.

Once a curse of gods and daemons habituating the marshes and wetlands, malaria is today recognized as a communicable disease caused by protozoan parasites and transmitted by mosquitoes. Although perceived by Marcus Tentius Verro as caused by a small animal as early as year 50 B.C. this was of course nothing but pure speculation.
from his side. The first real suggestion of the infectious nature of malaria was in 1879 when Klebs and Tomasi-Crudeli, inspired by the scientific fashion of the time claiming all diseases to be bacterial, isolated “Bacillus Malariae” from rabbits injected with marsh water (Garnham, 1966). Only a few years later the French army physician major Laveran drew some blood from one of his many malaria patients and when examining the sample in his microscope noted crescent formed bodies (now known to be the sexual form of *P. falciparum*) as well as rounded bodies surrounded by moving flagella (the exflagellation of the male gametocyte). Convinced of the living nature of the organism, Dr Laveran wrote a report to the French Academy of Medicine, only to find that the medical community at the moment was satisfied with the recently isolated “Bacillus Malariae” and thereby reluctant to embrace this new concept. It would take another 6 years before Leverans findings were commonly accepted and the organism was correctly identified as a protozoan parasite. Although suggested to be spread by mosquitoes already in the Vedic writings (1500-800 B.C.), the true route of malaria transmission would take yet another few years to resolve (Desowitz, 1991; Hoepli, 1956; Hoepli, 1959). The miasma theory was based on the accurate epidemiological observation of malaria, marshes and bad air, and could therefore, in the sense of a non-microbial and swamp associated theory of malaria transmission, stand almost unchallenged for more than 2000 years. One of the last one to advocate such a theory was the Swedish botanist Carl von Linné who in is thesis on malaria 1735 suggested clay contamination of drinking water to be the causative agent, based on the observations of black pigment in the blood of malaria patients (Linnaeus, 1733). By the beginning of the 19th century another correct epidemiological observation of malaria, marshes and mosquitoes was beginning to make way. The first proof of such a theory came in 1897 when military physician Dr. Ross discovered cysts on the exterior stomach wall of mosquitoes fed on malaria patients (Desowitz, 1991). One year later he was able to demonstrate the migration from stomach to salivary glands of the mosquito, thereby completing the lifecycle of the malaria parasite. In parallel, Italian professor Giovanni Battista Grassi followed the same trail as Dr. Ross which led to the same discovery at about the same time (Garnham, 1966).

### 2.3 Consequences & Perspectives

“Malaria has the disastrous effect of permitting human existence while precluding the possibility of human health and happiness”

Hugh S. Cumming

In the first World Malaria Report, presented by WHO and UNICEF within the Roll Back Malaria Partnership, it is stated that 3.2 billion people in 107 countries are at risk of malaria, and that up to 0.5 billion clinical episodes were recorded in 2004 (WHO, 2005). This means that the majority of all living humans are at risk and that almost 1 in 10 of all humans had an attack of malaria last year. The report further states that there were approximately one million deaths caused by malaria, translating into one person each 30 seconds. As if these figures were not terrifying enough there are indications that malaria mortality, when including indirect effects such as induced anaemia, maternal pathology, metabolic imbalances and enhancement of severity of other
childhood diseases, possibly sums up to three times the WHO estimate, reaching a staggering 3 million deaths a year (Breman et al., 2004; Snow et al., 2005).

![Image: The world as seen through the eyes of malaria – area and shade indicate level of malaria transmission]

This tremendous burden is in no way equally distributed: 56% of the cases occur in the poorest 20% of the world’s population. Africa alone is estimated to carry a least 60% of the total number of malaria attacks (75% of *P.falciparum* attacks) and 80% of the malaria mortality (Snow et al., 2005).

Although the absolute figures vary there is a general consensus that the global malaria situation has been deteriorating over the last decades and that this negative trend will continue, and accelerate, without prompt and effective counter measures (WHO, 2005). The population at risk of malaria has increased by around 10% the last ten years, and while the overall mortality in African children has continued to fall during the 90’s, malaria related deaths have increased both in relative and absolute numbers (Hay et al., 2004; Snow et al., 2001). Several reasons are suggested: Increasing drug and insecticide resistance, civil disturbances, climate instability and break down of the primary health systems in Africa (Greenwood et al., 2005; WHO, 2005).

Estimating the total impact of malaria on a society is an impossible task as it is likely to affect all aspects of social and economical behaviour (Sachs and Malaney, 2002). There is a clear correlation between hardship on a national level and malaria incidence, and the disease is probably best understood both as a cause and a consequence of poverty. Apart from the more obvious direct economic burden induced by malaria, as private and non-private medical costs and foregone income, there are several other, probably more severe, effects. Fertility rate is generally shown kept high when child mortality is high, actually over-compensating for the true loss in lives. As a consequence the investment in education for each children (especially daughters) drops and the employment of women is reduced. The intellectual capital of malaria endemic countries is negatively affected in many different ways: loss of school days due to sickness, direct and indirect effects of malaria on cognitive development, and brain-drain to non-malarious countries. Malaria has also been shown to have a direct and massive impact on the level of foreign investments and tourism (Gallup and Sachs, 2001).
It has been calculated that malaria accounts for a 1.3% reduction of yearly economical growth and that the negative accumulative effect on GNP is more than 50% in endemic areas (Gallup and Sachs, 2001). The recognition of the viscous circle connecting malaria and poverty opens up for the thought of creating a positive economic trend by the elimination or control of the disease. This has previously been seen in Greece, Spain and Portugal, all demonstrating an economic boom following the eradication of malaria.

Our home is without doubt a smaller planet today than ever before. Stating that the problems of the developing countries are our problems is no longer an expression of wishful thinking but hard facts as it is becoming increasingly obvious that there is in the end only one world. While failures in the 60’s and 70’s, including the WHO Malaria Eradication Program, led to a perception of Africa as a lost cause, there is today a growing optimism and a recognition of combating malaria as one of the most cost-effective measures of promoting economic growth in the poorest parts of the world.

While eradication still is far away, and most likely dependent on the future development of an effective vaccine, control of the malaria situation is achievable with the tools available. New artimisinin based combination therapies (ACTs) are highly effective in treating malaria and may prevent, or at least delay, further development of drug resistance (Yeung et al., 2004). Data from Asia even indicate mefloquine resistance to be reversed after introduction of ACTs (Nosten et al., 2000). Development of effective home based management systems and training of local shopkeepers can reduce delay in treatment of severe malaria. Intermittent Preventive Therapies (IPT), targeting risk populations such as pregnant women and children below the age of five with a combination of Sulphadoxine and Pyrethamine, have been shown to protect against anaemia and low birth weight (Rogerson et al., 2000; Shulman et al., 1999) and to reduce the number of malaria attacks (Schellenberg et al., 2005). The use of insecticide treated bed-nets (ITNs) has convincingly been shown to reduce malaria attacks and over-all mortality in children (Lengeler, 2004). All these interventions are shown to be highly attractive and cost effective ways of reducing the burden of malaria (Goodman et al., 1999). While this may sound straightforward, the establishment of effective malaria control based on ACTs, IPTs and ITNs is calculated to cost 2-5 US$ per person and year equalling a months income for the average African family. Increased global funding is desperately needed.

The statement of the World Malaria Report 2005 that “significant progress in the battle against malaria has been made in all malaria-affected regions” is questionable in view of the deteriorating malaria situation. There are however positive signs in the report: The ITN coverage has increased, home management and IPT for pregnant women are being adopted in the health policies of many countries, indoor residual spraying is expanding, and the use of ACTs is becoming more frequent (WHO, 2005).
3 THE DISEASE

Although Hippocrates, and later Galen, were nonsensical in their theories of the imbalance of the four bodily humors, they were correct in attributing the disease to disturbances in the blood. Clinical malaria is today understood as a disease of the circulatory system, restricted to the asexual replication of parasites in the erythrocytes.

The hallmarks of malaria are intermittent fever in combination with spleen enlargement. Hippocrates correctly described these conditions, and although the intervals later were named by the Romans, he also distinguished between different forms of malaria by their febrile periodicity: Every day (semi-tertian), every second day (tertian), and every third day (quartant) fever. Today we know that these distinct conditions correspond to infections with different species of malaria parasites infecting man.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Parasite</th>
<th>Fever</th>
</tr>
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<tbody>
<tr>
<td>Benign tertian malaria</td>
<td><em>P. vivax</em> and <em>P. ovale</em></td>
<td>Peaks every 2&lt;sup&gt;nd&lt;/sup&gt; day</td>
</tr>
<tr>
<td>Quartan malaria</td>
<td><em>P. malariae</em></td>
<td>Peaks every 3&lt;sup&gt;rd&lt;/sup&gt; day</td>
</tr>
<tr>
<td>Malign tertian malaria</td>
<td><em>P. falciparum</em></td>
<td>Peaks every 2&lt;sup&gt;nd&lt;/sup&gt; day / irregular</td>
</tr>
<tr>
<td>(Semi tertian malaria)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The somewhat confusing names (Tertian refers to three and Quartan to four) is due to measuring from the beginning of the first attack to the end of the second. *P. falciparum* often, especially in the initial phase of the disease, presents with an irregular fever pattern, hence the referral to every day (semi-quartant) fever. While the periodic fever pattern is an important feature, it is not enough to establish the diagnosis of malaria. Further, the presence or lack of parasites in the circulation is no foolproof evidence of malaria as symptoms may be present at very low peripheral parasitemias and asymptomatic carriage of parasites is a very common finding in African children. As a consequence of lack of diagnostic resources in combination with the potential severity of the disease, the current policy in many parts of Africa is to treat all febrile episodes in children as malaria. The following presentation will be restricted to the tertian malignant malaria caused by the *Plasmodium falciparum* parasite.

3.1 Symptoms

None of the clinical symptoms of malignant tertian malaria is specific – it can mimic a number of other medical conditions including influenza, hepatitis and encephalitis. The infection, initiated by the bite of an infected mosquito, starts off with a silent and non-symptomatic prepatent period lasting 8-15 days before onset of disease. Initial symptoms (prodromal symptoms) are flu like and will typically last for a few days before the first febrile attack – the malaria paroxysm. Although prodromal symptoms are present in infection with all four species of human malaria parasites they tend to be
more severe in *P. falciparum*, often presenting with combinations of acute headache, bone and muscle pain, nausea, coughing, vomiting and diarrhoea.

The malaria paroxysm has a sudden onset, lasts for 4-8 hours and may be divided into three distinct phases:

- **The Cold Stage** – Raising temperature with extensive chills lasting for up to one hour
- **The Hot Stage** – A sensation of heat in combination with headache, muscle pain and dizziness lasting for 2 - 6 hours. Vomiting and convulsions are common.
- **The Sweating Stage** – Declining temperature with profound sweating followed by exhaustion.

After an attack the individual often feels tired but well, and no symptoms may be present until the onset of the next paroxysm. As stated above, *P. falciparum* malaria paroxysms tend to be more or less irregular - every day, every second day or constant fever. This irregularity is more pronounced in the initial phase of the disease and in non-immune individuals. Other symptoms that may accompany the malaria attack include splenomegaly, hepatomegaly with slight jaundice and haemolytic anaemia. In most cases the infection is self-limiting even in non-immune individuals and resolves within a few weeks.

Separating *P. falciparum* malaria from the other human malarias is the general severity of the disease, with more profound paroxysms, frequent presentation of complication, and ultimately a high fatality rate. If untreated, the disease may be deadly in non-immune individual days after the first symptoms. The spectra of different severe complications associated with *P. falciparum* malaria will be presented in detail further on.

### 3.2 Clinical Immunity

Malaria in general, and severe disease in particular, is in endemic areas to a large extent a condition of the young child. While parasites remains a common finding in the circulation of older children and semi-adults, clinical signs, with the exception of pregnancy associated malaria, becomes increasingly rare with age. The reason for this is the acquisition of clinical immunity, a protection that needs years or decades of exposure to acquire and possibly never develops into a sterile state. This immunity is to be understood as an increasing capacity of the individual to control and tolerate the burden of circulating parasites without demonstrating severe clinical manifestations.

A clear temporal pattern in the acquisition of immunity can be recognized, where the most severe clinical manifestations disappear first, followed by a more complete protection from clinical episodes and lastly a reduction in the number of circulating parasites (McGregor, 1974). The time needed and how far the immunity will ultimately develop is dependent on the level of exposure; in an area of low transmission protection against severe disease will take longer to develop and episodes of clinical malaria may occur throughout life. The continuous boosting by new infective mosquito bites is
required to maintain the acquired protection; a person grown up in a malaria intense area that leaves for a non-malarious area is once again susceptible to the agony of malaria disease should he or she choose to returns to his home village years later (Riley et al., 1994).

Acquisition of natural immunity in a malaria endemic area. Protection from the severe manifestations is acquired first followed by protection from clinical disease. Adult immune woman become susceptible to severe malaria during pregnancy, a susceptibility reduced with subsequent expectancies.

Regardless of previous exposure women once again become susceptible to severe malaria in pregnancy (maternal malaria). This phenomenon is most pronounced in prima gravida; immunity is established with succeeding pregnancies, rendering each following child carriage less of a hazard for the mother as well as the child (Brabin, 1983). The newborn is protected from malaria during the first months of life. Although mechanisms such as behavioural pattern and haemoglobin F has been suggested, this protection is most likely to be mediated by the transfer of protective antibodies from mother to child (Campbell et al., 1980; Hviid and Staalsoe, 2004; Riley et al., 2000; Sehgal et al., 1989).

Epidemiological data propose the relative frequency of severe childhood disease (excluding cerebral malaria) to be lower in areas of high endemicity, possibly as a consequence of high exposure of the infant while still under the protective umbrella of maternal antibodies (Snow et al., 1997). There has been concerns raised that early and extensive use of bed nets or other protective measures would interfere with this early exposure of the protected new-born and thereby increase the frequency of severe malaria in the older child. However, no evidence of increased frequency of severe malaria episodes or shift in mortality from younger to older children has been noted in the areas where intense use of insecticide treated bed nets have been tried (Binka et al., 2002; Diallo et al., 2004).
3.3 Severe Manifestations

A few different syndromes, alone or in combination, account for the vast majority of malaria related deaths. However, you can be critically ill and even die from malaria without fulfilling any of the criteria of these specific severe manifestations; malaria is indeed a multi-system disease and may impair the function of virtually any vital organ (Bag et al., 1994). It is generally agreed that sequestration, the unique capacity of the *P. falciparum* infected erythrocyte (iRBC) to withdraw from the peripheral circulation by adhesive events, is a major contributor to the pathology of severe malaria.

While the level of acquired immunity of the individual is of most importance for the clinical manifestations there are a number of other host factors to be considered: the genetic predisposition, the general and nutritional status, age, co-existence of other diseases, and the socio-economic context. There are also, possibly even more important, a number of properties of the parasite such as replication rate and adhesive capacity to take into account; these factors will be further elaborated on in the context of the parasite.

Several genetic host factors influencing susceptibility to malaria and its complications have been identified (Haldane, 1949; Kwiatkowski, 2005). Point mutations in the β-chain of haemoglobin, resulting in HbS and HbC phenotype, have been shown to mediate protection, and in the case of HbC this has been suggested to be a result of abnormal display of iRBC surface molecules (Allison, 1954; Carlson et al., 1994; Fairhurst et al., 2005; Haldane, 1949; Williams et al., 2005). Further, regulatory variations of the globin genes, resulting in thalassemias, have been demonstrated to confer protection against severe malaria, and an association between certain MHC class I/II haplotypes and reduced risk of severe disease have been shown (Allen et al., 1997; Hill et al., 1991; Mockenhaupt et al., 2004). Although *P. falciparum* is capable of invading RBC of all blood groups, a correlation between blood group A and severity of disease has been suggested (Carlson and Wahlgren, 1992; Fischer and Boone, 1998). Cytokine secretion has been demonstrated important in malaria pathology, and an impaired secretion of tumour necrosis factor (TNF) is associated with protection from severe malaria (Day et al., 1999; McGuire et al., 1994). Several genetic traits, such as sickle-cell anaemia, thalassaeemias, MHC classes and blood group O, have been shown to be selected for in the African population, demonstrating the long and profound impact of the disease (Kwiatkowski, 2005). It also easily imagined that other host factors, such as abundance and distribution of endothelial receptors for parasite sequestration, influences the severity of disease (Kikuchi et al., 2001).

The relation between nutritional status and malaria is one of complicated nature as malnutrition is likely to represent a contributing factor to - and a consequence of - malaria. It has both been suggested that bad nutritional status might protect from malaria and that it contributes significantly to malaria mortality (Caulfield et al., 2004; Whitehead, 1977). Further it has been demonstrated that while iron supplementation significantly decreases anaemia, it also has a negative impact on infection rate (Caulfield et al., 2004). The causative role of malaria in malnutrition is more evident, recurrent fevers reduces the appetite, anaemia empties the iron deposits, vomiting and diarrhoea rids the body of nutrition, and the poverty accompanying malaria results in less food on the table.
Malaria manifests itself differently in children as compared to adults: severe anaemia is more commonly seen in children while renal failure and pulmonary oedema is almost completely restricted to adults, and coma, although not an uncommon complication in children, is more frequently seen in adults.

There is a growing recognition of HIV infection as a negative co-factor in malaria infection in pregnant women. The frequency of maternal malaria increase with HIV prevalence, and malaria in HIV positive pregnant woman results in more severe complications for the mother as well as the child (ter Kuile et al., 2004). Other infectious diseases, such as infection with intestinal helminths, may also have a negative impact on the course of malaria infection by contributing to the anaemia.

A brief presentation of the clinical features of the major manifestations constituting severe malaria will here follow.

3.3.1 Severe anaemia

Severe anaemia is possibly the malarial complication that in absolute numbers causes most deaths in endemic regions (Murphy and Breman, 2001). This is likely to reflect the number of children affected rather than the severity of the condition; a study performed in four malaria endemic countries demonstrated more than 80% of children of 4 months of age to suffer from anaemia and about 1/3 of the children to have profound anaemia with a Hb of less than 8 gram per cent (Breman et al., 2004). In malaria endemic regions, casualties in malaria anaemia are mainly seen in children less than five years of age, with a peak at very young age as the infant escapes the protection of maternal antibodies (Crawley, 2004).

While known to be associated with a dramatic increase in mortality, an Hb of less than 5 gram per cent (hematocrit of less than 33 %) has served as criteria for severe malarial anaemia (Lackritz et al., 1992; Lackritz et al., 1997). The condition has been suggested as a consequence of destruction of infected as well as uninfected erythrocytes, in combination with some level of bone marrow suppression (Ekvall, 2003). A degree of mutual exclusion of the two conditions of anaemia and cerebral malaria has been reported, and it was recently demonstrated that the adhesion of parasitized erythrocytes to endothelial receptors is reduced as the hematocrit drops, suggesting a possible pathway whereby severe anaemia could serve as protection from cerebral malaria (Flatt et al., 2005; White, 2005).

3.3.2 Cerebral malaria

This most dreaded manifestation of severe malaria, defined as deep unrousable coma in a malaria patient, or more strictly “the inability to localize a painful stimulus in a patient with a P. falciparum parasitemia in whom other causes of encephalopathy has been excluded”, has a case mortality rate of up to 30% and in addition possibly leaves as many as 10 % of the survivors with significant cognitive impairments (Marsh, 1999; Waller et al., 1995; Warrell et al., 1982). The progression of cerebral malaria is often dramatic with a sudden onset of coma and a rapid deterioration of the condition, not seldom resulting in death within hours or days. However, far from all cases demonstrate these striking features, and a number of different conditions have been
suggested as separating primary malarial coma, understood as the result of sequestration in cerebral vessels, from unconsciousness secondary to other malaria-related clinical manifestations:

**Prolonged post ictal state** - Although convulsions often mark the onset of cerebral malaria coma, there is reason not to use the term until at least 30 minutes of unconsciousness has past as it has been shown that prolonged post ictal periods is not uncommonly seen in children suffering from malaria (Crawley et al., 1996; Warrell et al., 1982). However, this prolongation could in itself be an indicator of involvement of cerebral vessels, perhaps caused by self-resolving transitory sequestration or incomplete blockage.

**Covert status epilepticus** – Prolonged and multiple seizures is a common clinical finding in cerebral malaria and shown associated with neurological sequelae (Bondi, 1992; Brewster et al., 1990; Jaffar et al., 1997). While this condition is predominantly seen in combination with unrousable coma, treatment with anti-epileptic drugs do not only terminates the seizures but also results in rapid return to full consciousness in a subset of patients, suggesting status epilepticus as a possible primary source rather than a consequence of the unconsciousness (Marsh, 1999). Cerebral hypoxia, probably at least partly due to sequestration of IRBC, and subsequent release of excitotoxic mediators, has been suggested as an important factor in the seizures (Crawley et al., 2001) and the coma seen as treatable with anti-epileptic drugs may, as above, be the result of self-resolving transitory sequestration or incomplete blockage of the vessels.

**Metabolic coma** – Metabolic acidosis is recognized as a frequent complication with high prognostic value in children with severe malaria (Day et al., 2000; Krishna et al., 1994; Marsh et al., 1996; Taylor et al., 1993). As elevated blood lactate levels is a prominent feature in these patients, anaerobic respiration in poorly oxygenated host tissue due to profuse sequestration has been postulated as a likely underlying mechanism. In some children, supported by the regain of consciousness after treatment with fluid resuscitation, coma has been suggested to be consequence of the acidosis rather than of primary cerebral pathology (Marsh, 1999).

These different conditions reflect the heterogeneity in the pathogenesis of the clinical condition of cerebral malaria. However, it should be noted that more than 50% of the children do not fall into any of these criteria, and hence are referred to as primary comatose (Marsh, 1999). Further, a unifying pathological theory of reduced tissue oxygenation may link the different conditions, suggesting blockage of capillary flow by sequestration of parasites as a common nominator. Although on many occasions demonstrated that cerebral malaria correlates with sequestration in the vessels of the brain, it should be remembered that these autopsy findings are from individuals who succumbed to the disease; it might be so that other causes of malarial coma resolves to a higher extent and hence that cerebral malaria originating from cerebral microvascular obstruction is over-represented in the examined autopsy material.
3.3.3 Respiratory Distress

Respiratory distress, in its widest definition as any form of altered breathing pattern, is a common and unspecific finding in patients with *P. falciparum* malaria. If restricted to severe respiratory distress, defined as documented chest recession or abnormally deep breathing, the condition still remains common but now has a highly significant prognostic value (Marsh et al., 1995). Once considered as a consequence of cardiac failure in anaemic children, there are today strong indications of respiratory distress as a secondary compensatory event in metabolic acidosis (English et al., 1996a).

However, the vessels of the lungs constitute a very large proportion of the total capillary system and iRBC sequestration, demonstrated to also occur in the vessels of the lung, may possibly introduce local endothelial damage and reduce perfusion, resulting in reduced oxygenation of the blood possibly contributing to a metabolic acidosis (MacPherson et al., 1985; Pongponratn et al., 1991).

![Venn diagram showing the relationship between severe anaemia, cerebral symptoms, and respiratory distress in children with severe malaria](image)


3.3.4 Other Complications

While severe anaemia, cerebral malaria and respiratory distress, or combinations thereof, constitutes the major clinical syndromes of severe malaria, there are a number of other conditions associated with the disease.

*Metabolic disorders* – While discussed above in association with cerebral malaria and respiratory distress, metabolic acidosis is in itself a common finding in malaria and carries a high prognostic value (Day et al., 2000; English et al., 1997; Marsh, 1999). While largely attributable to the production of lactic acid as a consequence of poor tissue perfusion, both impairment of renal function (Day et al., 2000; English et al., 1996b; Marsh, 1999; Sowunmi, 1996) and of hepatic function (Day et al., 2000) have been suggested to contribute to the condition.
Hypoglycaemia is found in 10-30% of children with severe malaria in sub-Saharan Africa, and has been shown associated with a fatal outcome (Dzeing-Ella et al., 2005; English et al., 1998; Waller et al., 1995; White and Pukrttayakamee, 1993). While the parasitic burden increases the peripheral glucose consumption and which to some extent could explain the phenomena, there are also indications that renal dysfunction, bringing about a decrease in glucose production, may play a crucial role (English et al., 1998).

**Organ failure** – Acute renal failure is a common presentation of severe *falciparum* malaria in non-immune adults and carries a mortality rate of 15-45% (Barsoum, 2000). Acute tubular necrosis is the most consistent histological finding, and although the pathogenesis is still unclear, sequestration is thought to contribute to the condition (Trang et al., 1992). As cerebral malaria is not associated with renal failure, this could indicate organ specific rather then general sequestration as the underlying pathology (Nacher et al., 2001). While acute renal failure is uncommon in children, impaired function of the kidneys has been demonstrated to be common and associated with acidosis and increased mortality (English et al., 1997; Jaffar et al., 1997; Molyneux et al., 1989; Waller et al., 1995).

Malarial hepatitis has often been used to describe jaundice in combination with severe malaria. However, whereas jaundice is frequently seen in non-immune adults, and the term fulminant hepatic failure sometimes have been used to describe jaundice in combination with malarial coma, confirmed acute hepatitis is a rare event (Anand and Puri, 2005; Badiaga et al, 2005; De Brito et al., 1969; Harris et al., 2001). Intravascular haemolysis, detected as elevated levels of unconjugated bilirubin, is recognized as the common reason for malarial jaundice, but as hepatomegaly and mild elevation of liver enzymes is noted in a significant proportion of the malaria patients, some degree of hepatocellular dysfunction is also indicated (Nacher et al., 2001; Waller et al., 1995; Wilairatana et al., 1994). While the pathogenesis of this hepatocellular dysfunction remains unclear, it is suggested to be a consequence of suppressed bilirubin excretion rather than of hepatitis (Anand and Puri, 2005; Nacher et al., 2001).

### 3.3.5 Maternal Malaria

While adults and adolescents in general in endemic areas develop protective immunity and hence enjoy a comparatively low frequency of malaria related complications, women once again become susceptible to severe malaria during pregnancy (Brabin, 1983). This well documented condition is known as maternal malaria and is associated with high level of mortality in both mother and child. An estimated 75,000 to 200,000 infants deaths are directly associated with maternal malaria each year (Steketee et al., 2001). Unaccounted indirect consequences might be even more terrifying as maternal malaria has been shown to limit antibody transfer to foetus, potentially severely restricting the protection of the new-borns from measles, pneumococcal infections and tetanus (Brair et al., 1994; de Moraes-Pinto et al., 1998).

The clinical presentation of maternal malaria differs with the number of previous pregnancies and the local level of transmission (Anya, 2004; Luxemburger et al., 2001; Nosten et al., 2004; Shulman and Dorman, 2003). Pregnancy-associated malaria in areas of high transmission carries an increased mortality risk of the mother due to post
partum bleeding, and a reduced average birth weight associated with neonatal mortality due to both pre-term deliveries and intrauterine growth retardation (Brabin, 1983; Guyatt and Snow, 2004; Nosten et al., 2004). In areas of low transmission there is a more acute risk of mortality in the mother and an increased risk of stillbirth and abortion (Luxemburger et al., 2001; Nosten et al., 2004; Shulman and Dorman, 2003). Previous pregnancies have been demonstrated protective; first time pregnant women are more frequently infected and show higher parasite density as compared to multigravid women regardless of the intensity of transmission (Brabin, 1983; Jelliffe, 1968).

As pregnancy is known to affect both the humoral and the cell mediated immunity, maternal malaria was initially though of as consequence of a general pregnancy induced immunosuppression. However, the relation between the number of previous pregnancies and the severity of the condition suggests that immunity to maternal malaria is acquired in the same way as to other severe malaria complications. Pregnancy associated malaria is likely a result of the introduction of a new organ (the placenta) in which infected erythrocytes can adhere, and the subsequent selection of a parasite population with appropriate adhesive properties (Beeson et al., 1999; Beeson et al., 2000). This scenario is supported by the histological finding that placentas from mothers experiencing maternal malaria are generally massively infested with parasitized erythrocytes while the peripheral blood may remain virtually free of parasites (Beeson et al., 2002)(Rogerson et al., 2003). The implications of these findings on our general understanding of the development of protective immunity will be further discussed in the context of parasitic immunity.

3.4 Malaria treatment

As seen from the description above, severe malaria does not constitute one homogenous entity but show several different manifestations. These manifestations need to be separately addressed in treatment and a brief presentation on the treatment and management of severe malaria will here follow.

3.4.1 Chemotherapy

In the first century A.D. the Roman physician Galen, agreeing with Hippocrates idea, wrote that the humoral balance of the malarious body must be restored by bleeding and purging. While the Chinese was already using the wormwood (active substance artemisinin; the stuff that makes vermouth taste like vermouth) to effectively treat the swamp fever, this was to be the standard practice in Europe for the coming 1600 years, until the discovery of Quinine (the stuff that makes G&T taste like G&T) in the 17th century.
**Quinine and its relatives** – The use of the bark from the quinaquina tree to treat febrile episodes was adapted from Indians by European settlers in South America in the 17th century. Quinine was isolated from the bark in the late early 19th century and the substance has served as a model substance for the development of modern synthetic anti-malarial drugs. While burdened with a plethora of side-effects, quinine has re-emerged in malaria treatment as a consequence of the wide spread resistance to chloroquine and is today recommended by the WHO as a drug of choice in intravenous treatment of severe and complicated malaria (WHO, 2000).

Chloroquine, the most prominent synthetic derivate of quinine, was developed during the last years the Second World War. As it was comparably cheap to produce and lacked most of the side effects associated with Quinine, chloroquine was considered a “magic-bullet” in the war against malaria. However, already by the end of the 1950s the first treatment failures were reported in South America and today all of Asia and sub-Saharan Africa is severely affected by drug resistance (Farooq and Mahajan, 2004).

All quinine derivates act by inhibiting the hemepolymerase and thus causing accumulation of toxic free heme killing the blood stages of the parasite. Two of the derivates also demonstrate additional effects; chloroquine inhibits the release and action of the cytokine TNF and primaquine also act on the liver stage of the parasite as well as on gametocytes. Antimicrobials (clindamycin, tetracyclines) act synergistically with the quinoline derivates and can be used to enhance their effect.

As a side note it can be mentioned that “Indian water” has been claimed as originally intended to be preventative against malaria. The legend has it that it tasted so awful that it needed to be mixed with gin to become drinkable and Voila! – the gin & tonic was born. And although the concentration of quinine in tonic water today is by far to low to have any protective effect when consumed in gin and tonic, the alcohol actually may; it has been shown *in vitro* that alcohol levels achievable by extensive drinking has strong inhibitory effect on parasite growth (Lell et al., 2000).

**Antifolates** – This group of substances act on blood- and liver stages of the parasite by inhibiting the enzyme dihydrofolate reductase. The result is a depletion of pyrimidines and a subsequent arrest of the DNA replication and death of the parasite. A combination of the antifolates sulfadoxine and pyrimethamine (SP; Fansidar®) has replaced chloroquine as first line drug in a number of countries with high chloroquine resistance (Farooq and Mahajan, 2004). However, resistance has been shown to develop rapidly with extensive use of this drug as well (Anderson et al., 2003). Proguanil, another antifolate commonly used, is well tolerated but has low efficiency when used alone and is therefore mostly used in combination with chloroquine.

**Artemisinin derivates** – Originally a 2000 year old Chinese haemorrhoid treatment, the herb *Artemisia annua* has been used to treat fever since at least the 16th century. In 1972 artemisinin (qinghaosu) was isolated and shown as an active substance with potent anti-malarial activity and the structure was resolved 7 years later (Group, 1979; Klayman, 1985). Artemisinin is active against blood-stages of the parasite including gametocytes and has been postulated to generate free radicals when reacting with Fe²⁺ thereby inhibiting the detoxification of haem, and to induce alkylation of essential parasitic proteins (Bhisutthibhan et al., 1998; Cazelles et al., 2002).
A number of semi-synthetic derivates have been developed (arthemeter, arteether, arthesunate) and increasingly used over the last 20 years. These drugs have so far proven safe, effective, and to act significantly faster than other anti-malarials (fever-clearance time of 32h as compared to 2-3 days), and are now recommended by WHO for treatment of severe malaria (WHO, 2000). Importantly, rectal applications of artemisinin derivates have been developed for treatment of small children in clinical settings where intra-venous treatment with quinine is not possible.

Because of the short plasma half-life of these drugs and in an attempt to avoid future resistance development, artemisinin and its derivates have preferably been used in combination with other drugs.

**Combination therapies** – The combination of several drugs with different modes of action is a frequent strategy when treating diseases such as tuberculosis, HIV and cancer. In malaria treatment this approach serves two purposes: to overcome partial resistance and to avoid, or at least delay, future development of resistance. Artesmisinin-based combinations treatments (ACT) have successfully been used in areas of high resistance in Asia and have even been reported to reverse resistance to the chloroquine derivate mefloquine (Mayxay et al., 2004; Mutabingwa et al., 2005; Nosten et al., 2000; Piola et al., 2005).

The main problems of implementing the use of ACT in Africa are the cost and the global availability of artemisinin. Both these problems could possibly be solved by the development of synthetic artemisinins (Mutabingwa, 2005; Vennerstrom et al., 2004). The non-artemisinin combination of sulfadoxine-pyrimethamine and amodiaquine is still effective in areas of Africa where resistance to the separate substances is moderate (Staedke et al., 2004). A summary of commonly used drug combinations in malaria treatment is provided in the table below.

### Non-artemisinin combinations

<table>
<thead>
<tr>
<th>Combination</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine + SP*</td>
<td>Long treatment, high cost and side effects makes this combination unsuitable for African market</td>
</tr>
<tr>
<td>Quinine + Doxycycline</td>
<td>As above. Mainly used in areas of SP resistance</td>
</tr>
<tr>
<td>SP + chloroquine</td>
<td>Policy in some African countries. Ineffective in areas where resistance to both drugs is high</td>
</tr>
<tr>
<td>SP + amodiaquine</td>
<td>Effective where amodiaquine resistance is low</td>
</tr>
</tbody>
</table>

### Artemisinin combinations (ACTs)

<table>
<thead>
<tr>
<th>Combination</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthemether + lumafantrine</td>
<td>Internationally licensed co-formulation. Available in Asia and Africa</td>
</tr>
<tr>
<td>Artesunate + amodiaquine</td>
<td>Policy in some African countries. Efficient where amodiaquine resistance is low</td>
</tr>
<tr>
<td>Dihydroartemisinin + piperaquine</td>
<td>Co-formulated drug used widely in Asia</td>
</tr>
<tr>
<td>Artesunate + mefloquine</td>
<td>Core of drug policy in southeast Asia. Considered to expensive for Africa</td>
</tr>
<tr>
<td>Artesunate + SP</td>
<td>Used in some Asian countries. Ineffective where resistance to SP is high</td>
</tr>
</tbody>
</table>

* Sulfadoxine-pyrimethamine
3.4.2 Clinical management

Apart from intravenous, or in settings where this is not possible intramuscular or suppository, treatment with appropriate anti-malarial drug, a number of separate conditions associated with severe malaria may need specific interventions. I will here briefly present some of these conditions and the current guidelines regarding their management.

**Hypoglycaemia** – This is as discussed a common finding in severe malaria, especially in children, and should according to the current WHO guidelines be corrected through intravenous infusion of dextrose solution (WHO, 2000).

**Acid-base balance** – Metabolic acidosis with high levels of lactate is common and associated with poor prognosis. Hypovolemia and reduced tissue perfusion has been suggested as contributing factors and WHO guidelines states that if there are signs of dehydration, perfusion should be improved by intravenous infusion of isotonic saline, and that poor oxygenation should be addressed by increasing the concentration of inspired oxygen (English et al., 1997; Maitland et al., 2003; WHO, 2000). Anaemia is often present, and the relative importance of transfusion is higher in children with metabolic acidosis (manifested as respiratory distress) as compared to anaemic children without acidosis, suggesting a more generous attitude towards transfusion in these children (Lackritz et al., 1992).

**Convulsions** – This a common feature in malaria and not necessarily a marker of severe disease. However, in cerebral malaria multiple and focal convulsions are a known risk factors for neurological sequelae and the WHO guidelines recommend treatment with diazepam or paraldehyde (Idro et al., 2004; WHO, 2000).

**Anaemia** – A common feature of severe malaria in children that might require transfusion. While a Hb of less than 4 g/dl is considered as an indication for blood transfusion regardless of the clinical condition, treatment of less severe anaemia should be considered in high-risk patients demonstrating respiratory distress (as discussed above), impaired consciousness or hyperparasitemia (WHO, 2000). These recommendations are in line with previous findings that anaemic children without complications had a threshold of Hb <3.9 g/dl where transfusion was associated with decreased mortality while the corresponding figure for children with respiratory distress was 4.7 g/dl (Lackritz et al., 1992).
3.4.3 Adjuvant therapies

“All adjuvant therapies tried so far have either worsened or failed to improve outcome”

Planche 2005

Adjuvant therapies of severe malaria have despite long-time efforts so far produced poor results when tried in clinical settings. This is well illustrated in the current WHO guidelines by the long list of previously tried treatments that now are advised against (WHO, 2000). I will here briefly present some of the more important candidates tested and focus on the use of glycans relevant to this thesis.

While anticonvulsant treatment is recommended by the WHO on the basis of the correlation between prolonged seizures and neurological sequelae in children with cerebral malaria, there is no actual evidence of the benefit of such treatment. In three relevant studies that have been performed with general administration of anticonvulsants in cerebral malaria, the incidence of convulsions were reduced but at the same time a significant increase in mortality was reported in two of the studies (Crawley et al., 2000; Meremikwu and Marson, 2002).

Severe malaria is has been postulated to be associated with elevated serum levels of inflammatory mediators, in particular TNF. Pentoxifylline, a substance shown capable of reducing the level of circulating TNF as well as improving perfusion, has been tried as supplementary treatment with poor results. While demonstrated to reduce the cytokine level, there was no clinical benefit observed (Looareesuwan et al., 1998; Wenisch et al., 1998). Treatment with polyclonal anti-TNF antibodies has shown similar results; no significant clinical benefits were observed although a tendency towards faster resolution of the disease and a reduction of fever was seen. Anti-inflammatory treatment has also suggested in cerebral malaria but has been shown either to lack effect or to be harmful (Hoffman et al., 1988; Warrell et al., 1982).

Corticoids were administered to cerebral patients on the assumption of cerebral oedema as a central factor in severe malaria. However, in a controlled clinical trial with one group receiving dexamethasone and one receiving placebo, it was shown that the ones receiving treatment did not only display unwanted side effects, such as secondary pneumonia and gastro-intestinal bleeding, but also had a prolonged duration of coma (Warrell et al., 1982).

Antibodies targeting the surface of the infected erythrocyte and thereby blocking sequestration is generally accepted as important in the development of protective immunity, and sera from immune individuals have been shown capable of inhibiting and reversing adhesion of iRBC, both in vitro to cultivated cells and in vivo in squirrel monkey (David et al., 1983; Udeinya et al., 1983; Udomsangpetch et al., 1989). However, when tried in children with cerebral malaria in Malawi, intravenous administration of antibodies prepared from a pool of local donors failed to improve the clinical condition as compared to a group receiving placebo (Taylor et al., 1992). Two reasons for the lack of success are suggested; the antibodies reversed cytoadhesion but
this event had no impact on the clinical course, or the antibodies were incapable of releasing the sequestered erythrocytes. The later explanation is supported by the fact that parasite clearance was not accelerated despite simultaneous treatment with quinine, and that no mature forms of parasites were seen in the peripheral circulation following treatment as would be expected following successful de-sequestration. One may speculate that antibodies recognizing the surface of the iRBC need to be present prior to sequestration in order to inhibit the adhesive event; at a later stage the accessibility for the antibodies may be limited and the sequestration may be more permanent, possibly as a result of fibrin deposition as suggested by histopathological and in vitro findings (Dondorp et al., 2004; MacPherson et al., 1985; Pongponratn et al., 2003; Treutiger et al., 1999).

Heparin has been tried as adjuvant therapy on the assumption of disseminated intravascular coagulation (DIC) as a key feature in the pathology of severe malaria, (Jaroonvesama, 1972). Although this treatment initially was reported to be successful (Mitchell, 1974; Munir et al., 1976; Munir et al., 1980; Smitskamp and Wolthuis, 1971) it was discontinued due to the risk of intracranial bleedings and the abandonment of the DIC theory (Vreeken and Cremer-Gooite, 1978). Two controlled clinical trials have more recently tried to evaluate the possible positive effect of administration heparin. An Indonesian study on a total of 62 patients diagnosed with cerebral malaria demonstrated heparin to have a significant effect on both the duration of coma and of fever (Hemmer et al., 1991a; Rampengan, 1991), while a German study on a total of 116 patients with both mild and severe malaria found no such effect. However, in the heparin dose used in the German study was substantially lower and the drug was administered subcutaneously as compared to the higher doses of heparin administered intramuscularly or intravenously in the Indonesian study as well as previous successful trials. Also, very few cases of cerebral malaria were included in the German study making it difficult to compare the results with previous the previous findings.

New knowledge demonstrating the heparin like molecule heparan sulphate (HS) as an important iRBC receptor present on both endothelial cells and uninfected erythrocytes (Vogt et al., 2003; Vogt et al., 2004), and the capacity of heparin to disrupt rosettes, suggest a new role for heparin as a potential receptor antagonist in the treatment of severe malaria (Carlson et al., 1992b; Rowe et al., 1994). Furthermore, while the DIC theory has been disproved, histological examination of brain tissue from patients deceased with severe malaria have shown fibrin deposits present in the microvasculature, and there are indications that fibrin thrombi might be a more frequent feature in cerebral malaria than previously expected (Dondorp et al., 2004; MacPherson et al., 1985; Pongponratn et al., 2003). Curdlan sulphate, another sulphated glycan, has in a recent clinical trial been indicated to reduce both the duration of coma and the proportion of patients with fever (Havlik et al., 2005). This is supported by a previous clinical study where the administration of curdlan sulphate resulted in higher parasitemias in the treated population, a possible consequence of the release of sequestered iRBC (Havlik, 1998).
4 THE PARASITE

“In marshes there are animals too small to be seen, but which enter the mouth and nose and cause troublesome diseases”

Varro (First Century B.C.), De re rustica

The malaria parasite is of the family *Plasmodia*, a subgroup of the order heamosporina within the phylum apicomplexa. Although close to 120 species of *Plasmodium* have been described, only four of them (*P. vivax, P. ovale, P. malariae* and *P. falciparum*) naturally infect man. Further, out of these four *P. falciparum* accounts for the greater part of the morbidity and the vast majority of mortality related to malaria.

4.1 The Life Cycle

The lifecycle of the malaria parasite is one of great complexity, involving a number of different asexual and sexual forms and developmental stages in man as well as in mosquito.

*In the human host* – *Plasmodium falciparum* malaria is transmitted to man as the infected female anopheles mosquito takes a blood meal and along with her saliva injects sporozoites into the blood stream. The injected sporozoites enter the systemic circulation where they rapidly invade hepatocytes, and a 5-7 day long asymptomatic interval of parasitic asexual intracellular replication follows. As the infected hepatocyte burst by the end of this period, thousands of infective merozoites are released into the bloodstream where they swiftly invade circulating erythrocytes. This constitutes the start of the repeated erythrocytic 48 hours lifecycle associated with the typical febrile intervals as well as all other clinical manifestations of malaria. After invading the erythrocyte, the parasite matures through ring and trophozoite stages into a segmented schizont that rupture the red blood cell and release 8-32 new merozoites into circulation. These merozoites in turn infect new erythrocytes and keeps the cycle going with an exponential increase in numbers of infected cells, an increase that will continue until the parasite is controlled by the host or chemotherapy. Parasite exodus from the human host is accomplished by the sporadic formation of gametocytes, male and female sexual forms of the parasites, which are taken up by a feeding mosquito and transmitted to a new human host.
The lifecycle of the *P. falciparum* parasite. Infection in the human host starts with the bite of an *Anopheles* vector injecting sporozoites (1) that rapidly infect hepatocytes. After 5 – 7 days of maturation and division, the hepatocyte bursts and release merozoites in the blood stream (2). The merozoit infect erythrocytes and hereby initiates a continous 48h lifecycle in which the parasite matures within the red blood cell that subsequently burst and releases new merozoites that in turn infect fresh erythrocytes (3). Male and female gametocytes are sporadically formed and sexual replication takes place after the ingestion of those by a mosquito taking a blood meal (4). Infected erythrocytes containing mature parasites are not found in the peripheral blood but sequester in the microvasculature by the means of rosetting and cytoadhesion (5).

*In the anopheles vector* – While over 400 species of *Anopheles* have been identified, only around 60 of these are important vectors of human malaria. As stated above, the parasites journey in the mosquito starts with the ingestion of mature gametocytes. Before fertilization the gametocytes are transformed into gametes; in the male gametocyte this is a dramatic event that within 20 minutes results in the production of eight gametes form each gametocyte. As a result of successful fertilization of the female gamete, immobile oocysts are produced in the gut of the mosquito and undergo morphological changes resulting in the transformation into motile ookinetes. The motile ookinetes in turn invade the epithelium of the midgut and are once again transformed forming the oocyst. After 7-10 days, hundreds of sporozoites are released from the oocyst and migrate to the salivary glands. The mosquito stage is now completed and the new sporozoites are ready to be injected into a new human host at the next encounter.
4.2 The Pathogenesis

60 years ago it was thought that there was nothing more to learn about the pathology of malaria (Maegraith, 1981). Today, the nature of malaria pathogenesis remains to a large extent unresolved and the search for a single explanatory factor triggering the development of severe disease have so far been in vain. There are two main reasons for this gloomy state of affairs; the lack of animal models capable of accurately reproducing the pathology of severe malaria and the revealed complexity of the disease. As a result, management of severe malaria, apart from anti-parasitic medication, is still limited to good nursing and treatment of symptoms as it was 50 years ago.

Two main hypotheses have previously offered unifying explanations of malaria pathogenesis. The “vascular permeability theory”, as formulated by Maegraith and his disciples, concluded that malaria pathology arise from non-specific inflammatory processes resulting in increased vascular permeability, oedema and subsequent anoxia in sensitive organs, and the “mechanical theory”, going back all the way to the findings of Marchifava and Bignami in 1894, stating that the pathological events result from an obstruction of blood flow and subsequent anoxia in vital organs as a consequence of mechanical blocking of the microcirculation by infected erythrocytes resulting in (Fletcher and Maegraith, 1966; Maegraith, 1981).

4.2.1 The vascular permeability theory

Maegraith has without doubt been the most prominent propagator of this inflammatory theory (Fletcher and Maegraith, 1966; Maegraith and Fletcher, 1972; Maegraith, 1981). The central dogma is that an unspecific inflammatory state, mediated by the release of soluble factors by the parasite causes increases vascular permeability. This increased permeability would in turn lead to the escape of proteins and water to the surrounding tissue, resulting in the formation of oedema and subsequent impaired circulation in the brain. It would further bring about vasoconstriction as a physiological response of the body with anoxia in vital organs as result. General circulatory disturbances were also implied to play an important role as a consequence of a general hyperactivity of the autonomous nervous system. The observed accumulation of parasitized erythrocytes within the micro-vessels of the brain was according to this theory a secondary event to the impaired circulation. While a number of substances implicated in the pathology of malaria in animals infected with different species of Plasmodium were suggested, the theory failed to identify the factor or factors responsible for the induction of the non-specific inflammatory response in human malaria (Desowitz, 1987). Further, in 1985 histopathological examination of brain tissue from patients deceased with cerebral malaria showed a lack of evidence for inflammatory or immune pathogenesis for human cerebral malaria (MacPherson et al., 1985). As a result of these and other findings WHO in 1986 stated that the permeability theory as well as the treatments based upon this theory should be abandoned (WHO, 1986). The more modern suggestions of inflammatory response as a crucial event in
malaria pathogenesis include cytokines and nitric oxide, and will be further discussed below.

4.2.2 The mechanical obstruction theory

This theory would in its simplest form relate all malaria pathology to the accumulation of infected erythrocytes in the microvasculature resulting in tissue hypoxia and organ damage. Sequestration is a well known feature of *P. falciparum* iRBC, where they by adhering to endothelial cells and associating with uninfected and other infected erythrocytes withdraw from the peripheral circulation to amass in the deep vascular bed. This thought to represent a protective measure in order to avoid being cleared by the spleen and other parts of the reticulo-endothelial system. The concentration of mature forms of the parasite in the microvasculature was first noted already by Marchifava and Bignami in 1894, and correlations between the abundance of parasitized erythrocytes in the cerebral vasculature and cerebral malaria was reported already in the early 1900s. These finding have since been confirmed on many occasions (MacPherson et al., 1985; Pongponratn et al., 1991; Silamut et al., 1999; Turner et al., 1994).

Two main objections have been raised against the mechanical obstruction theory; the lack of sequestration observed in some patients demonstrating clinical symptoms of cerebral malaria and the frequent recovery from cerebral malaria without signs of ischemic damage. The now commonly accepted definition of cerebral malaria as including the presence of sequestered parasites solves the first issue; however it leaves a group of patients with cerebral symptoms demanding another explanation than mechanical obstruction (Edington, 1974; Thomas, 1971; Turner et al., 1994). A number of plausible reasons have been put forward; the lack of well defined case definitions of cerebral malaria in older material, the possibility of other clinical conditions responsible for the cerebral symptoms, and the duration of time and response of the parasites to treatment between the initial coma and the time of autopsy (Berendt et al., 1994). As to the frequent lack of ischemic damage after an episode of cerebral malaria this may be a consequence of incomplete blockage of the vessels in combination with the presence of specific endogenous inhibitors of ischemic damage suggested present in the brain (Relton et al., 1991).

4.2.3 Modern perceptions of malaria pathogenesis

Although different investigators still tend to put different amount of emphasis on the obstruction or the inflammatory theory respectively, the dichotomy between the two has today in all essential aspects been wiped out. The more or less agreed fusion between the two schools may be summarized as the recognition of severe malaria as an uttermost complex state, in which parasites interplay with host inflammatory responses. Plasma levels of the pro-inflammatory cytokine TNF have been correlated with hypoglycaemia, coma, and death in African children (Grau et al., 1989; Kwiatkowski et al., 1990). The importance of TNF is further demonstrated as polymorphism in the promotor region has been shown to influence the clinical outcome; carriage of the TNF-2 allele is associated with susceptibility to severe disease (McGuire et al., 1994;
McGuire et al., 1999). Whereas these two findings establish a role for TNF in the development of severe disease, other findings indicate the relation to be one of complexity. It has been shown that *P. vivax* is capable of inducing circulating levels of TNF comparable to those seen in patients with cerebral malaria, without bringing about the lethal pathology (Karunaweera et al., 1992). It has further been demonstrated that while reducing the fever, administration of TNF-neutralizing monoclonal antibodies had no impact on the clinical outcome when tested on severely ill malaria patients (Kwiatkowski et al., 1993). Both these findings may possibly be explained by adding the *P. falciparum* specific feature of sequestration to the picture: first, TNF activates endothelial cells and has been shown capable of up-regulating the expression of endothelial receptors, and thus may exercise its pathological effect by increasing the amount of adherent iRBC (Ockenhouse et al., 1992; Rogerson, 2003). Secondly, induction of TNF production is possibly triggered locally at the site of sequestration, and the circulating level may be a poor reflector of the local concentration (Clark and Cowden, 1999); high local production in an area of reduced circulation also possibly limits the potential beneficial effect of neutralizing antibodies.

The cytokine lymphotoxin (previously known as TNFβ) has also been shown present in serum from malaria patients and hence implicated in malaria pathology. It shares the same receptors as TNF and is demonstrated a key mediator of cerebral malaria in the *P. berghei* ANKA mouse model of cerebral malaria (Engwerda et al., 2002). Interferon gamma (IFNγ) has also been implicated in pathology of *P. falciparum*; elevated levels have been demonstrated in severely ill patients and it is reported a mediator of cerebral malaria in experimental *P. berghei* ANKA infection of mice (Looareesuwan et al., 1999; Rudin et al., 1997).

*Suggested inflammatory and mechanical events synergistically bringing about severe malaria*
In addition to the up-regulation of endothelial receptors and induction of fever, TNF and lymphotoxin have been proposed to contribute to both hypoxia and acidosis by causing systemic inefficient use of oxygen (Clark and Cowden, 2003). They are also known inducers of nitric oxide (NO) production in endothelial cells, and while population studies support a general protective role of NO, increased local production have been demonstrated in cerebral malaria and may have a role in pathogenesis in parallel to what is implicated in sepsis (Clark and Cowden, 2003; Prada and Kremsner, 1997). Further, sequestration may also generate local production of NO by causing hypoxia and could hence enhance the effect of the cytokines (Melillo et al., 1995). TNF and lymphotoxin have also been reported to induce production of chemokines by macrophages in the placenta, resulting in the attraction of monocytes and further enhancement of the local inflammatory response (Abrams et al., 2003).

The glycosyl phosphatidylinositol (GPI) anchor of *P. falciparum* molecules has been proposed as the prime mover of inflammatory response. GPI has been demonstrated capable of inducing cytokine production and NO-expression in macrophages, and immunization with *P. falciparum* GPI has been reported to protect from severe symptoms in experimental *P. berghei* ANKA infection of mice (Schofield and Hackett, 1993; Schofield et al., 2002; Tachado et al., 1996). In addition, anti-GPI IgG levels have been shown associated with protection from cerebral malaria, even though not correlated with clinical outcome (Perraut et al., 2005).

Fibrin deposits is a typical histological feature of the *P. falciparum* infected placenta, and whereas DIC has been demonstrated uncommon in severe malaria, a general pro-coagulant state is a known feature of *P. falciparum* disease (Hemmer et al., 1991b; Horstmann and Dietrich, 1985; Pukrittayakamee et al., 1989). Microvascular fibrin deposits have been noted in the brain of patients deceased with cerebral malaria, and this feature is suggested more common than expected in African children with cerebral malaria (Dondorp et al., 2004; MacPherson et al., 1985; Pongponratn et al., 2003). Several explanations for the pro-coagulant state and the fibrin deposits have been proposed: macrophages of the placenta express the clotting initiator tissue factor (TF), possibly as a result of exposure to TNF and/or GPI, and TF have *in vitro* been reported expressed by monocytes when exposed to iRBC and by monocytes, macrophages and endothelial cells as a result of exposure to TNF (Grignani and Maiolo, 2000; Imamura et al., 2002; Pernod et al., 1992). Also, hypoxia is in itself also known to be an initiator of the pro-coagulant pathway (Yan et al., 1999).

### 4.3 Virulence factors

Setting the host factors aside and focusing solely on the parasite, there are a number of strain specific features that are shown or hypothesised to be important determinants of the clinical outcome. This suggests severe malaria to at least some extent to be the result of an infection with a more virulent strain, a concept further supported by available immunological data.
4.3.1 Sequestration

Only erythrocytes infected with early stages of the *P. falciparum* parasite are seen in the peripheral circulation whereas the iRBC containing mature parasites accumulate in the microvasculature. This capacity of the parasite to induce sequestration most likely represents an important virulence factor. Three different synergistic events is thought to bring about the accumulation of infected erythrocytes in the vascular microcirculation; the binding of iRBC to endothelial cells (cytoadhesion), the binding of iRBC to uninfected red blood cells and/or other iRBC (rosetting and autoagglutination respectively), and parasite induced rigidity of the infected erythrocyte.

4.3.1.1 Adhesive ligands of the *P. falciparum* infected erythrocytes

A number of parasite derived proteins have been shown associated with the membrane of the infected iRBC, and at least one of these has been demonstrated to be exposed on the erythrocyte surface and capable of interacting with host receptors.

*P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is a multi-domain protein of 200-350 kDa expressed on the iRBC surface that undergoes antigenic variation and thereby allows for the generation of diverse adhesive phenotypes. It is encoded by the highly diverse *var* gene family, consisting of approximately 60 genes distributed on all 14 chromosomes (Baruch et al., 1995; Fernandez et al., 1998; Howard et al., 1983; Leech et al., 1984; Roberts et al., 1992; Su et al., 1995). While multiple transcripts of the *var* gene family is seen in the early stages of intraerythrocytic parasite development only one dominant transcript is present in the later stages, and a single variant PfEMP1 determining the adhesive properties of the parasite clone is thought to be expressed at the surface of the trophozoite infected erythrocyte (Chen et al., 1998b). The PfEMP1
polypeptides, with a few exceptions, share a common structure with a N-terminal sequence (NTS) followed by a semi-conserved Duffy binding like domain (DBL-1α), a cysteine-rich interdomain region (CIDR-1), a DBL-2 region, and possibly 1-3 different DBL domains, sometimes interspersed by C2 and CIDR-2 domains. The complete proteins end at the C-terminal with a transmembrane region and a conserved acidic terminal segment (ATS). The function of PfEMP1 in mediating cytoadhesion and rosetting is demonstrated as mild trypsin treatment, that removes the polypeptide from the surface, as well as treatment with immune sera recognizing PfEMP1 inhibits cytoadhesion and rosetting (David et al., 1983; Leech et al., 1984; Udeinya et al., 1983). Further, expression of PfEMP1 has been shown to coincide in time with the adhesive capacity of the iRBC, and a large number of binding sites corresponding to the receptors implicated in cytoadhesion and rosetting have been mapped to different domains of the protein (Barragan et al., 2000a; Baruch et al., 1997; Chen et al., 1998a; Chen et al., 2000a; Reeder et al., 1999; Ruangjirachuporn et al., 1992; Smith et al., 2000a; Smith et al., 2000b).

Structure and adhesive events associated with different domains of the PfEMP1 polypeptide of the parasite clone FCR3S1.2

PfEMP1 expression is commonly restricted to specific electron dense protrusions of about 100 nm wide and 30-40 nm in height on the surface of the iRBC termed knobs. The knobs are localized at the iRBC outer membrane and demonstrated as the point of attachment of iRBC to endothelial cells and to erythrocytes (Deitsch and Wellems, 1996). Several proteins are localized to the knob structures such as knob-associated
histidine rich protein (KAHRP), *P. falciparum* erythrocyte protein 2 (PfEMP2) and *P. falciparum* erythrocyte protein 3 (PfEMP3), and the expression of KAHRP have been demonstrated indispensable for the knob-formation to take place (Coppel et al., 1988; Kilejian, 1979; Pasloske et al., 1993; Pologe et al., 1987). A plausible function for the knobs is the concentration, presentation and, through KAHRP, anchoring of the adhesin PfEMP1. While knobs are found on virtually all clinical isolates and are hypothesized as crucial for *in vivo* adhesion under flow conditions, some laboratory strains lacking the knob structures have been shown very adhesive, and a few knob-less clinical samples of *P. falciparum* have been isolated (Biggs et al., 1989; Udomsangpetch et al., 1989).

While PfEMP1 is generally considered the most prominent determinant of the adhesive capacity of the infected erythrocyte, a number of other iRBC membrane associated proteins have been suggested to participate in the binding events. The demonstrated inability of trypsin treatment to completely resolve the adhesion of iRBC to CD31 or block the agglutination of iRBC by immune sera does indicate the existence of such additional ligands (Fernandez et al., 1999).

RIFINs, encoded by the large *rif* (repetitive interspersed family) gene family, are clonally variant proteins demonstrated to be expressed at the surface of the infected erythrocyte (Fernandez et al., 1999; Kyes et al., 1999). In contrast to PfEMP1, several different RIFINs have been shown to be co-expressed on the surface of the late stage iRBC (Fernandez et al., 1999). The high gene copy number and the clonal variation clearly imply exposure to immune pressure, and RIFINs have been demonstrated immunogenic in natural infection. Furthermore, anti-RIFIN antibodies been suggested to be part of the acquired immunity (Abdel-Latif et al., 2003; Abdel-Latif et al., 2002). The stevor (subtelomeric open reading frame) gene family have been demonstrated closely related to the *rif* gene family in chromosomal location, gene structure and sequence homology (Cheng et al., 1998). Transcription of stevor genes has been reported but there is no data supporting the expression of STEVOR polypeptides at the surface of the iRBC. While RIFINs have been suggested directly or indirectly involved in adhesive events or transportation, the exact function of RIFINs as well as of STEVORs awaits further elucidation.

Pfalhesin is no parasite derived protein, but the result of a parasite induced modification of the abundant anion transporter (band 3) of the uninfected erythrocyte (Winograd and Sherman, 1989). Exposure of otherwise concealed regions of the band 3 protein is thought to result from an “accelerated aging” of the infected erythrocyte, and monoclonal antibodies directed to the newly exposed epitopes have been demonstrated to inhibit binding to CD36 expressing cells (Crandall et al., 1994; Crandall and Sherman, 1991; Winograd and Sherman, 1989). Further, targeting of modified band 3 with synthetic peptides has been reported to induce *in vivo* desequestration of iRBC in *P. falciparum* infected Aotus and Saimiri monkeys (Crandall et al., 1993).

Other suggested mediators of cytoadhesion include the product of the *clag 9* gene and the protein sequestrin. The aminosequence of the *clag 9* implies surface expression, and disruption of the gene resulted in abolishment of CD36 binding. Sequestrin is expressed in the late iRBC and proposed to bind to CD36 (Ockenhouse et al., 1991). Further, targeting of modified band 3 with synthetic peptides has been reported to induce *in vivo* desequestration of iRBC in *P. falciparum* infected Aotus and Saimiri monkeys (Crandall et al., 1993).

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multiple functions. The exact roles of CLAG, Sequestrin and SURFIN in adhesive events and natural \textit{P. falciparum} infection are still unclear.

Apart from the introduction of proteins involved in the interaction with host receptors of the endothelium or erythrocytes, the erythrocyte membrane undergoes fundamental changes as a result of \textit{P. falciparum} infection; parasite nutrients as well as waste products are transported through pores and channels and existing membrane components are digested or modified (Deitsch and Wellems, 1996).

4.3.1.2 Cytoadhesion and receptors

Cytoadhesion is likely to represent a fundamental feature of sequestration, and a large number of receptors present on vascular endothelial cells and the syncytial lining of the placenta have \textit{in vitro} been demonstrated as potential receptors for iRBC binding. While often proposed, the importance of adhesion to specific endothelial receptors as well as the whole concept of cytoadhesion to severe disease remains unproven.

CD36, a 88 kDa glycoprotein constitutively expressed on endothelial cells, platelets, and monocytes, was one of the first endothelial \textit{P. falciparum} iRBC receptors described (Barnwell et al., 1989; Ockenhouse et al., 1989). The vast majority of clinical \textit{P. falciparum} isolates have been demonstrated capable of adhering to CD36 and this binding has been shown stable and long lasting under flow \textit{in vitro} (Cooke, 1994; Hasler et al., 1990; Ockenhouse et al., 1991). While this likely indicate a fundamental importance of CD36 binding to the parasite, it also reduces its potential as a strain specific virulence factor associated with severe disease. This is further demonstrated as the capacity of CD36 binding is shown not to correlate with severe clinical manifestations (Newbold et al., 1997; Rogerson et al., 1999; Turner et al., 1994).

Thrombospondin (TSP) is a heparin and CD36 binding 450 kDa extracellular matrix glycoprotein secreted by endothelial cells, platelets and monocytes. It was the first protein to be identified as an iRBC receptor, and as in the case of CD36, most wild isolates are shown capable of adhering to TSP while no specific disease syndrome has been associated with the receptor (Roberts et al., 1985). Also, iRBC binding to TSP has been demonstrated unstable under flow conditions (Cooke, 1994).

Inter cellular adhesion molecule 1 (ICAM-1) is a 80 – 115 kDa member of the immunoglobulin superfamily expressed on endothelial cells, which supported by increased expression on vascular endothelium of brains from patients deceased with cerebral malaria, has been proposed as a receptor associated with severe disease (Turner et al., 1994). Furthermore, it has been shown that ICAM-1 expression is up-regulated by the malaria associated cytokines TNF and IFN\textgamma (Berendt et al., 1989; Dustin et al., 1986). However, the iRBC interaction with ICAM-1 has \textit{in vitro} been demonstrated to be feeble and in need of synergism with other receptors to be stabilized, possibly supporting a role for ICAM-1 in the initial rolling phase of iRBC attachment (Craig et al., 1997; McCormick et al., 1997).

The 130 kDa protein CD31, expressed on the surface of platelets, monocytes and neutrophils, is also a member of the immunoglobulin superfamily shown capable of mediating binding between iRBC and endothelial cells (Treutiger et al., 1997). While normally localizing to the junctions of the microvascular endothelial cells, CD31 have been shown to be redistributed to the luminal side of the endothelium by the malaria associated cytokines TNF and IFN\textgamma. Furthermore, pre-treatment with IFN\textgamma was
demonstrated to dramatically increase binding of iRBC to confluent human umbilical vein endothelial cells (HUVEC) (Romer et al., 1995; Treutiger et al., 1997). Although not associated with any specific clinical syndrome, CD31 binding is a common feature of clinical isolates (Paper I), and a polymorphism in the receptor gene have been reported to be associated with susceptibility to cerebral malaria (Kikuchi et al., 2001).

Heparan sulphate (HS) is a 10-70 kDa heparin like glycosaminoglycan demonstrated as a receptor involved in both cytoadhesion and rosetting (Vogt et al., 2003; Vogt et al., 2004; Paper I; Paper V). HS is produced by all cells, but shows tissue specific molecular characteristics (Kuppevelt et al., 1998; Ledin et al., 2004; Maccarana et al., 1996). While the global distribution of HS and the frequent binding of tested clinical isolates to heparin would suggest HS to be an important universal endothelial receptor involved in non-specific general sequestration, our data also show a correlation between iRBC heparin binding capacity and severe disease (Vogt et al., 2003; Paper I).

P-selectin is a 140 kDa glycoprotein involved in the initial tethering of leukocytes to endothelium (Geng et al., 1990; Ma et al., 1994). A similar role for P-selectin is suggested in the recruitment and initial contact between iRBC and endothelial cells (Senczuk et al., 2001; Udomsangpetch et al., 1997; Yipp et al., 2000). Further, while stimulation with the malaria associated cytokines TNF or IFNγ alone did not effect the expression of P-selectin, a combination of the two was shown to induce a tremendous up regulation on HUVEC (Raab et al., 2002). Immunohistochemical analysis of a possible early cerebral malaria case has implicated p-selectin in the interaction between iRBC and cerebral endothelium but no specific clinical condition has been associated with the receptor (Mori et al., 2000).

The 450 kDa glycosaminoglycan chondroitin sulphate A (CSA) has been demonstrated to be a receptor of iRBC capable of maintaining adhesion under flow conditions (Robert et al., 1995). While associated with clinical iRBC isolates from pregnant women, and hence implicated in placental binding, the phenotype is not restricted to maternal malaria (Beeson et al., 1999; Chaityaroj et al., 1996; Fried and Duffy, 1996; Rogerson et al., 1999). Indeed, soluble CSA was reported capable of reversing sequestration of P. falciparum in Saimiri monkeys (Pouvelle et al., 1997). The role of CSA as an important mediator of adhesion in maternal malaria is further supported by the demonstration that sera from multi-gravida women are capable of blocking adhesion to CSA (Fried et al., 1998; Maubert et al., 1999). Apart from CSA, hyaluronic acid (HA) and placental Fc-receptors have both been implicated as important iRBC receptors in the placenta (Beeson et al., 2000; Flick et al., 2001).

VCAM and E-selectin have been reported as potential endothelial receptors, but their role in sequestration in vivo is questioned as most clinical isolates investigated have been shown incapable of adhering to these receptors (Ockenhouse et al., 1992; Udomsangpetch et al., 1997).

4.3.1.3 Rosetting, autoagglutination and receptors

Rosetting and autoagglutination, defined as two or more uninfected RBC adhering to one iRBC and three or more iRBC adhering to each other respectively, are well established in vitro phenomena of laboratory strains as well as clinical isolates (Wahlgren et al., 1990a).
In contrast to cytoadhesion, rosetting has repeatedly been found associated with severe disease in the human host (Carlson et al., 1990a; Kun et al., 1998; Rowe et al., 1995; Rowe et al., 2002; Treutiger et al., 1992; Udomsangpetch et al., 1996; Paper I), although a few studies have reported a lack of clinical correlation (al-Yaman et al., 1995b; Rogerson et al., 1999). Interestingly, different forms of severe malaria have been reported to be associated with rosetting in different studies, reflecting the complexity of the parasite-host interaction and indicating rosetting as a common and unifying factor in the development of severe malaria pathology. Although clusters of iRBC and uninfected cells, likely to represent rosettes, have occasionally been observed in autopsy material, and rosettes have been spotted in samples of peripheral blood from patients with severe malaria, the occurrence of rosetting in vivo is a matter of some debate (Dondorp et al., 2004; Ho et al., 1991; Pongponratn et al., 2003; Riganti et al., 1990; Scholander et al., 1996). In an ex vivo model of perfusion of rat mesocecum vasculature, rosettes were observed in the venules and associated with microvascular obstruction (Kaul et al., 1991). A number of possible virulence functions have been stipulated; allowing for sequestration by decreasing flow, hiding the iRBC from immune cells, and making merozoite infection more efficient. Whether some, or possibly all, of these suggestions hold true, or if rosetting is a correlate rather than a cause of severe disease, remains to be further elucidated. However, in support of the idea of rosetting as a source of malaria pathology it has been demonstrated that sera from adults in malaria endemic regions disrupts rosettes, and that a lack of this capacity is associated with cerebral malaria (Carlson et al., 1990a; Wahlgren et al., 1990b). Furthermore, an association between polymorphism in the rosetting associated receptor CR1 and protection against severe disease has been demonstrated (Cockburn et al., 2004).

Although yet not completely resolved, the molecular mechanisms underlying the rosette formation are today better understood as receptors on the uninfected erythrocytes, as well as a need for human serum factors, have been demonstrated. Heparan sulphate has recently been reported present on uninfected human erythrocytes, and heparin and other sulphated glycans have been shown capable of inhibiting and breaking up rosette formation (Carlson et al., 1992b; Chen et al., 1998a; Rowe et al., 1994; Vogt et al., 2004). Further, the DBL-1 domain of the PfEMP1 is in FCR3S1.2 demonstrated to specifically bind to HS/heparin (Barragan et al., 2000a). Taken together this shows that HS is a receptor involved in rosetting.

Complement receptor 1 (CR1), a regulatory component of the complement mediated immune system, is found in varying copy numbers on erythrocytes and leukocytes. The observation that erythrocytes from CR1 deficient donors failed to form rosettes with a number of confirmed rosetting laboratory strains led to the notion of CR1 as a receptor involved in rosetting (Rowe et al., 1997). This finding was confirmed and expanded by showing that soluble CR1 inhibited rosetting in laboratory strains, and by demonstrating that a monoclonal antibody against CR1 was capable of reversing rosette formation in laboratory strains as well as fresh clinical isolates. It was further demonstrated that the DBL1α domain of the PfEMP1 of a rosetting, but not of a non-rosetting, parasite clone of was capable of mediating CR1 binding, implicating this domain in CR1 mediated rosette formation (Rowe et al., 2000). The importance of CR1 is indicated since polymorphisms in this receptor is shown common in malaria endemic areas of Papua New Guinea and associated with protection from severe disease.
(Cockburn et al., 2004). This finding may also explain the lack of correlation between rosetting capacity of the parasite and severity of disease previously noted in the same area (al-Yaman et al., 1995b).

Analysis of clinical isolates have demonstrated enhanced rosetting in patients of blood group A/B/AB relative to patients of blood group O, and a correlation between blood group A and severe malaria (al-Yaman et al., 1995b; Fischer and Boone, 1998; Lell et al., 1999; Udomsangpetch et al., 1993). It has also been shown that all laboratory strains as well as clinical isolates investigated display more intense rosetting when cultivated with either erythrocytes of blood group A or B (but not O), demonstrating a strain specific preference for a certain blood group (Barragan et al., 2000b; Carlson and Wahlgren, 1992). Further, A-trisaccharide can specifically inhibit rosetting in a blood group A preferring strain cultivated in blood group A/AB erythrocytes and the B-trisaccharide can specifically inhibit rosetting in a blood group B preferring strain cultivated in blood group B/AB, indicating blood group antigens as receptors involved in rosetting (Barragan et al., 2000b; Carlson and Wahlgren, 1992).

The role of the blood group antigen A as iRBC receptor has been confirmed by immunofluorescence, showing the binding of the blood group A-trisaccharides to the surface of the infected erythrocyte, and the capacity of the iRBC to adhere to blood group A antigen have been associated with severity of disease (Barragan et al., 2000b; Paper I).

Giant rosetting, featuring large aggregates with multiple uninfected as well as infected erythrocytes involved, and autoagglutination are common phenomena in clinical isolates and have both been associated with severe disease (Carlson et al., 1990a; Roberts et al., 2000) (Paper I). The molecular background of autoagglutination and giant rosetting is poorly understood; rosetting and autoagglutination are not completely overlapping phenomena suggesting specific molecular mechanisms (Roberts et al., 2000).

4.3.1.4 Rigidity of the infected erythrocyte

Other than specific interaction between the infected erythrocyte and receptors on endothelial cells and erythrocytes, enhanced rigidity of the iRBC has been demonstrated and proposed to contribute to sequestration (Cranston et al., 1984). The inflexibility is suggested as a result of membrane modification, the physical presence of a less deformable parasite, changes in the shape of the erythrocyte, or possibly combinations of these phenomena (Glenister et al., 2002; Nash et al., 1989). Membrane modification by the insertion of the parasite derived proteins KAHRP and PfEMP3 during intracellular maturation have been shown to induce major changes in the iRBC rigidity (Glenister et al., 2002). This is supported by the observation that strains with knob structures on their surface, a feature associated with the expression of KHARP, are less flexible as compared to knob-less strains (Paulitschke and Nash, 1993). The effect of these changes has elegantly been demonstrated in a microfluidic model for single cell capillary obstruction (Shelby et al., 2003). Interestingly, in this study it was shown that uninfected erythrocytes readily could pass a 6 μm channel despite the channel being blocked by an immobile schizont; this phenomena of non-complete obstruction could possibly explain the lack of permanent hypoxic damage in cerebral malaria and also suggests how local infection of new erythrocytes could be accomplished in an area of sequestration.
Membrane changes and enhanced rigidity of uninfected erythrocytes have also been reported in severe malaria and identified as a possible factor correlated to severe disease (Dondorp et al., 1997; Griffiths et al., 2001). This could possibly induce splenical clearance of uninfected cells and hence contribute to malaria related anaemia. Oxidative stress, perhaps mediated by the release of iron containing haemozoin from the bursting iRBC, has been suggested to be the mechanism underlying the changes in membranous properties of uninfected RBC (Griffiths et al., 2001; Omodeo-Sale et al., 2003).

4.3.2 Antigenic variation

The term antigenic variation refers to the mechanism whereby an infective organism dodges the host immune system by altering the exposed epitopes, resulting in a pathogen immunologically distinct from the parental strain. This may be a slow continuous process (antigenic drift) or a more rapid event with extensive changes in the antigenic material expressed (antigenic shift). The principle of antigenic variation is particularly important in pathogens dependent on long survival within a host and in those likely to infect the same host over and over again. Both antigenic drift and antigenic shift occur in the malaria parasite; the former as a result of recombination of the subtelomeric genes during sexual reproduction in the mosquito and the later as a result of the sequential expression of members of gene families within the parasite population.

Although the malaria parasite at large confines itself to the intracellular compartment during human infection, and thereby minimizes its encounters with host immunity, it still needs to interact with the environment. Sequestration is possibly the most apparent example of parasite-host interaction and, as stated above, shown to be mediated by the expression of var/PfEMP1 on the surface of the iRBC. Subsequent and mutual expression of PfEMP-1 in the population does not only confer virulence by protecting the parasite from host immunity, but also by altering the adhesive phenotype of the parasite and thereby determines the quantity and quality of sequestration. The var gene switching rate has been estimated to 1-2% per generation (Roberts et al., 1992).

While the regulatory events underlying the switching between different var genes are not fully understood, the expression patterns during maturation, with several genes expressed in the young ring and only one expressed at the surface of the iRBC of the mature trophozoite, indicate the presence of an efficient silencing mechanism (Chen et al., 1998b; Fernandez et al., 2002). A silencing element have also been identified in the intron region of the var gene, and the lack of this region have been associated with non-regulated expression of the gene (Calderwood et al., 2003; Deitsch et al., 2001; Kyes et al., 2003; Winter et al., 2003). It has further been demonstrated that the chromosomal position and sequence are unaffected by the activation of the var gene, implicating epigenic regulation of expression, possibly controlled at the level of chromatin assembly (Chen et al., 1998b; Freitas-Junior et al., 2005; Scherf et al., 1998). Apart from the var gene products, other variant gene families such as the rifins have been proposed to participate in antigenic variation.
4.3.3 Other possible virulence factors

Hyperparasitemia is a known risk factor of severe malaria, and it is conceivable that replication rate is an important virulence factor in parallel to other microbial infections. Whereas high parasitemia is likely to represent a result of multiple variables such as successful sequestration or immunological status of the host, it may also be a reflection of clone and strain specific replication and invasive capacity. This is supported by differences in growth rates observed both in laboratory strains and in *in vitro* adapted clinical isolates, although in the later this may reflect the adaptive capacity of the parasite rather than its indigenous replication rate. As discussed above, inflammatory mediators are also implicated in severe malaria, and it is plausible that different parasite strains elicit different immune responses resulting in specific syndromes and different levels of disease severity.
5 ANIMAL MODELS

“Patophysiological interpretations derived from animal models of falciparum malaria should be treated with caution, particularly as the animal disease manifestations under study do not correspond with events in man”

WHO, 1986

While experimental infection of animals has proven a prominent source of knowledge in many other infectious diseases, they have so far been of more limited value in the exploration of the pathological mechanisms of human malaria. The main reasons are that *P. falciparum* does not naturally infect any host but humans and a few primates, and that infection of different animals with *P. falciparum* or other species of Plasmodia have failed to completely reproduce the clinical and pathological features of the human infection. This said, a number of different animal models have been established for the study of malaria, and while fully agreeing with the of need of caution as stated in the WHO proclamation, animal models still have and will continue to contribute to our knowledge regarding the pathology of severe malaria as well as provide systems in which new drugs and vaccines can be evaluated.

The malaria animal models can be divided into systems replicating actual parasite infection, and more artificial non-infectious systems where only restricted areas of the host-parasite interaction can be studied. The infectious models can be further subdivided into those using the human parasites and those using other plasmodia. A brief orientation of the different approaches and the results produced follows below.

5.1 Infection with non-human parasites

Rodents are in regular use in experimental systems of infectious diseases and are susceptible to infection with several different species of malaria. Although, whereas sequestration is likely to represent a major contributor to pathology in humans, inflammatory processes are thought to be the main source of pathology in the rodent malaria models. In fact, the WHO statement above is at large a response to the increased vascular permeability theory, and the implications of this theory on treatment of severe malaria; a theory based on the findings in rodent malaria models (Fletcher and Maegraith, 1966; Maegraith and Fletcher, 1972; Maegraith, 1981; WHO, 1986).

The ANKA strain of *P. berghei* has been reported to introduce lesions comparable to those seen in human cerebral malaria in Balb/C mice (Finley et al., 1982; Polder et al., 1983). However, the situation in rodents infected with *P. berghei* differs from that of human infection in that lymphocytes, monocytes and platelets sequester – not iRBC – and the relation of this to the symptoms of cerebral malaria reported in this model is a matter of debate (Alger, 1963; Desowitz and Barnwell, 1976; Franke-Fayard et al., 2005; Hearn et al., 2000; Jacobs and Warren, 1967; Miller and Fremount, 1969; Neill and Hunt, 1992; Rest, 1982). It was indeed recently reported that the development of cerebral malaria in *P. berghei* infected mice is independent of sequestration, suggesting the pathology to be exclusively mediated by inflammatory substances (Franke-Fayard et al., 2005). The possible implications of this finding on the relevance of the model,
and possibly on the perception of human pathology, await further elucidation. Transgenic *P. berghei* parasites expressing the merozoite surface protein 1 (MSP-1) of *P. falciparum* have been generated, and a protective effect on parasitemia by anti-MSP-1 antibodies in mice have been reported using this system (de Koning-Ward et al., 2003).

*P. chabaudi* infection in mice does not result in any clinical sign of cerebral malaria but sequestration of late stage parasites have been reported in various organs including the brain, and a number of features suggest a relation to the sequestration of *P. falciparum* as seen in humans: adhesion to CD36 *in vitro*, enhancement of cytoadhesion by IFN\(\gamma\) stimulation, and an association between sequestration and antigenic variation (Cox et al., 1987; Gilks et al., 1990), (Mota et al., 2000)).

Non-human primates are susceptible to infection with a number of non-human species of plasmodia, and despite serious ethical and economical considerations they represent a precious resource. Infection of the experimental host rhesus monkey (*Macaca mulatta*) with *P. fragile*, *P. coatneyi* or *P. knowlesi* all result in severe disease. *P. coatneyi* infection demonstrates some striking similarities to human cerebral malaria; cerebral sequestration is present and the cytoadhesion is associated with knobs on the surface of the iRBC, the repertoire of endothelial receptors used for adhesion is comparable to that of humans, and the pattern of sequestration in the brain is similar to human findings (Aikawa et al., 1992; Sein et al., 1993; Smith et al., 1996). In addition, the cytokine production resulting from the infection has been demonstrated comparable to what has been shown in human *P. falciparum* infection, and a resulting local up-regulation of ICAM-1 and nitric oxide production in affected areas of the brain has been reported (Tongren et al., 2000; Yang et al., 1999). Successful infection of splenectomized Japanese monkey (*Macaca fuscata*) with *P. coatneyi* infection of have also been demonstrated and reported to induce cerebral sequestration with histological similarities to human cerebral malaria, while infection of non-splenectomized animals resulted in severe anaemia without cerebral engagement (Kawai et al., 1993; Kawai et al., 1995).

Infection with *P. fragile* has also been shown to result in knob associated sequestration in various organs including the brain (Fremont and Miller, 1975; Fujioka et al., 1994). The receptor usage was further demonstrated comparable to that of *P. falciparum*, the endothelial expression of receptors shown up-regulated and rosettes were found present in the cerebral vasculature of infected animals (Fujioka et al., 1994). In addition, *P. fragile* has in its natural host been demonstrated to undergo antigenic variation with sequential appearance of variant antigenic types in parallel to *P. falciparum* in humans (Handunnetti et al., 1987).

### 5.2 Infection with *P. falciparum* parasites

While a few non-human primates are susceptible to the infection of *P. falciparum*, the use of these systems is limited by serious ethical and economical considerations. *Aotus* monkeys are not naturally infected with *P. falciparum* but yet susceptible to experimental infection with adapted parasite strains (Collins et al., 1994a; Geiman and Meagher, 1967; Young et al., 1976). Whereas knob positive strains are shown to infect non-splenectomized animals and sequester, knob-less strains are reported to not sequester and in need of splenectomized animals in order to mount a successful
infection (Aikawa et al., 1990; Langreth and Peterson, 1985; Miller, 1969). Of importance is that the full lifecycle of *Plasmodium falciparum* can be reproduced, making these animals suitable tools for evaluation of different vaccine candidates.

Squirrel monkeys (*Saimiri sciureus*) have been demonstrated susceptible to experimental infection with adapted strains of *P. falciparum* (Gysin et al., 1980). Although splenectomy has been shown required in order to accomplish reproducible infections with high parasitemias, non-splenectomized animals can be infected but then demonstrate varying degrees of pathology (Gysin and Fandeur, 1983; Pye et al., 1994). A number of clinical features associated with severe malaria in humans, including cerebral involvement, anaemia and fever, have been reported in the squirrel monkey, and both sequestration in cerebral vessels and rosette formation have been shown to occur (Contamin et al., 2000; Contamin et al., 1998; Gysin et al., 1992; Pye et al., 1994; Tourneur et al., 1992). *In vitro* analyses of *P. falciparum* iRBC adhesion to *Saimiri* brain microvascular endothelial cells revealed CSA as a receptor, and sequestered iRBC were further shown to be released into circulation upon injection of CSA, demonstrating not only the importance of CSA *Saimiri* monkey in sequestration in the monkey but also proving the principle of receptor antagonist mediated desequestration (Robert et al., 1995). (Pouvelle et al., 1997).

Chimpanzees are also shown susceptible to infections with *P. falciparum* and capable of reproducing the full lifecycle (Lefrou and Martignoles, 1954; Taylor et al., 1985; Thomas et al., 1994).

### 5.3 Non-infectious models

The obstructive effects on microcirculation by *P. falciparum* human iRBC have been investigated by *ex vivo* perfusion of rat mesocecum (Kaul et al., 1991; Raventos-Suarez et al., 1985; Rock et al., 1988). In this system, knob structures at the surface of the infected cells were demonstrated as a required point of attachment in endothelial adhesion, and the dynamics of sequestration was exposed; from initial attachment to venular endothelium and subsequent recruitment of additional iRBC and uninfected cells to more complete obstruction (Raventos-Suarez et al., 1985). Pre-incubation of the iRBC with soluble TSP or sera from immune individuals was shown to inhibit accumulation of iRBC in this system, indicating TSP to be an important receptor and anti-sequestration antibodies to be part of naturally acquired immunity to *P. falciparum* malaria (Rock et al., 1988). Using same model, *in vivo* rosette formation in venules were also demonstrated and associated with increased microvascular congestion, an effect that could be inhibited by pre-incubation with heparin (Kaul et al., 1991).

The establishment of severe combined immunodeficient (SCID) mice capable of sustaining circulating human erythrocytes for shorter periods of time has made it possible to further study the interaction between endothelium and iRBC. Injection of radioactively labelled mature human iRBC has demonstrated organ specific and ICAM-1 dependent sequestration to take place in SCID mice (Willimann et al., 1995). Further, injection of mature *in vitro* cultivated human iRBC into an established model of human skin graft in SCID mice have visualized the rolling and adhesion of iRBC to human microvascular endothelium *in vivo*, supporting previous thinking of adhesion as a multi-step process with an initial contact followed by rolling and static adhesion (Ho et al., 2000). Administration of antibodies to CD36 and/or ICAM-1 in this model further
demonstrated the relative dependence of the two receptors in rolling and adhesion events, and confirmed rolling as a possible rate limiting step in sequestration (Ho et al., 2000). In the same model, it was also reported that a recombinant PfEMP1 peptide was capable of inhibiting and reversing sequestration of clinical *P. falciparum* isolates in vivo, indicating PfEMP1 as a possible target for therapies aiming at reducing sequestration (Yipp et al., 2003). In parallel to the short-time models, another model maintaining *P. falciparum* infection for days to weeks in SCID mice additionally treated with Cl\textsubscript{2}MBP liposomes and anti-PMN antibodies, has been established (Moore et al., 1995). However, in this set-up, all stages of iRBC were seen circulating in peripheral blood, indicating an absence of sequestration (Moore et al., 1995). Studies on anti-malarial drugs and on the effect of antibodies targeting MSP-3 and RESA have been performed using this system (Badell et al., 2000; Moreno et al., 2001).

Further development within the area of transgenic animals and parasites are likely to open up new avenues of investigation into the pathogenesis of *P. falciparum*. The expression of *P. falciparum* merozoite surface protein 1\textsubscript{19} (MSP-1\textsubscript{19}) in *P. berghei* have already been achieved, and experiments with these new transgenic parasites in mice have shown the titres of MSP-1\textsubscript{19} inhibitory antibodies to correlate with protection from subsequent infection (de Koning-Ward et al., 2003). In the near future we are likely to see other *P. falciparum* virulence genes expressed in species capable of infecting experimental animals, or possibly completely humanized animals capable of sustaining a *P. falciparum* infection and reproducing the human pathology.
6 VACCINES

6.1 Development of immunity

Severe malaria is mainly and above all a disease of the child and the pregnant women. The protection of the older child and the adult is commonly understood as the result of a slowly acquired immunity, that first shelters from the more severe symptom, and following continued exposure in time also reders protection from clinical disease and finally a reduction in frequency of infection (Bruce-Chwatt, 1963; McGregor, 1974). However, more recent data suggest that components of this protection may develop more rapidly than previously expected (Baird et al., 1991; Gupta et al., 1999). Although an important role has been attributed to CD4+ T-cells in protection from asexual blood stages (Troye-Blomberg and Perlmann, 1988; Troye-Blomberg and Perlmann, 1993), I will here focus on the humoral immunity, today generally agreed on as the most important in P. falciparum, and more specifically on the immunity to variable surface antigens (VSA) expressed on the iRBC surface.

Plenty of evidence support the importance of antibody mediated immunity in P. falciparum infection. Passive transfer of antibodies has not only been shown capable of conferring protection between residents in a local area, but sera collected in Africa has been reported capable of reducing the parasitemia of patients in Thailand (Bouharoun-Tayoun et al., 1990; Cohen et al., 1961; McGregor et al., 1963; Sabchareon et al., 1991). Antibodies correlating to protection were first shown capable of inhibiting rosette formation (Carlson et al., 1990a; Treutiger et al., 1992), and agglutination and immunofluorescence assays have subsequently demonstrated an association between antibody recognition of the surface of the iRBC and protection from severe disease (Bull et al., 2000; Bull et al., 1999; Bull et al., 1998; Dodoo et al., 2001; Giha et al., 2000; Marsh et al., 1989)

The humoral immunity in P. falciparum is possibly very complex as it seems likely that protection is a result of a combination of antibodies targeting several developmental stages, thereby reducing the parasitemia, blocking adhesive events, and mediating and cytotoxic events. While antibodies against conserved but poorly recognized antigens certainly can not be ruled out as an important part of immunity, the accumulation of antibodies against a repertoire of VSA is strongly suggested by available data (Bull et al., 2000; Bull et al., 1999; Bull et al., 1998; Carlson et al., 1990a; Giha et al., 2000; Marsh et al., 1989; Ofori et al., 2002; Treutiger et al., 1992). In a study in Ghana comparing the recognition of a variable and a conserved part of PfEMP1 demonstrated that only recognition of the variable part was associated woith clinical protection (Dodoo et al., 2001).

The first and most ample evidence of VSA as the primary target of humoral immunity is seen in maternal malaria, where the introduction of a new organ in which iRBC can sequester renders previously protected women vulnerable to severe malaria (Brabin, 1983; Jelliffe, 1968). This correlation between the appearance of parasites expressing a new VSA capable of mediating adhesion to placenta, and the loss of immunity, strongly suggest protection to be dependent on antibody recognition of VSA, as does the development of immunity to this disease state with subsequent pregnancies.
6.2 Vaccine candidates

The development of a vaccine against *P. falciparum* malaria has indeed proven as difficult as has been long expected. This is due to the lack of reasonable animal models, the tremendous complexity of the parasite, and the limitation of research funding associated with a disease that in all important aspects is restricted to the developing countries.

While we still do not have a vaccine at hand after more than 60 years of efforts there is still hope in the promising results seen with some experimental vaccines and the knowledge that natural immunity conferring protection from severe disease develops fairly quick in endemic regions.

Protection from *P. falciparum* malaria by the means of immunization was first reported as early as 1973 (Clyde et al., 1973). This was achieved by the bites of a great number of irradiated mosquitoes, an approach not easily transferable to mass vaccination. Since then a number of approaches to immunization have been investigated, with an escalating tempo over the last decades. At the moment, more than 50 different vaccine candidates are in development and approximately 20 of these have entered clinical trials.

The different focus areas and some of the specific proteins targeted by current vaccine development.
6.2.1 Pre-erythrocytic vaccines

The objective of a vaccine targeting the pre-erythrocytic forms of the parasite, sporozoites and infected hepatocytes, is to induce sterile immunity. If both sporozoites and hepatocytes are to be targeted, this requires a combination of high affinity antibodies competent of blocking the quick process of hepatocyte invasion and cytotoxic T-cells capable of killing the infected hepatocytes. Circumsporozoite protein (CSP) is the best characterized protein on the surface of the sporozoite, and a new vaccine candidate (RTS,S), based on fusion of portions of CSP to hepatitis B virus surface antigen, has in combination with the adjuvant AS02 recently demonstrated efficacies of 34% and 45% in Gambian adults and children from Mozambique respectively (Alonso et al., 2004; Bojang et al., 2001). Furthermore, in the Mozambique study a reduction of almost 60% in the incidence of severe disease was reported (Alonso et al., 2004). However, these results have been questioned: first of all, the true test of a pre-erythrocytic vaccine is the capability of inducing long-lasting sterile immunity which was not shown in these studies. Secondly, the reduction of the incidence of severe disease possibly only reflects the delay in acquiring the first infection as noted in the immunized as compared to the non-immunized group (Snounou et al., 2005).

In addition to CSP, the sporozoite protein thrombospondin-related adhesion protein (TRAP), shown naturally immunogenic in a malaria endemic area in Gambians, and the liver stage antigens 1 & 3 (LSA1/LSA3), are currently investigated as potential targets of pre-erythrocytic vaccines.

6.2.2 Blood stage vaccines

A blood stage vaccine need to target either parasite expressed epitopes exposed on the extracellular merozoite or epitopes exposed at the surface of the iRBC. The second group, targeting the iRBC, is sometimes referred to as anti-complication vaccines since they would optimally inhibit sequestration and hence reduce the severe clinical manifestations. However, by blocking sequestration, such a vaccine would also release the iRBC into circulation and thereby facilitate their elimination by the spleen and other parts of the reticuloendothelial system. Furthermore, targeting antigens at the surface of the iRBC would opsonise the cell and open up for antibody mediated cytotoxic event. The second group of vaccines, targeting the merozoite, would need to prevent invasion to halt any further development of the infection.

While ample evidence suggests naturally acquired immunity to be largely dependent on antibody recognition of VSA on the surface of iRBC, this vaccine approach has so far not attracted a lot of interest. The main reason for this is the high degree of variability and rapid switch rate of these antigens, in combination with the lack of feasible animal models in which the effect may be studied. However, immunization with the CD36 binding CIDR-1γ domain of a variant PfEMP has been demonstrated to confer protection against homologous challenges of Autos monkeys, correlating to the level of agglutinating antibodies (Baruch et al., 2002). This protection was shown persistent against new variant antigens expressed by the same parasite strain in recurrent waves of parasitemias, suggesting natural boosting to take place during the course of infection (Baruch 2002). In another study, immunization of mice with
plasmid DNA expressing the CIDR-1α domains of PfEMP1 of three different parasite strains was demonstrated to produce some cross-reactive antibodies, and this cross-reactivity was shown greatly enhanced by boosting with recombinant proteins of the three constructs (Baruch et al., 2003; Gratepanche et al., 2003). In addition, immunization of mice and Saimiri monkey with a recombinant DBL-3γ domain of PfEMP1 have been reported to generate adhesion blocking antibodies shown cross-reactive with iRBC of various CSA binding P. falciparum strains (Costa et al., 2003). These findings, together with the results presented in this thesis on inhibition of sequestration in vivo and cross-reactivity resulting from immunization with the DBL-1α of PfEMP1 (Paper IV and Paper V), indicate the development of a vaccine based on the main VSA PfEMP1 as both feasible and desirable.

Merozoite surface protein 1 (MSP-1) is the most well characterized antigen at the surface of the infective merozoite, and antibodies towards a 19 kDa C-terminal fragment of the protein (MSP-119) have been associated with clinical protection (al-Yaman et al., 1996; Egan et al., 1996). MSP-119 has also been demonstrated capable of inducing a high degree of protective immunity against P. falciparum challenge in Aotus monkeys (Egan et al., 2000). Merozoite surface protein 2 (MSP-2) and possibly ring-infected erythrocyte surface antigen (RESA) are also associated with the merozoite, and antibodies to these proteins have been linked to immune protection and inhibition of invasion in vitro (al-Yaman et al., 1995a; al-Yaman et al., 1994; Beck et al., 1995; Berzins et al., 1986; Perlmann et al., 1986; Riley et al., 1991). Immunization with a new three component vaccine, combining parts of MSP-1, MSP-2 and RESA, have been reported to significantly reduce the number of parasites in vaccinated children in Papua new Guinea (Genton et al., 2002).

Apical membrane antigen 1 (AMA-1) is located both on merozoites and sporozoites, and hence potentially constitutes both a pre-erythrocytic and a blood stage vaccine candidate (Silvie et al., 2004). Monoclonal antibodies, as well as anti AMA-1 antibodies isolated from sera of adults living in an malaria endemic area, have both been demonstrated capable of cross-reactive inhibition of merozoite invasion and a protective effect by AMA-1 immunization has been demonstrated in a number of experimental malaria models (Anders et al., 1998; Collins et al., 1994b; Crewther et al., 1996; Hodder et al., 2001; Kocken et al., 2002; Narum et al., 2000). In a recent clinical trial, recombinant AMA-1 protein in combination with the AS02A adjuvant was demonstrated safe and capable of eliciting functional antibodies as measured by in vitro growth inhibition (Heppner et al., 2005).

6.2.3 Sexual stage vaccines

This approach represents an altruistic alternative in the sense that the individual immunized would not be protected from malaria, but further transmission of the disease would be inhibited. The antibodies would be ingested together with the gametocytes during the mosquito’s blood meal, and there by target antigens specifically expressed during the sexual replication in the gut of the vector. Antibodies to the recombinant vaccine candidate antigen Pfs28 have been reported to block transmission and a combination with the antibodies to the candidate antigen Pfs25 have been demonstrated to enhance the effect in a synergistic fashion (Duffy and Kaslow, 1997). A recombinant fusion protein combination of Pfs25 and Pfs28 has been found immunogenic in an
animal model, and clinical trials of Pfs25 are in progress (Gozar et al., 2001). Introduction of a transmission blocking vaccine alone would likely impose an enormous pedagogic problem and would therefore better be combined with a pre-erythrocytic or blood-stage vaccine of benefit for the immunized individual.
7 THE PRESENT INVESTIGATION

7.1 Scope of this thesis

The general purpose of this work has been to investigate how the adhesive properties of the *Plasmodium falciparum* infected erythrocyte relate to human pathology (Paper I and II), and to explore the possibilities to interfere with these events (Paper III, IV and V). A great deal of the effort has been devoted to the development and evaluation of an animal model in which these questions can be addressed. The more specific aims were:

- To further characterize the relation between the adhesive profile of fresh clinical iRBC isolates and clinical manifestations.
- To investigate whether sequestration of iRBC could be studied *in vivo* in immunocompetent rodents.
- To characterize the histopathology induced in the rat by different parasite strains and clones with different adhesive properties.
- To evaluate the effect of a GAG based receptor antagonist on sequestration of iRBC *in vitro* and *in vivo*.
- To evaluate the effect of antibodies raised by immunization with the DBL-1α domain of PfEMP1 on iRBC sequestration events *in vitro* and *in vivo*.
- To investigate if immunization with the DBL-1α domain of PfEMP1 protects against sequestration of iRBC in an *in vivo* challenge model and whether this protection is cross-reactive.
7.2 Materials and methods

The materials and methods used are presented within the context of each specific experiment in papers I – V. In addition, some of the materials used in several experiments as well as the more important methods are briefly being presented here.

7.2.1 The parasites

Laboratory strains of *P. falciparum* were cultivated at 37°C in human O+ erythrocytes at 5% hematocrit in accordance with standard candle jar technique (Trager and Jensen, 1976). The medium used was RPMI-1640 with 25mM HEPES and supplemented with 25 Mm sodium bicarbonate, 2mM L-glutamine, 10μg/ml gentamicin and 10% heat inactivated human B+ serum. The parasites were subcultivated 2-3 times a week and never allowed to exceed 10% parasitemia. Viability and rosetting rate was studied each day by mixing a small amount of culture with acridine orange and examining the sample in UV-microscope. Clinical *P. falciparum* isolates were in all but few aspects *in vitro* adapted and cultivated in the same way; AB+ sera was used instead of B+ and gassing with a mixture of 90% NO, 5% O and 5% CO replaced the candle jar technique.

**FCR3S1.2:** A multiadhesive and highly rosetting and autoagglutinating clone originating from the picking of one rosetting iRBC of the FCR3S1 parasite by micromanipulation (Fernandez et al., 1998). The rosetting and autoagglutinating phenotype of this parasite was on a regular basis maintained by centrifugation on Ficoll-Paque.

**FCR3S1.6:** A low-adhesive parasite cloned from the FCR3S1 parasite by the picking and propagation of a non-rosetting iRBC by micromanipulation. Demonstrated to express feeble amounts of PfEMP1 on the surface.

**3D7AH1S2:** A highly CD36 binding parasite obtained by panning of the genome parasite 3D7AH1 on CD36 transfected Chinese hamster ovary (CHO) cells followed by micromanipulation cloning

**FCR3CSA:** A CSA binding parasite obtained by panning of the Gambian parasite FCR3 on CSA expressing *Saimiri* brain endothelial cells.

**R29:** A rosetting parasite clone demonstrated to adhere to complement receptor 1. Derived from the ITO strain by selection for the rosetting phenotype.

**UAS22, UAS29, and UAS31:** Ugandan *in vitro* adapted clinical isolates of children from the Apac district with severe malaria.
7.2.2 Enrichment of mature iRBC by magnetic cell sorting (MACS)

The difference in paramagnetic properties of iRBC of mature parasites (>20h) as compared to early stages of development or uninfected RBC was utilized in order to obtain high parasitemias of synchronous trophozoites. In brief, cultures were carefully pelleted and resuspended in PBS supplemented with 2% BSA before loaded on a column placed in a Vario-MACS super magnet. Rosettes, if present, were broken mechanically by multiple passes through a 0.6 mm needle prior to adding the culture to the column. While ring stages and uninfected cells freely pass through the magnetic field, trophozoites are sustained within the column. After excessive washing with PBS 2% BSA, the column was removed from the magnet and the content carefully eluted in PBS 2% BSA. The viability and parasitemia of the sample was evaluated by addition of acridine orange and inspection in an UV-microscope before and after each purification.

7.2.3 Rosetting, giant rosetting and autoagglutnation

Rosettes are defined as two or more uninfected RBC adhering to one iRBC. The number of uninfected erythrocytes in each rosette varies, as does the tightness of the rosette; this is further elaborated on in paper I. Autoagglutinates are composed of three or more iRBC attached to each other without the involvement of any uninfected RBC. As in rosettes, the number of participating cells varies widely. Giant rosettes constitute a combination of rosetting and autoagglutnation, as these often very large aggregates are composed of a mixture of iRBC and uninfected RBC. The rate of each of these phenomena is calculated as the number of late stage iRBC involved in the aggregates as compared to the total number of late stage iRBC and is given as a percentage.

7.2.4 Static, dynamic and suspension adhesion assays

Static adhesion assays were performed by letting parasites adhere to thrombospondin spotted on plastic, cryosections of rat lung, or living cells without any disturbing motion. In brief, transfected CHO or L-cells expressing CD36, ICAM-1 or CD31 were cultivated on plastic, overlaid with parasite culture at 2% hematocrit and incubated at 37°C. After washing and staining with 1% Giemsa, the number of adherent cells per 100 target cells was assessed in microscope at 1000x magnification. The same procedure was used for scoring the adhesion of iRBC to microvascular endothelial cells from rat lung with the exception that MACS enriched (see above) material was used at a hematocrit of 0.5%. Static adhesion assays to frozen cryosections of rat lung mounted on three-well glass slides were essentially performed the same way; MACS enriched iRBC at 0.2% hematocrit were overlaid on the sections and left to bind in a humidity chamber at 37°C before careful washing, Giemsa staining and microscopic assessment of the number of adherent iRBC/mm². Dynamic adhesion assays to frozen cryosections of rat lung were performed in the same way but with orbital shaking (50 rpm) during incubation.

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Suspension adhesion assays were performed by mixing fluorescent labelled soluble CD31, blood group A antigen, heparin or anti-human Ig antibodies with 30μl of parasite suspension at 5% hematocrit in RPMI. The sample was subsequently incubated 30 minutes in the dark (room temperature), washed three times in RPMI, and counterstained with ethidium bromide before examination in microscope. The binding rate was calculated as the number of surface fluorescent late stage iRBC (>20h) as compared to the total number of late stage iRBC.

7.2.5 Sequestration assays in vivo

MACS enriched in vitro cultivated iRBC were labelled with radioactive ⁹⁹ᵐ⁻technetium by subsequent incubation in stannous agent and the isotope. The samples were washed trice, and resuspended, in RPMI-1640. Sedated Macaca fascicularis monkeys or Sprague-Dawley rats were placed in the gamma camera and injected with the labelled iRBC through the tail vein (rats) or Vena saphena magna (monkeys respectively. Rats were typically injected with 10⁶-10⁷ iRBC in about 650 μl of RPMI whereas the monkeys received 10⁸ cells in a total volume of 5 ml. With the rats and a two of the monkeys, the process of sequestration was monitored dynamically for 30 to 60 minutes using by 2D imaging, while the rest of the monkeys were monitored tomographically for 60 minutes, rendering a three dimensional image of the activity. In addition, the lungs were excised by the end of the experiment and the activity was measured separately in the majority of the rat experiments. The relative amount of cells sequestered in lungs and other organs was calculated by comparing the count-rate of the lungs, either as measured separately or by determining the activity of areas imposed on the two- or three dimensional images, to that of the whole animal.

7.2.6 Modification of the anti-coagulant properties of heparin

Standard heparin was oxidized by the use of periodate. 2 mg/ml heparin was incubated in 50 mM sodium citrate, 0.2 M NaClO₄ and 20 mM NaIO₄, pH 3, at 4 °C for 2 h. The reaction was stopped by the extensively dialyzing of the samples against H₂O. NaBH₄ was added to the solution (20 mg/mg heparin in 1 ml H₂O) and incubated for additional three hours at room temp, followed by a second dialyze against H₂O.

7.2.7 Inhibition of sequestration in vivo

The in vivo sequestration assays were performed as described above with the addition of the modified heparin. In rats, the glycan was either mixed with the labelled erythrocytes directly before injection or injected into the tail vein three minutes after the administration of the iRBC and the animals were monitored for 30 minutes. In the monkeys, the glycan was injected into the Vena saphena magna 30 minutes after the administration of iRBC. The relative amount of cells sequestered in lungs was calculated as above.
7.2.8 Immunization of mice, rats, rabbits & monkeys

A prime-boost regimen injecting three times with Semliki Forest Virus particles followed by one immunization with a recombinant protein was used. Mini var-genes consisting of the functional domain (NTS-DBL-1\(\alpha\), DBL-1\(\alpha\), CIDR-1\(\alpha\) or DBL2\(\delta\)) of the FCR3S1.2\(\text{var}1\), FCR3\(\text{var}O\) or 3D7\(\text{var}5.2\), in combination with the transmembrane region and a part of the C-terminal ATS domain, were generated by separate PCR amplifications of the different domains. The ATS-TM fragment was ligated downstream of the DBL-1\(\alpha\), CIDR-1\(\alpha\) or DBL2\(\delta\). After assembling of the mini-var genes they were cloned into a SFV vector and transcribed into mRNA. Virus particles were produced by the transfection of baby hamster kidney (BHK) cells with the recombinant RNA in combination with two helper RNA (helper-S2 and helper-C), harvested and concentrated by centrifugation, and used for the immunization. Complementary fusion GST proteins of the different domains were produced in *E. coli* and the GST was cleaved off by thrombin digestion before immunization.

Mice, rats, rabbits and macaques were immunized subcutaneous with SFV particles three times followed by one boost with a recombinant protein combined with Freud’s incomplete adjuvant or Montanide ISO 720. Immune sera were prepared from blood collected 2-4 weeks after the last immunization and challenges of the animals with iRBC were performed within 1-4 weeks of the last immunization.

7.2.9 Direct and indirect challenge of immunized animals

Sequestration assays were performed as above. Rats immunized with the DBL-1\(\alpha\) domain of FCR3S1.2\(\text{var}1\) FCR3\(\text{CSA\,varO\,var}1\), or a mixture of the two plus the DBL-1\(\alpha\) domain of 3D7\(\text{var}5.2\) were challenged with the iRBC of the strains FCR3S1.2, FCR3CSA or R29. Rats immunized sequentially with all DBL-1\(\alpha\) constructs were only challenged with iRBC of the strains FCR3S1.2 or R29. The animals were followed for 30 minutes and the level of sequestration was calculated from the count-rate of excised lungs as compare to the count-rate of the whole animal. The protective effect was evaluated by comparing the level of sequestration to that of GST immunized control animals challenged with the same parasite.

Macaques immunized with the DBL-1\(\alpha\) domain of FCR3S1.2\(\text{var}1\) were challenged with the iRBC of the FCR3S1.2 clone and the level of sequestration was calculated from three dimensional images of the activity overlaid with two dimensional anatomic images generated by normal X-ray in the same camera. The animals were followed for 30 minutes and the protective effect was evaluated by comparison to GST immunized animals challenged with the same parasite.
7.3 Results & discussion

7.3.1 Specific adhesive phenotypes mediated by the DBL-1α domain of PfEMP1 correlate with severe disease.

Excessive sequestration, understood as the result of cytoadhesive events, rosetting, and increased rigidity of the iRBC, or combinations thereof, is by post mortem investigations demonstrated to be a central mechanism involved in the physiopathology of severe \textit{P. falciparum} malaria (Dondorp et al., 2004; MacPherson et al., 1985; Miller et al., 2002; Oo et al., 1987; Pongponratn et al., 1991; Pongponratn et al., 2003; Rogerson, 2003; Silamut et al., 1999). Due to the inability of the mature parasite to subsist in the peripheral circulation without being removed by the spleen, this most likely represents an unconditional prerequisite for parasite survival and hence is embodied by all clinical isolates. This is supported by the observed lack of any but early ring forms of the parasite in blood samples from \textit{P. falciparum} patients.

Yet, if sequestration is a central feature in the development of severe disease, and all clinical isolates sequester, an explanation of why only occasionally life threatening clinical manifestations are seen is needed. Apart from host specific factors such as genetic predisposition and immunological status, the quality and quantity of the sequestration are likely to play a major role. The quality would here represent factors such as specific location and level of vascular obstruction imposed, whilst the quantity would represent the total number of late stage iRBC that are sequestered at each time point. Both these aspects would ultimately depend on the adhesive capacity and receptor preference of the infecting parasites, and possibly also on the ability of the parasite to induce host responses such as elevated expression of endothelial receptors or deposition of fibrinogen at the site of iRBC sequestration.

In order to elucidate the relationship between the adhesive capacity and receptor preference of the parasite and the severity of disease, we analysed fresh iRBC isolates from a total of 111 Kenyan children for a number of adhesive events. Separating this work from previous efforts were the large number of potential receptors investigated, and the idea, originating from the ability of our laboratory strain FCR3S1.2 to adhere to a number of different receptors, that the capacity to adhere to multiple receptors rather than avidly to one specific receptor may represent a clinically important feature of the iRBC.

Several potentially important findings came out of this analysis. First, iRBC of different clinical isolates were commonly seen capable of adhering to some extent to a great number of the investigated receptors (98% and 88% of the samples were demonstrated some level of adhesion to CD36 / ICAM-1 or CD31 respectively). Second, the binding of soluble blood group A antigen and heparin to the surface of the iRBC were both significantly associated with severe disease, while the binding of soluble CD31 fell just short of being significant ($p=0.06$). Third, multiadhesion, defined as the capability of a clinical isolate to bind significantly to $>$4 of the investigated receptors, was also correlated to severe clinical manifestations. Fourth, rosetting was common (84% of the isolates) and shown to correlate with severe disease, confirming previous observations (Carlson et al., 1990a; Kun et al., 1998; Rowe et al., 1995; Rowe et al., 2002; Treutiger et al., 1992). In addition to the rosetting rate, we also scored the
tightness and the size of the rosettes, as well as those of giant rosettes. Both the frequency and the size and density of giant rosettes were found to be associated with severe disease, as were the size and density of the rosettes.

Whereas all these correlations were seen significant when comparing the total group of severe cases to the non-severe cases, it was found more complicated when comparing each specific clinical group to that of non-severe cases. In the severe anaemia cases the findings were similar those of the severe group as a whole, but the significance of blood group A binding was lost. In the group constituting severely ill patients who did not meet the criteria of cerebral malaria or severe anaemia (severe NUD), the significance of blood group A was also lost, and in addition the significance of rosetting, but not of giant rosetting. In the small group constituting cerebral malaria, all significances but that of blood group A binding was lost. This strengthens the role of blood group A in the bringing about of severe malaria. The significance of multiadhesion was also compared between the different clinical groups and the non-sever cases, and found significantly associated with both severe anaemia and severe NUD, but not with cerebral malaria. While some of the significances lost when comparing the subgroups of severe disease to that of mild most likely reflects the smaller number of patients investigated, it may also indicate true differences in iRBC adhesion to be associated with different clinical manifestations

| Correlations between the adhesive features of clinical isolates from patients with different clinical manifestation of severe malaria as compared to patients with mild disease. Darker shade indicates significance ($p<0.05$) while lighter shade indicate tendency ($p<0.2$) and unfilled areas indicate a lack of association ($p>0.2$) |
|---|---|---|---|---|---|
| **Adhesion Score** | **Rosetting** | **Giant rosetting** | **Heparin** | **Blood group A** |
| All severe cases (n=50) | | | | | |
| Severe anaemia (n=21) | | | | | |
| Severe NUD (n=18) | | | | | |
| Cerebral malaria (n=11) | | | | | |

The mean adhesion score, reflecting the total adhesive capacity of the isolate to the investigated receptor, was calculated and found to be significantly associated with severe disease as a whole, as well as with sever anaemia and severe NUD. However, the score was found to be similar between the cerebral malaria group and the non-severe cases. This was a somewhat suprising finding since sequestration and hence adhesive capacity would be expected to contribute relatively more to the pathology in these patients. However, this result mainly reflects the dependency of the calculated adhesion score on the binding to CD36 and CD31, receptors not associated with severe disease in any of the clinical subgroups. Another surprising finding was the high adhesion score noted with the iRBC of samples form patients with severe anaemia. This suggests adhesive capacity and thereby possibly sequestration as an important
feature in the bringing about of this condition. However, it has recently been suggested that severe anaemia may serve to protect from cerebral malaria by inhibiting sequestration (Flatt et al., 2005; White, 2005). This could result in highly adhesive iRBC, otherwise prone to cause cerebral malaria, ending up in the severe anaemia group and thus possibly partly explain our finding.

Several problems that might affect the outcome are associated with this kind of investigation. The classification of patients into distinct clinical subgroups is not uncomplicated, the iRBC found in the peripheral circulation may not fully represent those sequestered, and the adhesive profile of the iRBC sample might result from a mix of parasite trains simultaneously infecting the host.

The classification problem is most apparent in the group diagnosed with non-severe malaria as patients initially reported as non-severe may later develop severe disease. This was here accounted for by following the patients and reclassifying them as necessary. In addition, the classification of cerebral malaria relays on the clinical diagnosis of deep unrousable coma and, as discussed previously, malaria associated conditions probably unrelated to cerebral sequestration, i.e. metabolic disorders, are may contribute to an unknown number of coma cases.

The question of to which extent the circulating ring population represents the sequestered population of mature parasites remains unresolved. A simplistic view would be that as the sequestered iRBC burst and infect new erythrocytes they would be free to enter the circulation, and hence that the sequestered population, at least to some extent, would be represented in the circulating population. However, the possibility of infection and subsequent maturation in situ in severely clogged areas of the microvascuulture has been suggested and is supported by the fairly low parasitemia occasionally is seen in severely ill patients.

Infection with multiple strains is a common feature in malaria endemic areas and could explain why that the majority of the examined isolates demonstrated some level of binding to most of the investigated receptors (Beck et al., 1997; Färnert et al., 1997; Henning et al., 2004; Magesa et al., 2002; Zakeri et al., 2005). For instance, while some heparin binding was detected in more than 80% of the samples, only in less than 1/3 of these more then 12% of the individual iRBC demonstrated this capacity. Further, the binding to soluble CD31 was detected in 53% of the samples but only in 18% of these samples more than 12% of the iRBC bound to the receptor. This possibly suggests that the binding of the clinical isolate to a single specific receptor in some cases may be mediated by small populations of parasites of one or several strains different from the dominant one. In contrast, a high level of CD36 binding was recorded in the majority of the examined samples, suggesting most parasites capable of adhering to this receptor independently if they are from sever or mild malaria cases.
Mosaic representing the frequency and magnitude of the adhesion of different clinical isolates to the investigated receptors. The shades of squares indicate level of binding to the investigated receptor and correspond to a score of 1-5; darker shade equals higher binding. Asterisks indicate multiadhesive isolates.

Cytoadhesive events and rosetting are generally agreed to be mediated by the expression of the parasite derived adhesive ligand PfEMP1 at the surface of the iRBC. A number of receptor affinities have been mapped to the different domains of the molecule (Barragan et al., 2000a; Baruch et al., 1997; Buffet et al., 1999; Chen et al., 1998a; Reeder et al., 1999; Smith et al., 2000a; Smith et al., 2000b). Interestingly, when comparing the findings of the Kenyan study with this binding map, a pattern emerges; all the adhesive features associated with severe disease (rosetting, blood group A
antigen binding, and heparin binding) are associated with the DBL-1α domain of PfEMP1, suggesting this head structure of the protein to be an important mediator of severe malaria. A mosaic showing the binding to heparin, blood group A antigen and ICAM-1, not included in the original paper (Paper I) as these assays were performed only on parts of the study groups, has here been aligned with the original mosaic representing the frequency and magnitude of binding of each clinical isolate to the other investigated receptors. As can be seen from the figure, binding to heparin and blood group A antigen (represented by dark squares; darker shade indicates higher binding) is clearly over represented in the total severe group (41% and 40% of the isolates demonstrating more than 12% binding to each receptor respectively) as compared to the non-severe group (14% and 10% of the isolates demonstrating more than 12% binding to each receptor respectively).

DBL1α mediated adhesive events in FCR3S1.2

No association between the binding to CD36 and ICAM-1 and severe disease was found. In fact, binding to these two receptors was lower in all severe groups as compared to the non-severe group although this negative correlation was not found significant. This is in line with previous reports and possibly related to the recent finding that CD36 and ICAM-1 binding are not driving forces in the expression of the commonly recognized PfEMP1 believed to mediate severe disease (Bull et al., 2005; Newbold et al., 1997; Rogerson et al., 1999). Interestingly, this is also possibly further supported by the histopathological findings resulting from in vivo sequestration in the rat presented in this thesis (Paper II). While a CD36 binding parasite clone showed a high level of sequestration in the model, it failed to induce histopathological changes as seen with a multiadhesive parasite clone (Paper II).

If allowed to speculate, taken together this may represent part of the answer to the initial question of why severe disease is only occasionally seen despite sequestration
7.3.2 The FCR3S1.2 strain represents a potential model parasite for severe disease

The existence of variant strains more prone to cause severe disease is suggested from several different approaches. First, phenotypic features such as rosetting and giant rosetting have on several occasions been reported to be associated with severe disease (Carlson et al., 1990a; Kun et al., 1998; Rowe et al., 1995; Rowe et al., 2002; Treutiger et al., 1992; Udomsangpetch et al., 1996) (Paper I). Second, iRBC causing severe disease in children with little immunity have been shown to be more commonly recognized by the antibodies of semi-immune children and adults as compared to non-severe disease causing parasites (Bull et al., 2002; Bull et al., 1999; Nielsen et al., 2002; Ofori et al., 2002). Last, it has been demonstrated that parasites of the 3D7 strain that were in vitro selected for antibody recognition by semi-immune children tended to express a limited set of var genes (Jensen et al., 2004). All these data are consistent with the idea that a limited set of PfEMP1, mediating specific binding properties, are associated with severe disease. Our parasite clone FCR3S1.2 was derived from the FCR3S1 clone by micromanipulation. A single parasite involved in rosetting was picked under microscope and propagated, resulting in a highly rosetting and autoagglutinating clone (Chen et al., 1998b; Fernandez et al., 1998). Interestingly, a number of other adhesive features were also found to be enhanced as a result of this cloning. In contrast, when the same FCR3S1 clone was panned on CD36 transfrectant CHO cells, a decline in rosetting rate was seen (Scholander et al., 1996). These findings suggest rosetting to be linked to cytoadhesion and once again indicate CD36 binding as possibly negatively associated with severe disease.

A number of different findings suggest FCR3S1.2 as good model for severe disease causing parasites: (1) in Gambia more than 50% of the sera samples taken from children were capable of disrupting rosettes formed by FCR3S1 iRBC (Carlson et al., 1990a), (2) the adhesive profile of the FCR3S1.2 clone fits well with the binding phenotypes associated with severe disease in Paper I; a high rosetting rate with large and tight rosettes, a high giant rosetting rate, high levels of binding to heparin, blood group A and CD31, and a multiadhesive phenotype, (3) infected erythrocytes of the FCR3S1.2 clone were found equally or better recognized than a number of local clinical isolates when tested against a panel of Kenyan plasma samples from semi immune children (Paper V), and (4) antibodies raised against the PfEMP1 of FCR3S1.2 preferentially recognized clinical isolates from children with severe malaria as compared to mild disease (Q. Chen, personal communication)
7.3.3 Sequestration can be studied in the lungs of unmodified rats

Despite numerous animal models being used in the study of *P. falciparum* pathology there is to date no easy and practical system for the *in vivo* study of sequestration established. The main limitation is the inability of the parasite to infect any erythrocytes but human and those of a few primates. The models used today are either dependent on delicate and rare primates or on surgically or genetically modified animals, making them technically complicated, ethically sensitive and costly (Collins, 1992; Contamin et al., 2000; Gysin et al., 1980; Ho et al., 2000; Kaul et al., 1991; Raventos-Suarez et al., 1985; Tourneur et al., 1992). In addition, since adaptation of the parasites is required before infection of primates they are not suited for the study of clinical isolates.

We therefore set out to establish a new system using human *P. falciparum* infected erythrocytes in immunocompetent rodents. The specific aims was to create an inexpensive and feasible *in vivo* system in which the pathophysiology of sequestration and the results of different anti-sequestration approaches could be studied in laboratory strains as well as with clinical isolates (Paper II).

In *vivo* challenge of rats with iRBC. Parasites grown in vitro and enriched by passing over a MACS column or uninfected human erythrocytes are labelled with $^{99m}$Tc prior to injection in the tail vein of the rat. In the rat the material enters the right side of the heart and passes through the lung microvasculature prior to entering the main circulation.
Parasites were propagated \textit{in vitro} in O+ human erythrocytes and used as mature trophozoites 24-28h post invasion. A MACS magnet was used to enrich for infected erythrocytes, rendering a small sample with parasitemia of 70% or more containing only mature stages of the parasite. The sample was labelled \textit{in vitro} with a gamma radiating isotope of technetium ($^{99m}$TC) and injected into the tail vein of normal immunocompetent Sprague-Dawley rats and the distribution of the material in the animal was followed dynamically for 30 minutes by the use of a gamma camera. The proportion of injected material present in various organs during the last 10 minutes of the experiment was calculated by the imposing ROI (region of interest) areas onto the generated gamma camera images and comparing the activity in these areas to that of the whole animal.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{gamma_images.png}
\caption{Gamma images of a rat injected with labelled uninfected (to the left) or \textit{P. falciparum} infected erythrocytes (to the right).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{organ_diagram.png}
\caption{An illustration of the different organs in which accumulation of labelled material can be seen (to the left). The level of sequestration was determined by comparing the activity of different regions of interest (ROI) superimposed on the gamma camera images (to the right).}
\end{figure}
As our parasite clone FCR3S1.2 adheres to a great number of different receptors and represents a possible severe disease causing parasite strain (see discussion above), it was considered a good starting point and was hence the first to be tried in the new model. Administration of these iRBC resulted in a promising accumulation of radioactive material in lungs, spleen, liver and kidneys of the rats injected. A number of different control experiments were subsequently made in order to examine if the observed accumulation of FCR3S1.2 infected erythrocytes in the animals was iRBC and PfEMP1 specific.

First of all, uninfected human erythrocytes were administered in order to account for possible effects by the somewhat larger size of human erythrocytes and the anticipated immunological reactions to human cells. Equal proportions of the injected material were found in the liver, spleen and kidneys as compared to what was seen in the animals injected with FCR3S1.2 iRBC. This was demonstrated to be the case in all subsequent experiments and the accumulation in these organs were accordingly regarded as non-specific. However, the accumulation of material in the lungs was found to be significantly higher in the animals that received FCR3S1.2 iRBC as compared to the RBC controls, indicating sequestration as a possible event in the model. The amount of uninfected RBC in relation to FCR3S1.2 iRBC recorded in the lungs was substantially reduced in subsequent experiments by measuring the activity of excised lungs. This demonstrates a large part of the observed lung activity seen with uninfected erythrocytes to be a result from background activity of non-lung tissue.

Second, we administered iRBC of FCR3S1.6 clone, a poor expresser of PfEMP1 and accordingly a modestly rosetting and cytoadhering parasite, to the rats. While this resulted in some accumulation of cells in the lungs as compared to uninfected erythrocytes, the level was significantly lower than seen with FCR3S1.2 iRBC. This suggested several possibilities: The low amount of PfEMP1 still managed to confer a certain level of cytoadhesion, unspecific trapping was increased due to the higher rigidity of the infected erythrocyte, some adhesion was mediated by other surface proteins than PfEMP1, or a combination of one or more of these events.

Third, we used mild trypsin treatment of the iRBC prior to labelling and injection in order to further investigate the specificity of the accumulation of FCR3S1.2 in the rat lungs. This method has previously been demonstrated to remove the PfEMP1 from the surface of the iRBC and thereby inhibit rosetting and other adhesive features (Fernandez et al., 1999). The administration of trypsin treated FCR3S1.2 iRBC to the rats resulted in a level of accumulation in the lungs that was significantly lower as compared to untreated FCR3S1.2 iRBC and comparable to that previously seen with FCR3S1.6 iRBC. It was however still significantly higher than what was seen with uninfected iRBC and as we have later demonstrated that a somewhat higher level of inhibition of FCR3S1.2 iRBC accumulation is achievable with anti-PfEMP1 antibodies, this remaining accumulation is likely to be partly resulting from non-complete removal of the PfEMP1 (Paper IV and V).

Taken together, these experiments demonstrate that the sequestration of FCR3S1.2 iRBC in the rat lungs is specific and that this interaction is mediated at least in part by PfEMP1.
Box plot showing the differences in the proportions of the injected material accumulating in the lungs of rats injected with different clones of iRBC, trypsin treated iRBC or uninfected erythrocytes. Level of binding in the lungs measured by ROI technique and given as the percentage of total activity in the animal.

Infected erythrocytes of two other parasite strains were subsequently tested in the new model. The injection of iRBC of the CSA binding clone FCR3CSA resulted in an accumulation of material in the lungs comparable to that previously seen with FCR3S1.2 iRBC, while administration of iRBC of the CD36 binding clone 3D7AH1S2 resulted in an accumulation of material in the lungs significantly exceeding that of FCR3S1.2 iRBC. These findings suggest that CSA as well as CD36 capable of acting as receptors for human iRBC are present in the microvasculature of the rat lung. The role of CD36 was subsequently further confirmed by the inhibition of sequestration seen as a result of co-injection of FCR3S1.2 with soluble CD36. The role of the DBL-1α domain of FCR3S1.2 PfEMP1 was investigated by incubation of the FCR3S1.2 iRBC with antibodies against the DBL-1α, previously shown capable of recognizing the surface of FCR3S1.2 iRBC, prior to injection (Paper V). These antibodies profoundly inhibited sequestration in a concentration dependent manner, indicating a major role for PfEMP1 in the cytoadhesive events.

While all rats independent of being injected with iRBC or uninfected material at a superficial glance seemed to display the same dynamic pattern, with a high initial level of material present in the lungs that declined rapidly in the first minutes and levelled out towards the end of the experiment, profound differences were found when the dynamics were further analysed. The decrease in accumulated material during the first minutes of the experiments was discovered to be much more rapid in the animals injected with uninfected erythrocytes as compared to those injected with FCR3S1.2 iRBC. This difference in adhesion dynamics between uninfected and infected erythrocytes indicates a time frame in which anti-sequestration therapies can be tested in the model and this finding was subsequently used in experiments with a receptor antagonist (Paper III).

Further, a significant difference in tempo of decline of material in the lungs was also noted between rats injected with iRBC of FCRS1.2 and of 3D7AH1S2. The most obvious explanation for this observation is differences in the stability of the binding as
CD36 have previously been demonstrated highly competent in sustaining binding under flow conditions (Cooke, 1994).

By the use of freshly isolated rat lung endothelial cells and cryosections we further analysed the functionality of the rat equivalents to human receptors in iRBC adhesion. By blocking the binding of iRBC of FCR3S1.2 and 3D7AH1S2 with specific antibodies and enzymatic treatments in different combinations, we demonstrated that at least CD36, CD31 and heparan sulphate are able to act as iRBC receptors in same way as the human receptors.

Lung sections from animals injected with FCR3S1.2, FCR3CSA or 3D7AH1S2 were embedded in paraffin, stained, and examined by microscopy. Apart from the verifying the presence of iRBC in numbers as predicted by the radiological analysis, major differences in the histopathological appearance were also revealed. While iRBC of the CD36 and the CSA binding parasites were predominantly found one by one in capillaries, iRBC of the multiadhesive clone FCR3S1.2 were commonly found in clusters, clogging the lumen of venules and smaller veins. In addition, strands of pink degenerate material possibly representing fibrin deposits were frequently seen in conjunction with iRBC of FCR3S1.2 clone, and while the iRBC of FCR3CSA and 3D7AH1S2 were mainly found to be intact, FCR3S1.2 iRBC were in commonly in different stages of degeneration. The overall picture of the histopathological changes induced by FCR3S1.2 iRBC demonstrates similarities to previous findings in human cerebral malaria (Dondorp et al., 2004; MacPherson et al., 1985; Pongponratn et al., 2003).

While these pathological changes specifically induced by a parasite demonstrating all the adhesive features we previously showed associated with severe disease is potentially a very interesting finding, caution is needed in the interpretation. First, the histopathological findings seen in humans are generally believed to result from a prolonged infection. However, it is not know how fast the pathological changes occur in humans; one may for instance speculate that the often quite rapid onset of coma may depend on the sudden appearance of a novel phenotypic variant parasite followed by a fairly swift induction of pathology. Second, naturally occurring anti-human antibodies have previously been reported in rodents and could be partly responsible for the pathology. This would however not explain why these changes are not seen with the CD36 binding parasite albeit present in larger amounts in the lungs. Also, apart from the problems with possible rat specific pathological events, there are also potential organ specific problems. While the pathological observations are made in the lungs of the rat, profuse sequestration have not been reported in the lungs of humans. Further investigation is needed to elucidate whether the pathology seen in the lungs of the rat is relevant for other organs in humans.
7.3.4 Sequestration is inhibited \textit{in vivo} by a non-anticoagulant GAG receptor antagonist

Heparin was tried as an adjuvant treatment in severe malaria during the late 1970s and early 1980s based on the belief that severe malaria was caused by disseminated intravascular coagulation (DIC) (Jarrowesama, 1972). Whereas a few sporadic reports of almost miraculous recovery were published (Mitchell, 1974; Munir et al., 1976; Munir et al., 1980; Sheehy and Reba, 1967; Smitskamp and Wolthuis, 1971), no systematic and controlled studies were performed and the use of heparin in severe malaria was discontinued in 1986 after a proclamation by the WHO (WHO, 1986). This proclamation was brought about by the demonstrated risk of intracranial bleedings resulting from the anti-coagulant activity of heparin. While DIC is today regarded as a rare event in severe malaria (Vreeken and Cremer-Goote, 1978), new knowledge on the role of glycans as adhesion receptor has once again made heparin an attractive drug candidate.

The DBL-1$\alpha$ domain of PfEMP1 has been shown to mediate binding of the iRBC to heparan sulphate (HS) (Barragan et al., 2000a), and as HS has been demonstrated present on both endothelial cells and uninfected erythrocytes (Vogt et al., 2003; Vogt et al., 2004) it is thus implicated as an iRBC receptor involved in both rosetting and cytoadhesion. Rosetting rate as well as the capacity of the iRBC to bind to heparin has been associated with severe disease, and heparin treatment has been demonstrated to disrupt rosettes in laboratory strains as well as clinical isolates (Carlson et al., 1990a; Rowe et al., 1994; Rowe et al., 1995; Rowe et al., 2002; Treutiger et al., 1992)(Paper I). Taken together this suggests a role for heparin as a receptor antagonist in adjuvant treatment of severe malaria.

The administration of an antagonist capable of inhibiting and reversing iRBC adhesive events would serve two purposes. As severe \textit{P. falciparum} malaria manifestations are thought at least partly to represent consequences of local and general sequestration, reversal of iRBC binding would potentially have a direct impact on the clinical condition. Further, an antagonist capable of inducing desequestration would contribute to the clearance of the infection as circulating iRBC containing mature parasites are removed by the spleen and other parts of the reticuloendothelial system would.

To circumvent the unwanted and potentially life-threatening side effects of heparin, heparin devoid of anti-coagulant activity was generated by targeting the anti-thrombin binding pentamer with periodate treatment (Fransson, 1978). The generated product, depolymerised glycosaminoglycan (dGAG), was found to have no or very low anti-coagulant activity and to bind to recombinant DBL-1$\alpha$ as well as to intact iRBC of the laboratory strain FCR3S1.2 and of three different clinical isolates. The anti-rosetting capacity of dGAG on FCR3S1.2 did not differ from that of standard heparin, and the variability in the sensitivity to dGAG treatment seen with the wild isolates was in line with previous results with standard heparin. dGAG was further demonstrated capable of inhibiting as well as reversing the binding of iRBC of FCR3S1.2 and isolates to cryosections of rat lung.
The effect of dGAG on *in vivo* iRBC sequestration was evaluated in our new rat model. Inhibition of the sequestration of iRBC by co-injection with dGAG was demonstrated using the FCR3S1.2 clone as well as clinical isolates. Whereas 80% of the FCR3S1.2 iRBC sequestration could be blocked by dGAG, the corresponding inhibition with the clinical isolates varied between approximately 35% and 40%. The injection of dGAG three minutes after the administration of iRBC resulted in comparable levels of inhibition for the clinical isolates, although a higher concentration of the glycan was required, while about 50% inhibition was seen with FCR3S1.2. These findings strongly support the idea of heparan sulphate as a receptors used by the parasites for adhesions *in vivo* in the rat similar to what is thought to occur in the human situation human situation (Paper I). While the post injection of dGAG three minutes after the iRBC most likely demonstrate desequestration to take place, it can not be ruled out that the effect to some extent may reflect an inhibition of continued recruitment of iRBC still in circulation as human erythrocytes previously have been reported to have a half-life of about 7 minutes in the circulation of immunocompetent rats (Fabry et al., 1989).
Macaca fascicularis monkeys were used to further evaluate the effect of dGAG on sequestration in vivo. This new model represents a complete transfer of the rat model to monkeys, and the obtained results in the different systems were very similar: iRBC specific accumulation was only seen in the lungs, while accumulation in liver, spleen and kidneys was extensive and comparable in animals injected with uninfected and infected erythrocytes. In addition, possible sequestration in the bone marrow, previously reported to occur in humans infected with malaria, was noted in the monkeys but not further investigated. Whole body tomography rendering a three dimensional image in combination with conventional X-ray mapping of the organs was used in order to be able to measure the activity of the lungs non-invasively. The dGAG was injected 30 minutes after the administration of iRBC and resulted in a reduction of the amount of the injected material present in the lungs of approximately 50% as compared to non-treated controls. The results are likely to represent true desequestration as the level of injected material present in the lungs was totally static and very little activity was seen in the circulation at the time of dGAG injection. In one animal the process of sequestration and the effect of the injection of dGAG were monitored dynamically. The findings of relatively larger amounts of iRBC in the heart of the dGAG treated monkeys and the fluctuations in the amount of material in the lungs noted in the dynamically monitored animal post injection possibly reflect the reoccurrence of previously sequestered iRBC in the circulation and thereby further support the interpretation of the experiments.

While the reduction in sequestration resulting from the administration of dGAG may seem modest it should be kept in mind that the situation in the models is rather artificial. In a real life, sequestration most likely represents a dynamic event with continuous recruitment of new iRBC to the endothelium situation and the impact of an antagonist already present in the circulation when these events takes place may be hypothesized to be much larger than reflected in our experiments.
In addition to the discussed capacity of dGAG to act as an antagonist of HS binding, it may also affect the recruitment of iRBC to the endothelium by binding to p-selectin, a receptor previously shown important in the recruitment of leukocytes to a site of inflammation (Geng et al., 1990; Ma et al., 1994). HS has recently been suggested as a potential ligand for p-selectin and thereby to have a regulatory effect in the inflammatory response by blocking the adhesion of leukocytes to p-selectin (Gotte, 2003; Parish, 2005; Wang et al., 2002). The adhesion of iRBC to the endothelium has previously been shown to mimic the recruitment of leukocytes, and p-selectin has been demonstrated to be important in the initial contact between iRBC and endothelial cells (Senczuk et al., 2001; Udomsangpetch et al., 1997; Yipp et al., 2000). While interesting, as it would suggest a possible way in which heparin can inhibit sequestration of non-HS binding parasites, this would need further investigation.

In conclusion we have here shown modified heparin to act as a receptor antagonist and thereby inhibit the sequestration of the FCRS1.2 clone in vivo. Apart from suggesting dGAG as promising candidate for adjuvant treatment of severe malaria these findings also further strengthens the relevance of the new animal model and confirms HS as an additional functional receptor in the microvasculature of the model.

7.3.5 Immunization with the DBL1α domain of PfEMP1 generates functional and cross reactive antibodies.

The vaccine approach investigated within the scope of this thesis is in important aspects one of mimicking (Paper IV and V). First, it is an attempt to reproduce part of the protective immunity as seen in malaria endemic regions, and secondly an attempt to present the antigen to the immune system in a manner similar to that of the malaria infected erythrocyte. By mimicking these events we also hope to reproduce an important result of naturally developed immunity – protection from severe malaria.

Antibody recognition of the surface of the infected erythrocyte have been shown to correlate with protective immunity, and in their capacity to disrupt rosettes these antibodies have been demonstrated to modulate adhesive events (Bull et al., 2000; Bull et al., 1999; Bull et al., 1998; Carlson et al., 1990b; Nielsen et al., 2002; Ofori et al., 2002; Treutiger et al., 1992). Further, as a result of the expression of a new variant antigen, adult immune women become susceptible to severe disease during pregnancy, a susceptibility shown to be reduced by the antibody production associated with subsequent pregnancies (Beeson and Brown, 2002; Duffy, 2003; Duffy and Fried, 2003; Khattab et al., 2004; Megnekou et al., 2005; Staalsoe et al., 2004). These observations strongly indicate that antibodies targeting the variant antigens at the surface of the iRBC are an important feature in protective immunity and that they to some extent exercise their effect by reducing the adhesive capacity of the iRBC.

PfEMP1 is the most studied variant surface antigen (VSA) expressed on the iRBC. It has been shown capable of mediating binding to a great number of receptors present on human endothelial cells and uninfected erythrocytes, and the expression of sub-sets of variant PfEMP1 have been indicated to correlate with severity of disease (Barragan et al., 2000a; Baruch et al., 1997; Buffet et al., 1999; Chen et al., 1998a; Chen et al., 2000b; Reeder et al., 1999; Smith et al., 2000a; Smith et al., 2000b; Vogt et al., 2003;
Vogt et al., 2004) (Hvid, 2005; Jensen et al., 2004; Lavstsen et al., 2005; Staalsoe et al., 2003). Further, the different domains of the protein have been mapped to have affinities for different receptors, and the adhesive features associated with the DBL-1α domain of the parasite clone FCR3S1.2 are implicated in severe disease as previously discussed (Chen et al., 2000b)(Paper I). The DBL-1α domain has been shown present in the vast majority of the sequenced PfEMP1 and the sequence is reported more conserved than that of other domains (Flick and Chen, 2004; Smith et al., 2000b). The combination of conferring adhesive properties associated with severe disease, being present in all but a few PfEMP1, and demonstrating a relatively conserved gene sequence, all makes DBL-1α an attractive target for a vaccine aiming at VSA recognition.

Mini var-genes, consisting of one of three functional domains (DBL 1α, CIDR-1α or DBL2δ) of the FCR3S1.2var1, the transmembrane region, and a part of the C-terminal ATS domain, were generated and cloned into a Semliki Forest Virus (SFV) vector. The constructs were transcribed into mRNA and packed into virus particles by co-transfection of baby hamster kidney (BHK) cells, and the particles were used to immunize mice, rats and rabbits. A prime-boost schedule in which the animals were immunized three times with virus and boosted once with E. coli expressed recombinant protein was used. This resulted in the production of antibodies capable of recognizing the iRBC surface and disrupting rosettes of the FCR3S1.2 parasite strain. In contrast, sera from animals immunized three times with the recombinant protein alone showed no surface reactivity. This discrepancy is most likely explained by differences in how the antigens are presented to the immune system in the different approaches. In the virus system, the protein is anchored to the cell membrane by its transmembrane region and exposed to the immune system in a way similar to that of natural infection. This is likely to influence the immunogenicity and possibly also result in a more correct folding of the protein. Also, while the recombinant approach uses Freuds adjuvant to stimulate the immune system, the virus method has a more innate approach, where the processing and presentation of a foreign protein at the host cell surface and the subsequent apoptosis serves as more natural stimulators of the immune response.

Sera from animals immunized with virus particles encoding for DBL-1α only demonstrated more surface reactivity and higher rosette breaking capacity as compared to sera of animals immunized with the other constructs or a with combination of all constructs. The sera from the DBL-1α immunized animals were shown to recognize both E. coli expressed DBL-1α and FCR3S1.2 derived PfEMP1. The protective capacity of the immunizations was evaluated in vivo in our new rat model (Paper II). Animals immunized with DBL-1α encoding virus particles were challenged by the injection of purified and radio labelled FCRS1.2 iRBC as previously described (Paper I) and a significantly reduced level of sequestration was seen in the immunized animals as compared to GST-controls.

To confirm and expand these results with DBL-1α, a new set of immunizations were performed in rats and primates (Paper V). SFV particles were generated the in the same way as previous but this time encoding for the DBL-1α polypeptide of three different parasite stains (FCR3S1.2, FCR3varO or 3D7var5.2). The rats were immunized with either the FCR3S1.2 or the FCR3varO DBL-1α encoding particles, or a combination of
all three different particles, and the primates were immunized with either the FCR3S1.2 DBL-1α or the GST encoding particles. As before, a prime-boost regimen with 3 injections of SFV particles and one boost with recombinant protein was used. While sera from the rats immunized with the FCR3S1.2 or the FCR3varO DBL-1α encoding particles alone showed strong recognition of the homologous recombinant protein in ELISA and Western blot, only a weak cross-reactivity was seen with the other two recombinant DBL-1α polypeptides. The sera from the rats immunized with all three constructs, either simultaneously or one after another, recognized all three domains but reacted more strongly with the FCR3S1.2 DBL-1α. The immunized rats were challenged with iRBC of the different strains FCR3S1.2, FCR3CSA and R29, all previously demonstrated to sequester in the lungs of the rat microvasculature.

Apart from confirming the previous results of FCR3S1.2 DBL-1α immunization conferring protection from homologous challenge, the sequestration of iRBC of the R29 strain where also demonstrated to be considerably inhibited by this immunization as well as by the mixed ones. No significant protective effect was seen with FCR3CSA iRBC in any of the groups, and the animals immunized with FCR3varO encoding SFV particles only showed a minor reduction of the amount of sequestered FCR3S1.2 and R29 iRBC. The lack of protective effect against the sequestration of FCR3CSA is in line with previous findings that sera from infected children do not recognize placental iRBC isolates (Hviid, 2005; Recker et al., 2004).

Pre-incubation of iRBC with sera from DBL-1α immunized rats was used to confirm the protective effect to be mediated by surface reactive antibodies. The infected erythrocytes were incubated for 45 minutes with the sera prior to injection in the rats, and whereas the results from these assays at large confirmed the findings of the direct challenge, some differences were noted. The protective effect of the sera from rats immunized with FCR3S1.2 DBL-1α on the homologous parasites was enhanced, while the effect was reduced on the heterologous R29 parasites, and a minor inhibition of the sequestration of FCR3CSA now was present. The sera from rats immunized with mixed particles reproduced the result from the direct challenge when used with FCR3S1.2 and R29 iRBC, but also demonstrated a slight protective effect against FCR3CSA. Titration of the sera was performed, and the inhibitory effect on the sequestration of FCR3S1.2 and R29 was found to be concentration dependent.

This possibility to use naïve rats to evaluate the effect of the immunizations in vivo opened up for more extensive investigation of the protective capacity of the generated antibodies and was explored by using three different in vitro adapted clinical isolates from Ugandan children with severe malaria. Pre-incubation of iRBC of these three parasites with sera from rats immunized with FCR3S1.2 DBL-1α prior to injection into rats showed no or very little protective effect in two of the isolates, while a modest inhibition of sequestration was noted in the third. However, one of these isolate was shown not recognized by the sera form immunized rats at any time point, and the other two isolates, while initially demonstrated well recognized, were no longer recognized when retested subsequent to the challenge experiment. This suggests a switch in var-gene expression of the main iRBC population, an event not unexpected to occur in clinical isolates as result of in vitro adaptation and prolonged cultivation.
Sera from the FCR3S1.2 DBL-1α immunized primates were *in vitro* demonstrated to react with recombinant DBL-1α as well as native PfEMP1, and to be capable of breaking rosettes and induce agglutination in iRBC of the FCR3S1.2 strain. Challenges of the monkeys with FCR3S1.2 iRBC were performed in parallel to the challenge of the rats (discussed above; Paper III) and a reduction of the level of the sequestration as compared to GST immunized animals was found in four of the five monkeys.

Antigenic variation constitutes the main challenge for a vaccine targeting VSA and is here well illustrated by the result seen with the Ugandan clinical isolates. However, there are indications that severe disease is caused by a relatively small number of different PfEMP1 conferring specific adhesive properties, and that recognition of these is an essential part of the induced humoral immunity (Bull et al., 1999; Bull et al., 1998; Carlson et al., 1992a; Carlson et al., 1994; Chattopadhyay et al., 2003; Jensen et al., 2004; Lavstsen et al., 2005; Nielsen et al., 2002; Nielsen et al., 2004; Staalsoe et al., 2003) (Rowe et al., 1995; Rowe et al., 1997) (Paper I). If this holds true it opens up for vaccines against PfEMP1 as well as antagonistic targeting of adhesion, two approaches investigated within the scope of this thesis (Paper III, IV and V). But even if assuming the number of PfEMP1 bringing about severe manifestations to be limited and the sequences of these to be relatively conserved, a high level of cross reactivity would most likely be required from a vaccine targeting severe disease through these molecules. By using a mix of DBL-1α domains from three different phylogenetically distant PfEMP1 we here tried to induce a cross reactive response to PfEMP1, but while a certain degree of cross reactivity was seen with FCR3S1.2 DBL-1α both in IFA of clinical isolates and in rats challenged with iRBC of R29, this cross reactivity was not shown enhanced in the groups receiving a mix of the DBL-1α. Interestingly, the FCR3S1.2 DBL-1α sera also preferentially recognized clinical isolates from children with severe as compared to mild disease (Q. Chen, personal communication), suggesting this domain to be a good candidate for a VSA specific vaccine. The result with the sera from animals receiving a mix of different DBL-1α domains indicates that the picking of other PfEMP1 to include in such a vaccine will need careful consideration.

The reduction in sequestration by approximately 50% as seen as a result of the FCR3S1.2 DBL-1α immunization corresponds surprisingly well with the reduction achieved by the administration of the HS antagonist (Paper III). As the relative contribution of PfEMP1 mediated adhesion as compared to other possible mechanisms have not been assess in the accumulation of iRBC in the lungs of the macaque monkeys, these two results possibly suggest that a proportion of the adhesion is mediated by other domains of PfEMP1 or other means.

The data presented here demonstrate that a vaccine aiming at accelerating the development of induced humoral immunity by targeting the DBL1α domain of PfEMP1 may be feasible. It also confirms the sequestration in the rat to indeed be mediated by PfEMP1, and for the first time confirms the DBL-1α domain of PfEMP1 to be directly involved in sequestration *in vivo*.
7.4 Summary

- Clinical isolates from children with severe malaria are multiadhesive in that they are capable of simultaneous binding to several different host receptors present on endothelial cells and uninfected erythrocytes.

- The capacity of iRBC binding to heparin and blood group A antigen as well as the rosetting rate are significantly associated with severe disease and hence implicate a role for the DBL1α domain of PfEMP1 in severe malaria.

- Rosettes and autoagglutinates are significantly larger and tighter in isolates from children with severe malaria as compared to those from children with mild disease.

- PfEMP1 mediated sequestration occurs in vivo in the lungs of immunocompetent rats and monkeys and the event can be monitored by imaging in a whole-body gamma camera.

- The receptor repertoire used by the parasite in adhesion to human and rat endothelium is similar or identical.

- The level of sequestration and the induced histopathological changes seen in the rat are strain and clone specific.

- A new glycosaminoglycan (dGAG) devoid of anticoagulant activity has been generated and demonstrated to be capable of inhibiting the rosetting and cytoadhesion of different parasites in vitro.

- dGAG inhibits and reverses sequestration in vivo in rats and monkeys.

- The DBL1α domain of the FCR3S1.2var1 is immunogenic in rats and monkeys when delivered by the Semliki Forest Virus system and induces antibodies capable of recognizing live iRBC.

- Rats and monkeys immunized with the FCR3S1.2var1 DBL1α domain are protected from the sequestration of iRBC of laboratory strains in vivo.
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9 REFERENCES


Reeder, J. C., Cowman, A. F., Davern, K. M., Beeson, J. G., Thompson, J. K., et al. (1999): The adhesion of *Plasmodium falciparum*-infected erythrocytes to


