Sequestration and the infected-erythrocyte surface in
*Plasmodium chabaudi* malaria infection

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Abstract

Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP-1 coded by a gene family - var) is involved in sequestration, antigenic variation and acquired immunity. Much of this information has been gained from the study of parasites maintained in vitro. The major objective of the current work has been to characterize a model where these phenomena can be studied in vivo in a context of a dynamic host-parasite relationship.

P. chabaudi chabaudi AS infected erythrocytes have been analysed in sequestration and adhesion assays and we have studied the antibody response in mice to parasite-derived erythrocyte surface antigens. Results demonstrate that P. c. chabaudi AS sequesters primarily in the liver and spleen of these mice and the molecular basis for this phenomena is very similar to that seen with P. falciparum. Antibody response during the acute phase of infection is directed against parasite-line specific erythrocyte surface antigens promoting the phagocytosis of homologous infected-erythrocytes. Therefore, this model presents many similarities to P. falciparum infection of humans.

As with P. falciparum in humans, sequestration and antigenic variation appear to be intimately linked in P. c. chabaudi AS infections indicating that the protein responsible for both phenomena may be a homologue of PfEMP-1. In an attempt to identify the P. c. chabaudi AS homologue of Pfvar, a gene family was identified that is expressed during the parasite erythrocytic life cycle. The deduced protein sequence contains a cysteine rich-domain. Antibodies raised against it recognize a protein with similar size to PfEMP-1 that is partially insoluble in Triton X-100 and is localized around some mature parasites.

These results are discussed in the context of validating a rodent model to study sequestration, antigenic variation and acquired immunity in vivo.
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**Most Used Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APP</td>
<td>Acute phase plasma</td>
</tr>
<tr>
<td>$A_x$</td>
<td>Absorbance measured at a wavelength of $x$ nanometers</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoadsorbent assay</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>gramme</td>
</tr>
<tr>
<td>x g</td>
<td>Relative centrifugal force (gravities)</td>
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<tr>
<td>HIS</td>
<td>Hyperimmune serum</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo dalton</td>
</tr>
<tr>
<td>KGS</td>
<td>Kreb’s glucose saline</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mAmp</td>
<td>Milliampere</td>
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<tr>
<td>mg</td>
<td>Milligrammes</td>
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<tr>
<td>ml</td>
<td>Millilitres</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>NP</td>
<td>Normal plasma</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
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<tr>
<td>PfEMP-1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein-1</td>
</tr>
<tr>
<td>Pfvar</td>
<td><em>P. falciparum</em> var genes</td>
</tr>
<tr>
<td>KGS</td>
<td>Kreb’s glucose saline</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per unit volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per unit volume</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight per unit weight</td>
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CHAPTER ONE

Introduction

Malaria is an infectious disease caused by organisms of the genus *Plasmodium*-phylum Apicomplexa - and transmitted by female mosquitoes of the genus *Anopheles*. All members of the Apicomplexa are parasites, and the invasion of host cells is believed to be mediated by the contents of a complex set of organelles present at the apical end of the invasive stages of these parasites; hence the name Apicomplexa. *Plasmodium* belongs to the family PLASMODIIAE that represents organisms with pigment production, blood schizogony and have blood-sucking dipteran arthropods acting as vectors.

More than 100 species of the genus *Plasmodium* are found in the blood of reptiles, birds and mammals (reviewed by Garnham, 1966). Under natural conditions of transmission four of these species can infect man: *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*. The first three, chiefly *P. vivax*, cause much morbidity but are rarely fatal. *P. falciparum* is responsible for most of the mortality and morbidity associated with malaria.

1.1. Malaria history versus human history

*Plasmodium* is older than man but the origins of the four human malaria parasites are obscure. They probably evolved from tropical Africa, although an Asian site of origin is still proposed by some (Coatney, 1968).

If Africa was truly the "cradle" of *Plasmodium*, the transcontinental migrations of monkeys, apes and prohominids from at least the Miocene through to the Pleistocene period must have been accompanied by the distribution of parasites to Europe, across Asia to the Pacific, and probably across the Bering land bridge in the late Pleistocene. While *Plasmodium* thrived and speciated in Asia, it failed to do so in the New World. Malaria as a disease does not appear to have been recognized in the New World in pre-Columbian times and may have been taken there by the early European invaders.
Throughout history malaria has plagued travellers to the tropics - merchants, missionaries and soldiers. It has affected the course of history many times.

Hippocrates was the first to describe the manifestations of the disease, and relate them to the time of year and to where the patients lived. The association with stagnant waters led the Romans to begin drainage programmes, the first intervention against malaria.

The first recorded treatment dates back to 1600, when the bitter bark of the cinchona tree in Peru was used by the native Peruvian Indians. By 1649, the bark was available in Europe, as “Jesuit’s powder” so that those suffering from malaria might benefit from the quinine it contained, and this was used for many centuries. However, ignorance of malaria's cause and mode of transmission continued to prevail, and it remained one of the principal causes of morbidity and mortality in the tropics.

The last 100 years have seen an explosion of knowledge about malaria, as science and medicine have advanced. In 1889, Laveran identified *Plasmodium* (protozoan) parasites as the causative organism of the disease and, in 1897, Ronald Ross discovered that Anopheles mosquitoes transmit malaria. Pilot schemes to control malaria by attacking mosquitoes succeeded. At the beginning of this century, the pharmaceutical industry started looking for synthetic anti-malarial agents (eg. Mepacrine, 4-aminoquinolines, sontochin and chloroquine). Insecticide development also received a great stimulus with the discovery of DDT (dichloro-diphenyl-trichloroethane). In 1948, Shortt and Garnham described the missing part of the malaria parasite’s life-cycle, the pre-erythrocytic liver schizont. The main challenge appeared now to be operational, to establish the most effective ways of using the highly effective and safe drugs and residual insecticides. At first, these attempts appeared to be successful, with malaria being eliminated from many parts of Europe, Asia and North America. However, resistance to both anti-malarials and insecticides soon developed, and in most of the developing world, malaria has returned, in some cases leading to disastrous epidemics.

From the early 1970’s the malaria situation has slowly and progressively deteriorated. In an attempt to combat this situation, much emphasis has been placed on the identification of parasite-encoded components of different stages of the parasite life cycle as "vaccine candidates", in their characterisation and production by molecular biology and genetic engineering techniques leading straight into vaccine trials. However,
most of these antigens are relatively poorly defined in function. Recently, there is a tendency to go “back to basics” as many believe that only by understanding the basic biology of *Plasmodium* will it be possible to maximise the effectiveness of intervention measures in the longer term.

In addition, much emphasis has also been given to new measures of vector control. The understanding of different epidemiological patterns of malaria lie in the behaviour and interaction of anopheline mosquitoes with man. Only the female has the haematophagous habit, each blood meal being related to one gonadotrophic cycle and as a major protein source. The males live by sucking plant nectars for which they are equipped with much less formidable mouth parts (Harwood *et al.*, 1979). Not all *Anopheles* species find human blood acceptable, and some will not feed on man at all. In fact only some 85 of the approximately 400 known species and subspecies of *Anopheles* transmit malaria under natural conditions and not all of them transmit all the species (Harwood *et al.*, 1979). Initially, insecticide control was a cheap and effective, though ecologically damaging, strategy. However, insecticide resistance in *Anopheles* and increased ecological awareness has limited their effectiveness. New techniques of vector control are, thus, urgently required, and the creation of transgenic mosquitoes incapable of transmitting malaria shows promise (Crampton *et al.*, 1992). Finding ways to spread the genes involved in natural resistance to *Plasmodium* throughout wild populations of mosquitoes (Collins *et al.*, 1997; Richman *et al.*, 1997) is also under investigation. The recent discovery that an essential factor (xanthurenic acid) is required for the formation of gametes inside mosquitoes (Billker *et al.*, 1998) may provide a novel method for breaking the transmission cycle.

1.2. Malaria - the current situation

Malaria is still one of the most prevalent infectious diseases in the world. According to W.H.O, malaria is estimated to kill between 1.5 and 2.7 million people every year. Another 300 to 500 million people have the disease, and one third of all humanity lives in zones where they risk catching it. Ninety percent (90%) of the cases occur in Africa where malaria accounts for up to a third of all hospital admissions and up to a
quarter of all deaths of children under the age of 5. Two-thirds of the remaining 10% of the cases are concentrated in just six countries. These are, in decreasing order of prevalence, India, Brazil, Sri Lanka, Vietnam, Colombia and the Solomon Islands.

1.3. Life cycle of the malaria parasite within a mammalian host

*Plasmodium* species have a complex life cycle involving passage through both invertebrate and vertebrate hosts (Figure 1.1). In the case of human malaria, man is the intermediate host of *Plasmodium*, with the sexual stages of the life cycle taking place in the body of the mosquito. Infection is initiated by the bite of an infected female *Anopheles* mosquito and the injection into the bloodstream of sporozoite stages contained in the insect's saliva. Shortly after injection, the sporozoite arrives in the liver and enters hepatocytes. There, the parasite undergoes growth and asexual reproduction (schizogony) to form a large pre-erythrocytic schizont. Rupture of the infected cell releases thousands of merozoites, which then penetrate erythrocytes to initiate the erythrocytic cycle. After a period of growth, during which the parasite passes through the ring and trophozoite stages, there is schizogony - production of the erythrocytic schizont, with division of cytoplasm and nucleus to form a relatively small number of merozoites. The infected cells then burst, releasing merozoites. Repeated cycles of schizogony and red blood cell infection ensure that a high proportion of available erythrocytes become infected.

As well as repeated cycles of asexual division some parasites switch to the sexual phase of the cycle. Some merozoites invade erythrocytes and produce male or female gametocytes rather than a further generation of schizonts. No further development can occur unless the cells containing gametocytes are taken up by a suitable mosquito. Production of male gametes and fertilization of the female gamete occurs in the stomach of the mosquito. The motile zygote (ookinete) then penetrates the stomach wall where it develops into an oocyst on the outer lining. Within the oocyst there is repeated division (sporogony) to produce large numbers of sporozoites and, when the oocyst ruptures, the sporozoites migrate anteriorly in the insect to enter the salivary glands, for injection into another host.
Figure 1.1. Life cycle of *Plasmodium* spp. There are two distinct phases: sexual (in the mosquito) and asexual (in the mammalian host). An infected mosquito feeds and injects sporozoites into the bloodstream. Sporozoites invade hepatocytes and undergo growth and asexual division to form exoerythrocytic schizonts. Infected liver cells burst and release merozoites into the blood stream. These invade erythrocytes, develop through ring and trophozoite stages to form the multi-nucleate mature erythrocytic schizonts that then burst and again release merozoites that invade new erythrocytes. After invasion, some merozoites undergo sexual differentiation to form gametocytes and these are taken up by feeding mosquitoes to produce female and male gametes in the mosquito gut. After fertilisation, the motile zygote (ookinete) penetrates the stomach wall and an oocyst develops. Inside the oocyst repeated divisions (sporogony) produce many sporozoites. These migrate to and mature in the insect salivary glands from where they can be transmitted during feeding.
Growth of the parasite in the erythrocyte is fuelled partly by the intake and digestion of erythrocyte cytoplasm. Haemoglobin is digested, to obtain amino acids, but the haem is stored in the form of an insoluble pigment. When the infected erythrocytes bursts, pigment and other metabolic products resulting from parasite maturation/replication are released into the circulation, inducing a number of changes in the host, of which fever is the most characteristic.

The pathological consequences of malaria infection are primarily associated with the destruction of erythrocytes, which can lead to anaemia and vascular collapse. In *P. falciparum* malaria, the most dangerous form, there is an additional primary cause of pathology. Erythrocytes containing mature parasite forms (schizonts) are sequestered in capillaries of internal organs. If the brain is involved, the damage caused by blockage of capillaries can lead to the potentially fatal condition known as cerebral malaria. Other pathological manifestations arise as secondary consequences of infection, for example the nephrosis that follows immune complex-mediated damage to the kidney.

1.4. Parasite-host interaction

1.4.1. Innate resistance

In human populations, which are genotypically and phenotypically very diverse, innate resistance may play a role in host specificity observed in various *Plasmodium* parasites. The innate factors which operate at the level of the erythrocytic cycle include the presence or absence of appropriate receptors for the parasite (Miller *et al.*, 1975), the age of the red blood cell (Craik, 1920), enzyme activity (Allison, 1960), and haemoglobin type (Allison, 1954). Thus, individuals whose erythrocytes lack the Duffy blood group determinant on their surface membrane appear to be completely resistant to infection with *P. vivax* (Miller *et al.*, 1976; Miller *et al.*, 1978). Closely related primate species such as *P. knowlesi*, *P. inui* and *P. cynomolgi* are similarly unable to invade Duffy negative cells *in vitro*. Innate resistance, however, is not always absolute and genetically determined factors can play a role in reducing the severity of infection and improve survival. In some rodent and human malarias the age of the red blood cell is a particularly critical factor: although certain parasites invade both mature and immature red blood cells (e.g. *P.*
chabaudi and *P. falciparum* (Chwatt, 1948; Jarra *et al.*, 1989) others invade reticulocytes preferentially (e.g. *P. vivax*) (Kitchen, 1938). It was first proposed by Haldane (1949) that the high frequencies of thalassemia and other hemoglobinopathies observed in malaria endemic areas resulted from the fact that these genetic disorders offered some degree of protection against the parasite. Further evidence that malaria could select for certain phenotypes and determine gene frequencies in human populations was presented by Allison (Allison, 1954) who demonstrated that the sickle-cell trait exists in a state of "balanced polymorphism" in malaria endemic areas where the disadvantage of a defective hemoglobin was balanced by the improved survival that it conferred to the carrier. Other red cell disorders implicated in innate resistance against *P. falciparum* include haemoglobin C (HbC), HbE, glucose-6-phosphate dehydrogenase (G6PD) deficiency, ovalocytosis and spherocytosis (reviewed in Pasvol *et al.*, 1982). Even though most studies on innate resistance have been concerned with the ability of host erythrocyte variants to inhibit parasite growth, innate resistance can potentially affect the growth of the parasite at any stage in its life cycle. More recently, the influence of HLA class I and II variation on susceptibility to *P. falciparum* infection was described (Hill *et al.*, 1991). These authors have shown that an HLA class I antigen, HLA-B53, and an HLA class II haplotype, DRB1*1302-DQB1*0501, are associated with reduced susceptibility to severe malaria and that both these haplotypes are much more frequent in West Africans than other racial groups. Further work has shown that, among malaria-immune Africans, HLA-B53-restricted cytotoxic T lymphocytes recognized a conserved nonamer peptide from liver-stage-specific antigen-1 (LSA-1) (Hill *et al.*, 1992). This work suggested a possible molecular basis for the association of HLA-B53 with resistance to severe malaria.

**1.4.2. Immune mechanisms used by the host**

It is generally accepted that immunity to malaria is gradually acquired by individuals living in endemic areas as judged by both changes in the degree of parasitaemia and the severity of clinical symptoms in relation to age. Although some of the population of such regions are particularly susceptible to the more virulent or severe aspects of the disease (such as cerebral malaria, severe anaemia and renal failure), the majority (who are almost continuously exposed to reinfection during the transmission seasons) will survive initial infections and gradually reach a stage where they are clinically immune. It takes some
time (several years) and many infection episodes before this level of immunity develops and even then individuals are often only protected against clinical illness. Although the acquired immune response changes a potentially lethal pathogen into little more than a commensal, it is not usually capable of complete parasite elimination or sterile immunity. This type of immunity, called “premunition”, does not prevent the more or less continuous presence of parasites in the blood during the transmission seasons: indeed this may actually be required in order for an effective level of immunity to be maintained. The mechanisms involved in premunition immunity are not yet clearly understood, neither is the identity of the plasmodial antigens that elicit it.

The parasite is (potentially) exposed and vulnerable to immune attack at many stages of its life cycle. All stages are associated with the expression of potentially immunogenic antigens; as a sporozoite, before and while infecting the hepatocyte, as an exo-erythrocytic trophozoite/schizont, as a merozoite prior to erythrocyte invasion and while developing in the erythrocyte. For the purpose of this review I shall focus on asexual erythrocytic stages.

Originally, immunity to malaria was thought to be mediated solely by phagocytic cells. Many of the earlier workers (over 100 years ago) such as Laveran, Golgi, Metchnikoff, Marchiafava and Celli recognized the importance of phagocytes, and particularly of macrophages, in defence against malaria (see Taliaferro et al., 1937). Experimental data on how macrophages participate in the host response to malaria were first obtained by Taliaferro and Cannon (Cannon et al., 1931; Taliaferro et al., 1936) in studies using canaries and monkeys as experimental hosts. Their, mainly histological, observations indicated that there was an initial rise in parasitaemia during which parasites were slowly phagocytosed, primarily in the spleen and to a lesser extent in the liver and bone marrow. As the parasitaemia approached high or acute levels, a "crisis" occurred in which the parasitaemia first peaked and then sharply dropped, with the remaining parasites appearing pyknotic (crisis forms). The infected erythrocytes appeared to be held in the cords of Billroth in the spleen. Within the following few days the parasites were ingested increasingly more aggressively as the parasitaemia was controlled. Upon reinfection, with homologous parasites, infected erythrocytes were phagocytosed within hours rather than days. In addition, in some primate malarias, crisis also resulted in
changes in the length and synchrony of the malaria cycle as well as in the number of merozoites released per schizont (Taliaferro et al., 1944).

At the end of the 1930's, Coggeshall and Kumm (Coggeshall et al., 1937) demonstrated that immunity was not only cellular but that a serum factor, presumed to be antibody, played a significant role in controlling the parasitaemia. The effectiveness of this arm of the immune response was demonstrated in the now classical experiment of Cohen and colleagues (Cohen et al., 1961), who showed that the transfer of purified Immunoglobulin G (IgG) from clinically immune Gambian adults into infected children was followed by a rapid decrease in parasitaemia. Cohen's results have been confirmed by numerous other studies in mice and man (Diggs et al., 1969; Phillips et al., 1972; McDonald et al., 1980; Freeman et al., 1981). With experiments that use total sera it is difficult to quantify the contribution that antibody makes. However, the observation that a monoclonal antibody (IgG3 isotype) protects mice from both lethal and non-lethal lines of Plasmodium yoelii, demonstrated that in some situations antibody alone can be sufficient to confer protection (Majarian et al., 1984).

Protective immunity has a high degree of specificity in man (Jeffery, 1966). Using rodent malaria infections of inbred CBA/Ca mice, other authors have examined the specificity of immunity in single and mixed infections (Jarra et al., 1985; Jarra et al., 1989b; Snounou et al., 1989; Snounou et al., 1992). As early as days 11 or 12 in P. c. chabaudi AS or P. y. yoelii A infections i.e. during the "crisis" period, when parasite numbers are falling dramatically, protective responses, effective in the clearance of parasites, are relatively ineffective against a superimposed infection with parasites of a heterologous parasite line or species. Thus, the direct action of the immune effector mechanisms on the parasite, are both species- and largely line-specific.

The nature of the lymphocyte subset(s) responsible for transferring protection has been investigated and a role for T lymphocytes in protection has been established. Some protection has been transferred, in some systems, by immune T cells alone, but transfer of B and T cells was found to provide optimal protection against infection with P. berghei in rats (Brown et al., 1976a). Meding and Langhorne (1991) also noted that both B cells and T cells were essential for the effective transfer of immunity to P. c. chabaudi in severe combined immunodeficient (SCID) mice. Some authors concluded that T cells probably mediate their effect by working as helper cells for antibody production (Brown
et al., 1976a and b; Gravely et al., 1976; McDonald et al., 1980a and b). Others have suggested T cells exert their effect(s) against the malaria parasites through different mechanisms (Cavacini et al., 1990). Weinbaum (1976) and Roberts (1979), showed that BALB/c mice \( \mu \)-suppressed by treatment with heterologous anti-\( \mu \) chain antibody, resulting in a B-cell deficiency, are not able to control a (normally self-limiting) primary \( P. y. yoelii \) infection. In contrast, such mice are able to control primary infections with \( P. chabaudi adami \) (Grun et al., 1981). In this case however, and in contrast to normal mice, the \( \mu \)-suppressed animals were unable to completely eliminate the parasites. This observation was interpreted by these authors to suggest that a T cell dependent, non-antibody but cell-mediated immune mechanism(s) could mediate resistance to blood-stage malaria. Similar results were obtained by Weid and Langhorne (1993); \( P. c. chabaudi \) AS infected mice which are B cell deficient can apparently control acute infection, but require these cells to eventually clear the infection. However, van der Heyde and collaborators (1994), using genetically B-cell depleted mice (J\( _{1/2} \)D mice) demonstrated that such animals are able to control \( P. c. adami \) and \( P. c. chabaudi \) CB but not \( P. c. chabaudi \) AS infections. Although B cell deficient mice can resolve an acute infection with these parasites, these animals develop a chronic low-grade parasitaemia, thus indicating a role for antibody in final or complete elimination of the parasites.

Specific protection through cytotoxic action by Class I MHC-restricted CD8\(^+\), cytotoxic, T cells on infected erythrocytes seems unlikely since erythrocytes lack the organization normally required to produce the appropriate presentation of internally derived foreign peptides (Brown, 1991). However, MHC class I has been detected in small amounts on the surface of mouse reticulocytes and in greater amounts on \( P. yoelii \) infected reticulocytes (Jayawardena et al., 1983). This finding has been discussed as a possible way by which cytotoxic CD8\(^+\) cells could recognize and lyse intraerythrocytic malaria parasites (Jayawardena et al., 1983; Mogil et al., 1987), although the apparent protection conferred by CD8\(^+\) cells, observed by Mogil and collaborators, has been explained by others as contamination of the CD8\(^+\) preparation with a small number of CD4\(^+\) cells (Vinetz et al., 1990). CD8\(^+\) cells are thought to be involved with immunity to the exo-erythrocytic stages of malaria (Schofield et al., 1987). It is clear that the outcome of acute infection is multifactorial and reflects several interactions between many different components of the immune system.
1.4.3. Parasite evasion of malaria immunity

While capable of producing severe disease and death, particularly in the young, *Plasmodium* parasites characteristically develop stable long-lasting relationships with their vertebrate hosts, often in the absence of clinical disease. These chronic malarial infections that evolve after the host overcomes initial acute attacks clearly demonstrate the host's capacity to restrict overwhelming parasite proliferation; however, they also demonstrate the parasite's capacity to evade host-defense mechanisms. This is approaching an ideal state of parasitism.

Probably the most hostile environment that *Plasmodium* species have to face is the bloodstream of the vertebrate host where they are exposed to an array of resistance, and immune defence mechanisms. Four separate developmental stages have the capacity to survive in both naive and immune individuals; sporozoites, asexual intraerythrocytic parasites, merozoites and gametocytes. In the case of invasive stages (sporozoites and merozoites), direct exposure to plasma lasts only for minutes and seconds, respectively. The brevity of this extracellular period is itself capable of promoting the survival of a proportion of parasites. By contrast, infected erythrocytes have to circulate for between 24 and 72 hours, depending on the parasite species, in order to complete the asexual division cycle. Those parasites which differentiate into gametocytes need to survive for up to a week to permit development to occur. Once inside the erythrocyte it would seem that the parasite is in an ideal position, shielded from antibodies and multiplying in a cell unable to process and present antigens to T cells. However, because they reside in a cell which has very little metabolism apart from glycolysis, intra-erythrocytic parasites have to bring with them all the biosynthetic functions that they require as well as the capacity to import and export metabolites from, and to, the surrounding plasma.

It is clear now that modifications in the erythrocyte surface must accompany parasite growth and development (Figure 1.2). The advantage to the parasite of an intracellular existence now becomes a problem due to the fact that parasite growth modifies the erythrocyte to the extent that it becomes a target for a specific immune response to the infected cell surface. Functional changes must therefore include the ability of the parasite to survive in the face of a vigorous immune response.
Figure 1.2. Schematic representation of the membrane skeleton of a normal erythrocyte (A) and a *P. falciparum* infected erythrocyte (B) to show the alterations believed to occur in the membrane of a *P. falciparum*-infected erythrocyte. Knob protrusions are presumably localized to the spectrin-actin-protein 4.1 junction. Localization of PfEMP-1 into knobs might occur by association with knob-associated histidine-rich protein (KAHRP), a major and essential component of knobs. Receptors in cryptic regions of band 3 become exposed at the surface with the intracellular growth of the parasite inside the erythrocyte. Each PfEMP-1 molecule consists of several Duffy binding-like (DBL) domains, a cysteine-rich interdomain region (CIDR), a transmembrane (TM) domain and an acidic terminal segment (ATS). Other parasite proteins thought to be present at the knob region are mature parasite infected erythrocyte surface antigen (MESA) and *P. falciparum*-erythrocyte membrane protein 3 (PfEMP-3).
- **Sequestration.** Regardless of the state of immunity, only immature asexual forms or mature gametocytes are usually found circulating in the blood of humans infected with *P. falciparum*. The reason for this was first described just prior to the turn of the century by Bignami and Bastianmelli who noted the sequestration of erythrocytes in patients infected with *P. falciparum* (cited in Berendt et al., 1994). Subsequently, it was realized that erythrocytes parasitized by certain plasmodial species acquired adhesive properties which allowed them to adhere to the endothelia of blood vessels in various tissues of the infected host (Howard et al., 1984). Sequestration has been considered to be of great significance in man as it seems to be the key initiating event of cerebral malaria, the principal cause of death in severe disease. *P. falciparum* is the only human malarial parasite which sequesters. It has been suggested that sequestration allows parasites to avoid passage through the spleen at a stage in their life cycle when they would be most susceptible to destruction by immune mechanisms activated in the spleen. Furthermore, the parasite’s preference for the relatively anoxic environment of the deep vasculature in order to complete its development to schizogony may be facilitated by sequestration (Scheibel et al., 1979). None of these possible advantages has been formally proved, although parasites cultured *in vitro* prefer hypoxic conditions and the presence or absence of the spleen modulates sequestration (David et al., 1983). Characterization of the molecular mechanisms of sequestration has principally involved the use of *in vitro* models. Several cell types were assayed as targets for the adhesion of infected erythrocytes. Three cell types were found to mediate adhesion: human umbilical vein endothelial cells (HUVEC), amniotic epithelial cells and the C32 amelanotic melanoma cell line. The first studies were done using the melanoma cell line as it was very easy to culture (Udeinya et al., 1985). These studies showed that adhesion (i) like sequestration *in vivo*, is parasite stage-specific; (ii) occurs at the same time as the expression of several parasite-derived neo-antigens; and (iii) presents itself concurrently with the development of morphological alterations called knobs (only visible by electron microscopy) in the infected cell membrane. These consist of an electron-dense cup-shaped structure that underlies a protrusion of the erythrocyte membrane that follows the contour of the cup. Electron microscopy of autopsy sections of the brains of cerebral malaria patients also shows knobs as attachment points of infected erythrocytes to endothelial cells (MacPherson et al., 1985). *In vitro*, cultured parasites that express knobs usually bind to
endothelial or melanoma cells (Raventos-Suarez et al., 1985). Thus, it was believed that cytoadherence is dependent on the formation of knobs at the surface of the infected erythrocyte. However, exceptions are known and it is recognized that parasite lines exist which do not display knobs and yet are cytoadherent (Biggs et al., 1989). Several parasite proteins are present at the cytoplasmic face of the knob structure, including the knob-associated histidine-rich protein (KAHRP) (Culvenor et al., 1987; Pologe et al., 1987). More recently, the disruption of the gene encoding KAHRP has shown that this protein is essential for knob formation. These knobless parasites did not lose their adhesive properties in static assays although adhesiveness was reduced 5-fold when tested under flow conditions that mimic those of post-capillary venules. Furthermore, the knobless cells that adhere were 3 times easier to detach. This suggests that knobs have a role in the adhesion of infected cells in the haemodynamic environment of the microcirculation (Crabbe et al., 1997).

Working with the melanoma cell line model, Barnwell and collaborators (1985) showed that a monoclonal antibody (OKM5) inhibited adhesion of infected erythrocytes. The antigen recognized by this antibody is now known to be the platelet and endothelial membrane glycoprotein CD36. Furthermore, these and other authors showed that (i) adhesion to cells correlates with the expression of CD36, (ii) infected erythrocytes can adhere to purified protein, (iii) soluble CD36 blocks the adhesion of infected erythrocytes to melanoma cells (Barnwell et al., 1989; Ockenhouse et al., 1989) and (iv) transfection of cDNA encoding CD36 into heterologous cells induces the ability to bind infected cells and this too is blocked by anti-CD36 monoclonal antibodies (Berendt et al., 1989; Oquendo et al., 1989). Using purified platelet glycoprotein thrombospondin (TSP) it was shown that this protein could also work as an adhesion receptor (Roberts et al., 1985).

Thus, although there was a significant appreciation of the adhesive properties of *P. falciparum* there was still no correlation between the cerebral form of the disease and an increased binding capacity of infected erythrocytes. A panel of parasite isolates taken from children with cerebral and non-cerebral malaria was therefore examined (Marsh et al., 1988). There was no difference in the binding between the 2 groups when melanoma cells were used. This result could have two explanations: (i) the difference between severe and non-Severe disease is a function of the host instead of the infected erythrocyte or (ii) the melanoma assay was unable to detect critical differences between the binding of
different parasites. HUVEC cells express a phenotype believed to be more representative of the endothelium in vivo and it was found that the ability of parasite isolates to adhere in the melanoma model did not correlate with adhesion to HUVEC. Only a minority of field isolates adhere well to HUVEC and, in subsequent studies designed to find new receptors on endothelial cell surface for infected erythrocytes, it was shown that intercellular adhesion molecule-1 (ICAM-1) was responsible for a novel adhesion pathway (Berendt et al., 1989). The fact that not all parasite strains can bind to ICAM-1 may help explain the relative rarity of severe disease. It was also shown that parasites could be selected to bind to endothelial leucocyte adhesion molecule-1 (formerly ELAM-1, now designated E-selectin) and vascular cell adhesion molecule-1 (VCAM-1) in vitro (Ockenhouse et al., 1992).

Infected erythrocyte-endothelial cell adhesion interactions must be capable of occurring with sufficiently rapid kinetics to capture moving cells from shear flow and be of great enough affinity eventually to anchor the cell (Wick et al., 1991; Nash et al., 1992). Studies of adhesion of infected eryocytes under shear flow have indicated that different receptors may have complementary roles in modulating adhesion in microvessels. Initial interaction at high wall shear stress may be of a rolling type, mediated by ICAM-1 or other receptors, with immobilization and stabilization occurring via CD36 and/or TSP (Cooke et al., 1994).

More recently, chondroitin sulphate A (CSA) has been proposed to have a role in the adhesion of infected red blood cells to the human placenta (Fried et al., 1996). Maternal malaria is a major cause of pregnancy-related complications in endemic areas (McGregor, 1984). After years of exposure to malaria, women that have acquired immunity to P. falciparum, become uniquely susceptible to malaria infection when they get pregnant. Maternal malaria was attributed for a long time to the generalised immunosuppression associated with pregnancy (Mutabingwa, 1994). However, susceptibility to infection is markedly lower in multigravid women than in primigravid women so immunosuppression per se cannot entirely explain the phenomenon (McGregor et al., 1983). The placenta is a preferential site for sequestration of infected erythrocytes and can experience high parasite densities while the peripheral circulation is free of parasites (Walter et al., 1982). Infected erythrocytes obtained from the placenta consistently bind to CSA but not to other receptors indicating that a parasite
subpopulation, infrequently occurring in non-pregnant hosts, preferentially sequesters and multiplies in the placenta. With successive pregnancies, women develop increasing immunity to this parasite subpopulation reducing the frequency and severity of malaria infection (Fried et al., 1996). However, the involvement of CSA protein seems not to be limited to cytoadhesion in the placenta. The injection of CSA protein into *P. falciparum*-infected *Saimiri* non-pregnant monkeys was shown to reverse cytoadhesion of infected erythrocytes (Pouvelle et al., 1997).

- **Rosetting.** Erythrocytes infected with mature stages of the malaria parasite are not only capable of adhering to endothelial cells but also to two or more uninfected erythrocytes to form rosettes (David et al., 1988). Like sequestration, rosetting may be protective for the parasite because (i) it may conceal the infected cell and protect it against parasite specific antibodies and/or phagocytic cells, or (ii) by juxtaposing infected and uninfected cells, rosetting may facilitate the transfer of merozoites into uninfected cells within the rosette especially under conditions of flow (Wahlgren et al., 1989). Though none of these possible advantages has been formally proven and recent data has suggested that rosetting does not play a role in the invasion of uninfected erythrocytes (Clough et al., 1998) this phenomenon receives lots of attention because it has been very strongly associated with severe malaria (Carlson et al., 1990; Treutiger et al., 1992; Rowe et al., 1995). It has been suggested that rosetting, by causing aggregates of cells, leads to microvasculature obstruction (Kaul et al., 1991). Sera from malaria immune individuals has been shown to disrupt rosettes (Carlson et al., 1990). Rosetting seems a heterogeneous phenomenon in which a number of erythrocyte ligands, such as ABO blood group (Carlson et al., 1992), CD36 (Handunnetti et al., 1992), soluble ligands, such as immunoglobulins (Scholander et al., 1996) and sulphated glycoconjugates (Rowe et al., 1994) differentially modify the degree of rosette formation in different isolates. More recently, it was reported that complement-receptor 1 (CR1) on erythrocytes plays a role in the formation of rosettes and that CR1 polymorphisms in Africans, that influence the interaction between uninfected and infected erythrocytes, may protect against severe malaria (Rowe et al., 1997).

- **Antigenic variation.** Antigenic variation is the process by which a clonal parasite population can switch its antigenic phenotype and the parasite, by expressing a new variant type, is able to escape the host response directed to the previous antigenic
variant type. This important mechanism of immune evasion was first described for *Trypanosoma* and in this case is the result of the expression of alternative genes for the variant surface glycoprotein (VSG) (reviewed by Borst *et al.*, 1998; Cross *et al.*, 1998). The first suggestion of antigenic variability by a malaria parasite during the course of an infection in the blood was by Cox (1959) with *P. berghei* in mice. However, antigenic variation in malaria was only demonstrated by Brown & Brown (1965) in studies using the schizont-infected cell agglutination (SICA) test with *P. knowlesi* infection of pre-immunized rhesus monkeys. Each recrudescence of parasitaemia was associated with a distinct parasite variant type which stimulated the production of a specific agglutinating antibody, but which was not agglutinated by antibodies to early variants. The agglutinating antibodies themselves have no *in vivo* protective function, but there is, in addition, production of opsonizing variant-specific antibodies which are parasiticidal (Brown *et al.*, 1974). Since this work, antigenic variation has been also observed in *P. c. chabaudi* (McLean *et al.*, 1982a), *P. falciparum* (Hommel *et al.*, 1983) and *P. fragile* (Handunnetti *et al.*, 1987). Variation in *P. c. chabaudi* has been detected directly by measuring the degree of immunity transferred against recrudescence populations by immune sera taken from mice after earlier parasitaemias. In these experiments the starting inoculum was a mosquito passaged and cloned population. This was used to ensure a high degree of uniformity in the parasites and to reduce the possibility that recrudescence(s) were due to the multiplication of antigenically-distinct forms present initially (McLean *et al.*, 1982a). Proof of antigenic variation in *P. falciparum* was achieved *in vitro*. Thus, in the absence of selective pressure, a culture derived from a clone could be sub-cloned to give parasite lines which were serologically distinct from the parent line (Biggs *et al.*, 1991; Roberts *et al.*, 1992). In contrast with other organisms studied so far, *Plasmodium* variant antigens are expressed on, or at, the host's cell (erythrocyte) surface rather than on the organism itself and were named variant erythrocyte surface antigens (VESA). In *P. knowlesi*, they could be labelled by surface-specific iodination, were trypsin sensitive, insoluble in non-ionic detergents, and of high molecular mass that varied between serotypes (Howard *et al.*, 1983).

In the *P. knowlesi* model, serial passage of cloned organisms in splenectomized animals gave rise to parasites which no longer expressed VESA on their surface. On transfer back to an intact animal, these parasites were capable of re-expressing VESA
(Barnwell et al., 1983). Data from the rodent parasite *P. c. chabaudi* in inbred mice (Gilks et al., 1990) and from *P. fragile* in its natural host (Handunnetti et al., 1987) indicated that the same held true for these parasites and it became clear that loss of VESA expression was accompanied by loss of sequestration. Very similar behaviour was evident when *P. falciparum* was passaged in splenectomized *Saimiri* monkeys. Modulation of the adhesive phenotype occurred whereby all stages of the parasite appeared in the peripheral circulation and was accompanied by a change in the surface antigenicity. As with *P. knowlesi*, transfer back to an intact animal resulted in a return to normal sequestration (Barnwell et al., 1983; David et al., 1983; Hommel et al., 1983). Therefore, it appeared that there is co-modulation of VESA expression and adhesive behaviour. Using a modification of the SICA assay, it was shown that erythrocytes infected with *P. falciparum* express a serologically diverse parasite-specific antigen (Marsh et al., 1986; Forsyth et al., 1989). In vitro surface labeling studies then showed that *P. falciparum* expresses a parasite line-specific molecule at the surface of the erythrocytes equivalent in biochemical properties to the VESA antigen described for *P. knowlesi* (Leech et al., 1984). This protein was named *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP-1) (Howard et al., 1988). It was noted that *P. falciparum* frequently lost its capacity to bind to endothelium when placed in in vitro culture and this loss was always accompanied by the loss of PfEMP-1 expression. Selection of clones for particular adhesive characteristics was accompanied by changes in the molecular mass of PfEMP-1 (Magowan et al., 1988). The antigenic switching was also accompanied by size changes in PfEMP-1, as demonstrated by immunoprecipitation (Biggs et al., 1991), as well as changes in cytoadherence specificity (Biggs et al., 1992; Roberts et al., 1992). Furthermore, all these features were shown to be phenotypic since they could all be features of a line derived from a single clone (Roberts et al., 1992). The studies in this area suggest that a single molecule, PfEMP-1 expresses variant-specific epitopes and also mediates adhesion to endothelium in *P. falciparum* infections (Leech et al., 1984; Howard et al., 1988; Howard et al., 1989; Biggs et al., 1991; Biggs et al., 1992; Roberts et al., 1992). Evidence that the two functions reflect properties of the same molecule has been presented in two sets of experiments (Baruch et al., 1996; Gardner et al., 1996). These workers demonstrated a role for PfEMP-1 in both antigenic variation and adhesion properties, however some authors concluded that PfEMP binds to CD36, TSP and
ICAM-1 (Baruch et al., 1996) while others concluded that PfEMP-1 mediates adhesion to CD36 and ICAM-1 but suggested that an invariant molecule may mediate adhesion to TSP (Gardner et al., 1996). In fact, results from other laboratories have provided evidence that other receptors on the surface of the infected erythrocyte may mediate adhesion to endothelium. One of the molecules proposed was Band 3. This molecule aggregates during erythrocyte senescence, revealing novel epitopes that are involved in the removal of aging erythrocytes from the circulation. It has been shown that Band 3 undergoes further changes during intra-erythrocytic parasite growth, which are related to adhesive properties (Crandall et al., 1991; Crandall et al., 1993; Crandall et al., 1994a and b). Another molecule that has been correlated with adhesive properties is sequestrin (Ockenhouse et al., 1991). A group of parasite encoded low-molecular-mass proteins called rosettins have been described as potential parasite ligands in the surface of the infected-erythrocyte for the rosetting phenotype (Helmby et al., 1993). More recently, it has been reported that PfEMP-1 is a parasite ligand for rosetting in a P. falciparum clone (Rowe et al., 1997).

A number of genes that encode the different PfEMP-1 variants have been cloned and named var (for variation) (Baruch et al., 1995; Su et al., 1995). The switches in expression of P. falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes (Smith et al., 1995). Switching from one variant type to another is thought to occur at high frequency, and it was estimated that 2% of parasites switched to a new variant type in every erythrocytic cycle in vitro (Roberts et al., 1992). There has been some speculation was made that the rapid switching of the expression of PfEMP-1 may be a consequence of the sub-telomeric position of var genes on almost all P. falciparum chromosomes, a particularly unstable chromosomal area where genes are frequently reorganized (Thompson et al., 1997). However, more recently, it was shown that var gene expression is not linked to a specific expression site, but rather occurs in situ, irrespective of a particular chromosomal location - subtelomeric or central (Scherf et al., 1998).

- **Antigenic diversity.** The malaria parasite exists as a series of genetically and biologically distinct species and lines. Isolates of parasites collected in the wild from the natural host may represent (often) complex mixtures of different lines and/or species (Thaithong et al., 1984). Analysis of cloned parasite lines, derived from such isolates by
dilution or by micro-manipulation, has allowed the recognition and definitive characterization of parasite bio-diversity. Antigenic diversity can be defined as the expression of antigenically different forms of parasite gene products by parasites of different genotype within any given isolate. That this heterogeneity is maintained by genetic recombination, in addition to mutation, was shown in *P. c. chabaudi* (Walliker et al., 1975) and later with *P. falciparum* (Walliker et al., 1987). Gametocytes of two different clones of *P. falciparum* were mixed and mosquitoes fed on those mixtures. Genetic recombination was unequivocally demonstrated when the resulting progeny were screened in chimpanzees.

As a result of intensive studies, such as comparison of DNA and amino acid sequences from well characterized parasite genes and gene products from different parasite isolates, there is now a considerable body of data on diversity among antigens of *Plasmodium*. In contrast to antigenic variation that has only been noted in erythrocyte surface-expressed late stage-specific antigen (PfEMP-1), antigenic diversity occurs in many different antigens, e.g., the circumsporozoite protein - CSP - in mature sporozoites (Jongwutiwes et al., 1994); merozoite surface antigens - MSP-1, MSP-2 and MSP-3 (Miller et al., 1993; Farnert et al., 1997; McColl et al., 1997), rhoptry antigens (Howard et al., 1996), infected erythrocyte-associated antigens - RESA and MESA (Contreras et al., 1988; Forsyth et al., 1989) and secreted parasite antigen (S-antigen) in erythrocytic stages (Saint et al., 1987); gamete/gametocyte antigens and zygote/ookinete antigens in sexual stages (Foo et al., 1991). The diversification of antigens may effectively confer on the parasite the ability to infect a host which has only experienced infections with parasites of a different genotype as the immunity directed at these parasites is species and strain specific. So, antigenic diversity represents an advantage to the parasite (Petersen et al., 1989).

**Immunosuppression.** McGregor and collaborators (1962) first reported that the antibody response to immunization with tetanus toxoid was greater in children receiving malaria prophylaxis than in untreated children. Children with acute *falciparum* malaria have depressed responses to a variety of vaccine antigens (e.g., meningococcal C polysaccharide, *Salmonella typhi* O antigen and typhoid and poliomyelitis vaccines) while responses to other antigens remain unaffected (e.g., *S. typhi* H antigen, measles, and diphtheria, pertussis, tetanus and Bacille Calmette-Guerin vaccines) (Greenwood et al.,
1972; Williamson et al., 1978; Bradley-Moore et al., 1985). Immunosuppression may extend to specific responses to malarial antigens since decreased lymphocyte responsiveness to plasmodial antigens in vitro has been observed by several groups in subjects undergoing acute infection with *P. falciparum* (Ho et al., 1986; Riley et al., 1988; Webster et al., 1988). The cellular basis for immunosuppression in acute falciparum malaria remains to be established but several factors have been pointed out as possible modulators of the immune reactivity (e.g., overloading of macrophages with haemazoin (Morakote et al., 1988), or a defect in IL2 synthesis (Lelchuk et al., 1984). Immunosuppression, probably, only has a minor role in clinically immune adults. However, it may be important during acute infections.

- **Others.** Studies with *P. falciparum* merozoite surface protein-1 (MSP-1) have revealed a mechanism by which the parasite can avoid the action of protective antibodies. These protective antibodies act by completely inhibiting the secondary processing of MSP-1, a step suggested to be necessary for the invasion of the erythrocyte by a merozoite. However, it was shown that a group of antibodies, which do not affect processing, interfere with the activity of the protective antibodies by competing with them for binding to the merozoite surface (Patino et al., 1997).

Recently, it was shown that two *P. falciparum* parasite lines cooperate in order to escape the host's immune response. CTLs restricted by the commonest HLA class I molecule in the Gambia (HLA-B53), often recognize a polymorphic epitope in a variable region of the circumsporozoite (CS) protein. Two allelic variants (cp26 and cp29) are CTL epitopes that bind HLA-B53 and are octamers that differ only at the second amino acid position. However, in the CTL responses to these malaria epitopes, cp29 was able to antagonize cp26-specific CTLs, and cp26 was able to antagonize cp29-specific CTLs (Gilbert et al., 1998).

### 1.5. Aims of the project

It is clear that acute infection, severe disease and some mortality are characteristic of malaria in many parts of the world. It is also clear that the induction of potent protective immune responses, particularly against the asexual forms of the parasite, can
lead to control of parasitaemia. In individuals that survive the acute phase of their infection, malaria tends toward chronicity and many factors, such as antigenic variation and diversity, may contribute significantly to this situation. In order to intervene most effectively in this dynamic host-parasite interaction we need to know much more about the different factors involved.

For obvious reasons, research involving malaria in the human host is limited and clinicians have an ethical obligation to initiate treatment when an infection is detected. The distribution of malaria parasites in mammalian hosts is unusual. Although about 40 mammalian species have been recorded as potential hosts' overall very few orders are parasitised. Thus, a remarkable concentration of different malaria parasites occurs in just a few mammalian species with the majority being found in primates. The reason for this apparent host-parasite restriction is not known but it causes serious problems in research since it makes the study of human parasites \textit{in vivo} extremely difficult. \textit{P. falciparum} can be cultured and parasites are often adapted to, and subsequently maintained \textit{in vitro}. However, they represent selected populations and differ in certain aspects from the original isolate. Although several species of non-human primates are partly susceptible to infection with human plasmodia, the ethical, methodological and economical problems involved in using these animals are very controversial. The more so, because almost all susceptible non-human primates are rare, or even endangered species. On occasion, however, parasites of primates in their natural host can be a valuable alternative, but the use of such models is difficult to justify on a regular basis. The difficulties of working with primate malaria parasites have meant that numerous species of rodent and avian malaria parasites have been widely used as laboratory models to study the biology of plasmodia. The best animal model should be relevant to: (i) human malaria and (ii) the study of the parasite biology at the cellular and molecular level. The main criticism of rodent and avian models is their uncertain phylogenetic relationship with human plasmodia. Despite certain similarities between \textit{P. falciparum} and avian plasmodia (eg. \textit{P. relictum} and \textit{P. gallinaceum}) differences in their life cycles, vectors and in the host's immune system limit the usefulness of avian malaria parasites as models. Maintenance of the rodent malarial \textit{in vivo} is not difficult and, for most laboratories, rodent malaria parasites therefore continue to be the logical first choice for studies on the basic biology of \textit{Plasmodium} in the vertebrate host.
Careful selection of the host-parasite combination in rodent malaria can provide model(s) capable of providing much useful information. *P. c. chabaudi* is found naturally in the thicket rat (*Thamnomyys rutilans*) in Central Africa. Natural infection may last for up to two years in wild-caught animals (though it is not known if this is with marked recrudescences or not) and chronically infected animals are well (Garnham, 1966). Transfer of the infection to laboratory mice occurs readily without adaptation. Carter and Walliker (Carter *et al*., 1975) have extensively characterised this parasite with material obtained from wild-caught thicket rats and established a number of cloned lines. Cyclical transmission is straightforward for most clones using laboratory bred *Anopheles stephensi*. Infection of adult mice of several inbred strains with relatively low numbers of certain *P. c. chabaudi* parasite lines produces an initial acute synchronous primary parasitaemia. After peaking (with approximately 40% of the erythrocytes parasitized) at around 9-11 days post infection in most mice (this time is host/parasite line dependent) the parasitaemia is reduced from acute to low or subpatent levels by day 16-18 during a period referred to as "crisis". This period is characterized (in many of the experimental models examined so far) by the appearance of morphologically abnormal/damaged parasites in the blood and reflects the first potential turning point in the course of a malaria infection in a range of hosts including man. After a short period of low grade or subpatent parasitaemia, *P. c. chabaudi* recrudesces producing a second, generally lower, peak of parasitaemia. It forms a chronic infection without liver-stage relapses, but with marked recrudescences (McLean *et al*., 1982b). The parasites from this second peak were shown to be antigenically different from those in the first peak (McLean *et al*., 1982). After a few more days of patent parasitaemia, the recrudescence is itself controlled and the mice are effectively immune. *P. c. chabaudi* has many similarities with *P. falciparum* in man. It is capable of invading both mature and immature red cells (Jarra *et al*., 1989a). A synchronous erythrocytic infection occurs, although the erythrocytic cycle is only 24 hours, unlike the 48 hour cycle of *P. falciparum*. Mature schizonts are found circulating in the peripheral blood, unlike the almost total absence and sequestration of *P. falciparum*. However, some peripheral withdrawal of schizonts does occur and the parasites sequester, particularly, in the liver (Gilks *et al*., 1990). It is believed that both *P. falciparum* and *P. c. chabaudi* AS, use antigenic variation and sequestration to evade the immune response of their respective hosts. In *P. falciparum* the same molecule (PfEMP-1
encoded by the var gene family) that is involved in adhesion undergoes antigenic variation. In *P. chabaudi*, it was shown that the two phenomena are also intimately linked suggesting the involvement of a common molecule (Gilks et al., 1990) that it is still unknown. However, it should be noted that knobs are not a feature of *P. chabaudi* schizont-infected erythrocytes. Parasite-line specific immunity occurs (Jarra et al., 1985; Jarra et al., 1989b) and the same seem to be truth to *P. falciparum*. None of the other rodent malarias are as similar to *P. falciparum* in these respects.

Antigenic variation and sequestration are believed to be the principal mechanisms by which some *Plasmodium* spp. escape the host’s immune response and sequestration is thought to be a major factor in cerebral malaria. In addition, it has been suggested that the immune response against the variant antigen PfEMP-1 play an important role in the acquisition of immunity to malaria (Bull et al., 1998) and might be determinant for parasite population structure (Gupta et al., 1994). The complete understanding of the role and regulation of these phenomena in *Plasmodium* infections is a crucial goal in malaria research.

The overall aim of this project is to investigate the host-parasite dynamics, specifically in terms of the parasite and host cell binding properties and the parasite line specificity of the anti-parasite immune response. In this context, the identification of the protein(s) in the surface of the erythrocytes infected with *P. c. chabaudi* AS that is involved in antigenic variation/sequestration in this organism will receive special attention.
CHAPTER TWO

Material and Methods

2.1. Parasites

*P. c. chabaudi* (AS & CB) originally isolated from a natural host (*Thamnomys rutilans*) in the Central African Republic was supplied by Professor D. Walliker (WHO Registry of Standard Strains of Malaria Parasites, Department of Animal Genetics, University of Edinburgh, Scotland) as a cloned, recently mosquito transmitted line (Carter, 1978; Walliker, 1983; Jarra et al., 1985). Parasite reference populations (stabilates) were prepared at no more than six syringe passages from the original material supplied by Professor Walliker. Blood stage parasites were collected from mice under ether by cardiac puncture in 200 µl heparinized Kreb's saline pH 7.4 (see Jarra and Brown, 1985) containing 0.2% glucose (KGS) and 50 U ml⁻¹ of heparin. Analar glycerol to 10% (v/v) was added and, after mixing, the stabilates were snap frozen in liquid nitrogen and maintained in this medium or stored at -60°C.

2.2. Infection of experimental animals

Cryopreserved parasite stabilates were thawed at 37°C and promptly syringe passaged into 16-18 g (4 week old) male Specific Pathogen Free bred (NIMR) CBA/Ca mice intraperitoneally (i.p.). Water and 41B diet (Quest Nutrition - Grain Harvesters Ltd, Canterbury, UK) were provided *ad libitum* (Gilks et al., 1989). Parasitaemia was monitored by light microscopy of air dried, methanol fixed, thin tail-blood smears stained with 10% Giemsam's stain in phosphate buffer pH 7.4 (Garnham, 1966). When the parasitaemia reached approximately 20%, infected blood in KGS + 25 U ml⁻¹ heparin was syringe passaged into further 16-18g or 23-27g (11-15 week old) recipient mice, depending on the experiment. Parasites were then syringe passaged routinely every 5-7 days for no more than 10 passages before the line was discarded. Where exact numbers
of infected erythrocytes were to be injected, i.e. for the preparation of infection plasma or in cases where the infection was followed, total erythrocyte counts were made using a haemocytometer (Neubauer chamber) and the parasitaemia determined from a thin smear. Blood was then diluted in KGS + 20% normal CBA/Ca mouse serum (NMS) to the appropriate concentration of parasitised erythrocytes and then injected in 200 μl per mouse i.p.. Where appropriate, parasitaemia counts are presented as plots of the log geometric mean parasitaemia against time post-infection.

2.3. Preparation of plasma from *P. c. chabaudi* AS infected and normal mice

A group of mice were infected with 5x10⁴ *P. c. chabaudi* AS or CB parasitized erythrocytes i.p., and their parasitemia monitored on thin smears. A control group was injected i.p. with KGS. On days 11 to 12 post-infection (approximately 1-2 days after peak parasitaemia), mice from both the infected and the control (sham infected) groups were bled into 100 μl KGS + 25 U ml⁻¹ heparin at 4°C. The blood was then centrifuged (2000 x g at 4°C for 1-2 min), the plasma removed and snap frozen in liquid nitrogen. APP was obtained from the infected mice and NP was obtained from the sham infected mice. In some experiments, plasma samples obtained on sequential days of infection were used.

2.4. Plasma fractionation

A Protein G Sepharose 4 Fast Flow column was washed at 4°C with 300 ml of phosphate buffered saline (PBS) followed by 30 ml of binding buffer (0.02 M Na₂HPO₄/NaH₂PO₄, pH 7.0). Six ml of NP or APP were loaded onto the column followed by 30 ml of binding buffer. The unbound non-IgG fraction was collected and the IgG-rich protein retained on the column was then eluted with 30 ml of 1.0 M glycine-HCl, pH 2.7. Both fractions were dialyzed against PBS and concentrated to the initial volume of plasma using Centriprep concentrators of 3 kDa cutoff (Amicon).
2.5. Maintenance of a rat endothelial cell line

Rat endothelial cells were obtained from Dr. Ann Ager (NIMR). The cells were grown in RPMI 1640 medium supplemented with NaHCO₃, Hepes, Sodium pyruvate, penicillin, streptomycin and monothioglycolate (RPMI 1640 COMP/ABS; Gibco BRL, USA) and 10% Fetal Calf Serum (FCS). Cells were passaged routinely every 4 days for no more than 20 passages before the line was discarded. Cells were stored in RPMI 1640 COMP/ABS supplemented with 10% FCS and 10% Dimethylsulphoxide (DMSO) at -70°C.

2.6. Electron microscopy

The brains, kidneys, livers and spleens were removed from 3 *P. c. chabaudi* AS infected CBA/Ca mice at days 7, 10 and 13 post-infection and were immersion fixed in 2% glutaraldehyde (v/v) plus 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (SCB), pH 7.2 for 12-48 hours. Following fixation, the samples were washed in SCB for 15 minutes, post-fixed in 1% osmium tetroxide in SCB for 90 minutes and washed again as before. Samples were en-bloc stained with 1% aqueous uranyl acetate for 90 minutes, dehydrated through a graded ethanol series (50% - 5 minutes, 75% - 10 minutes, 90% - 10 minutes and absolute ethanol - 3 times for 30 minutes) to propylene oxide (2 times for 20 minutes) and embedded in araldite cyziz. Sixty nm sections were placed on a grid, stained with saturated uranyl acetate in 75% ethanol (30 minutes), washed in distilled water and stained in Reynold's lead citrate (5 minutes). Samples were viewed in a Jeol CX100 electron microscope. The same protocol was used for TEM studies of parasitized erythrocytes in peripheral circulation.

2.7. Immunofluorescence analysis of expression of adhesion molecules

Rat endothelial cells were plated out in 6 well plates (Nunc, USA) at a density of 10⁵ cells per well and grown for 72 hours in the presence or absence of 100 U ml⁻¹ IFN-γ. Cells were detached with 0.1% EDTA for 5 minutes and washed twice (7000 x g for 2
Material and methods

Minutes) in PBS containing 1% (w/v) BSA (PBS-B). Samples containing $1 \times 10^6$ cells in 50 μl of PBS-B were first incubated for 30-45 minutes at 4°C with 25 μg ml$^{-1}$ of anti-rat ICAM-1 and anti-VCAM-1 monoclonal antibodies and then washed twice in PBS-B, as described above. Anti-mouse IgG FITC conjugated antibody (Sigma) was added to a final concentration of 25 μg ml$^{-1}$ and the incubation continued for a further 30 minutes at 4°C after which the samples were again washed in PBS-B. The final pellet was resuspended in 1 ml of PBS-B and each sample was then analyzed in a Becton-Dickinson FACStar Plus fluorescence activated cell sorter (FACS) using the Innava 90 Argon-ion laser at 488 nm. Using pre-determined counting parameters for forward scatter and fluorescence intensity a total of 10000 events was recorded. The data were analyzed using FACSplot analysis software.

2.8. In vitro cythoadherence assays

Rat endothelial cells were plated into 8 well chamber slides (Nunc, USA). The cells were seeded at a density of $2 \times 10^4$ cells/well and grown for 72 hours in the presence or absence of 100 U ml$^{-1}$ IFN-γ. The culture medium was then replaced with 200 μl of erythrocytes from a P. c. chabaudi AS infection (2% hematocrit) resuspended in binding medium (RPMI 1640, 25 mM HEPES, 25 μg ml$^{-1}$ gentamycin, 2 mM glutamine, 2 mM CaCl$_2$, 10% FCS). The slides were incubated at 37°C for 90 minutes with gentle resuspension of the settled erythrocytes every 10-15 minutes. After incubation, unbound cells were rinsed off by washing three times with PBS with gentle rotation. The adherent infected erythrocytes were fixed with 1% glutaraldehyde in PBS for 30 minutes at 37°C. The slides were stained with 1% Giemsa for 30 minutes, and the number of infected erythrocytes per 100 target cells determined by light microscopy (Figure 2.1).
Figure 2.1. Schematic representation of the *in vitro* adhesion assays and visualization of the quantification method. Endothelial cells (EC) were seeded out onto chamber slides and allowed to grow for 72 hours in the presence or absence of IFN-γ. The adhesion assay was performed at 37°C for 90 minutes after 200 μl of parasitized erythrocytes (PE) was added to the endothelial cells. The results were quantified as Relative Adhesion (%) = number of bound infected erythrocytes per 100 target endothelial cells.
In some experiments the adhesion of infected erythrocytes to soluble recombinant human CD36 (a gift from Dr. Chris Newbold, Oxford University) was measured. In these assays, triplicate spots of CD36 (12.5 ng ml\(^{-1}\)) or PBS-B (as a control) were added to plastic dishes and incubated at 4°C overnight after which the dishes were washed to remove unbound protein. Then, the dishes were blocked with PBS-B at 4°C overnight and subsequently washed three times. Then 7.5 μl of \(P. \text{ c. chabaudi}\) AS infected blood at 2% hematocrit was added and incubated for 1 hour at 37°C. The subsequent washes, fixation and Giemsa staining were performed as before. The number of bound cells per spot were counted.

### 2.9. ELISA

\(P. \text{ c. chabaudi}\) AS infected CBA/Ca mice (40% parasitaemia) were bled into KGS/heparin at +4°C to provide parasitized erythrocytes. The blood was passed through a CF11 cellulose powder (Whatman, UK) column to remove leukocytes and eluted cells washed 3 times with KGS by centrifugation at 750 x g for 15 minutes at 4°C. The final cell pellet was resuspended to 5 ml in KGS and approximately 3 μl of 10% (w/v) saponin in KGS was added to lyse the erythrocyte membranes. After centrifugation at 18000 x g for 5 minutes at 4°C the supernatant was removed, the pellets lysed with 3-4 volumes of a detergent buffer (1% Triton X-100 (w/v), 5mM EDTA, 100 mM Tris-HCl (pH 8.0)), centrifuged at 20000 x g for 5 minutes at +4°C and the supernatant retained. Each well of a 96-well microtitre plate was coated with 50 μl of an appropriate predetermined dilution of this parasitized erythrocyte antigen in coating buffer (100 mM Tris-HCl, pH 8.0). After overnight incubation at 4°C the plates were washed with Tris buffered saline + 0.05 Tween 20 and blocked with BLOTTO (Tris buffered saline, 0.05% Tween 20 (v/v), 5% dried milk) for 2 hours at room temperature. Serial dilutions in BLOTTO of NP and plasma from different days of a \(P. \text{ c. chabaudi}\) AS infection were then added. After incubation at room temperature for 2 hours the plates were washed 3 times as described above. An anti-mouse Ig affinity purified biotin conjugated antibody (The Binding Site Co., UK) was then added (in different experiments conjugated antibodies directed against different mouse Ig isotypes were also used). The plates were incubated for 40 minutes at
37°C, washed 6 times and exposed to Streptavidin conjugated alkaline phosphatase (Sigma) for 40 minutes at 37°C. The plates were then washed as above, incubated for 15 minutes with substrate buffer (10 mM diethanolamine (pH 9.5), 0.5 mM MgCl₂), exposed to the substrate p-nitrophenyl phosphate (Sigma) and read using a Titertek Multiskan MCC/340 reader with a 405 nm filter. All samples were tested in triplicate. Background values were obtained by using 96-well microtitre plates where only coating buffer (without antigen) was added and the binding of antibody from each sample was assayed as described above. These values were subtracted from the respective values obtained for anti-parasitized erythrocyte binding.

2.10. Surface-immunofluorescence-antibody assay

Parasitized erythrocytes and erythrocytes from CBA/Ca infected and non-infected mice respectively were washed 3 times by centrifugation at 1500 x g in KGS containing 1% (w/v) BSA (KGS-B) and 1 µl of the final cell pellet placed in a 0.5 ml tube. Ten µl of homologous APP (from a P. c. chabaudi AS infection), heterologous APP (from a P. c. chabaudi CB infection) or NP diluted in KGS-B (1:4 to 1:10) was added and the cells gently resuspended before incubation at 37°C for 30-60 minutes. Anti-mouse IgG FITC conjugated antibody (Sigma) was added to a final concentration of 25 µg ml⁻¹ and the incubation continued for 15-30 minutes at 37°C. Between each addition and following the final incubation, the cells were washed twice with ice cold KGS-B. The final pellet was resuspended in 1 ml of KGS-B and each sample was then analyzed in a Becton-Dickinson FACStar Plus fluorescence activated cell sorter (FACS) as previously described (see 2.7)

2.11. Phagocytosis assay

Macrophages were obtained from CBA/Ca mice by peritoneal lavage with 3-4 ml/mouse of ice cold RPMI 1640 medium supplemented with 5 U ml⁻¹ of heparin. An erythrocyte-free leucocyte preparation of 1-2 x 10⁶ cells ml⁻¹ was usually obtained. One ml of this suspension was added to Leighton tissue culture tubes (Wheaton) containing
coverslips, gassed with 7% CO₂/5% O₂/88% N₂ and incubated for 1-2 hours at 37°C. Cells non-adherent to the coverslips were removed by washing the coverslips in situ with RPMI 1640 medium. One ml of RPMI 1640 containing 10% (v/v) FCS was added to the adherent cells, the tubes gassed as above and incubated at 37°C for 2 hours. During this period aliquots of \textit{P. c. chabaudi} AS parasitized erythrocytes (1 x 10⁸ infected erythrocytes per ml containing mature trophozoites/schizonts at 40-50% parasitemia) or uninfected erythrocytes in RPMI 1640 + 10% FCS (v/v) were incubated with homologous APP, heterologous APP, NP, KGS or homologous APP (Protein G fractionation) fractions at 37°C for 1 hour at 70-80 rpm (G24 environmental incubator shaker - Edison, USA). The cells were then pelleted by centrifugation, washed 3 times with RPMI 1640 + 10% FCS (v/v), resuspended in the same medium and 1 ml added to each Leighton tube. After a further incubation step of 1 hour, non-adherent or non-ingested parasitized erythrocytes and erythrocytes were removed by gentle aspiration and the coverslips washed three times with 1 ml of PBS. Non-ingested, but adherent parasitized erythrocytes and erythrocytes were then lysed by a brief (20 sec) treatment with cold distilled water, followed by an additional wash with 1 ml of PBS. This treatment had no detrimental effects on the integrity of the macrophages. The adherent cells were then fixed with methanol, stained with Giemsa's reagent and the numbers of internalized parasitized erythrocytes and erythrocytes assessed by light microscopy. The results are presented as the Phagocytic Index (percentage of macrophages with infected or uninfected erythrocytes inside) for the different treatments and are further broken down by the numbers of cells inside individual macrophages (Figure 2.2).

\textbf{2.12. Statistical analysis}

These were performed by using Student's \( t \) test, with \( P < 0.05 \) considered to be significant.
Figure 2.2. Schematic representation of the phagocytosis assays and visualization of the quantification method. Mouse peritoneal macrophages were plated onto coverslips and allowed to adhere for 1-2 hours at 37°C. Non-adherent cells were washed out and the assay performed by incubating parasitized erythrocytes (PE) in the macrophage culture for 1 hour at 37°C with gentle rotation. Non phagocytosed cells were washed out and the results were quantified as the Phagocytic Index (%) = Percentage of macrophages (Mϕ) with infected erythrocytes inside.
2.13. Isolation of free parasites

Infected CBA/Ca mice were bled into KGS/heparin at 4°C and washed 3 times with ice-cold PBS by centrifugation (750 x g, 4°C). The blood was depleted of leukocytes by passage through CF11 columns (see 11.9) and then washed three times with PBS by centrifugation at 750 x g for 5 minutes at 4°C. The final erythrocyte pellet was resuspended into 5 volumes of PBS and, approximately, 3 μl of 10% white saponin (w/v) (Sigma, UK) in KGS was added, to lyse the erythrocytes. After centrifugation at 18000 x g for 5 minutes at 4°C, the supernatant was removed and the dark area of the pellet (parasites) removed and stored at -20°C or used immediately as in the case of chromosomal DNA preparation.

2.14. Preparation of parasite DNA and plasmids

2.14.1. Preparation of intact P. c. chabaudi AS chromosomal DNA

Free parasites were obtained as described before and the pellet dispersed in PBS to obtain a concentration of $10^{10}$ cells ml$^{-1}$. Then, 1 ml of 50 mM EDTA (pH 7.5) was added and gently mixed with the parasites. After equilibration at 42°C, the parasite suspension was mixed with 2 ml of 1.25% low melting temperature SeaPlaque agarose (FCM BioProducts, USA), prepared in PBS, at the same temperature. The mixture was poured into pre-cooled moulds (20 minutes at -20°C), and set at 4°C. The strips of agar were then removed using a spatula, cut into approximately 7.5 mm pieces and incubated in lysis buffer (0.5 M EDTA, 0.1 M Tris-HCl, 1.0% Sarcosyl (w/v), 1 mg ml$^{-1}$ Proteinase K) at 37°C overnight. The incubations was repeated two times more but with reduced Proteinase K concentration of 500 μg ml$^{-1}$ and 250 μg ml$^{-1}$, respectively. Finally, the blocks were washed 5 times using distilled water and stored in 0.5 M EDTA, pH 7.5 at 4°C.
2.14.2. Preparation of *P. c. chabaudi* AS genomic DNA

Parasites were purified as described above. Frozen parasites were thawed on ice, fresh parasites were used directly. The parasite pellet was diluted in 10 volumes of proteinase K buffer (10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 150 mM NaCl, 0.5% SDS (w/v), 100 µg ml⁻¹ proteinase K) and incubated in a 50 ml Falcon tube for 3 hours at 56°C. The solution was then extracted once with 1 volume phenol, twice with 1 volume phenol/chloroform and once with 1 volume chloroform. For each extraction the phases were mixed gently on a rotator for 5 minutes to avoid shearing high molecular mass DNA. After each extraction the phases were separated by centrifugation in Corex tubes (10 min, 10000 x g, 4°C). The upper aqueous phase was transferred each time with a wide-bore pipette into a fresh tube. After the second phenol/chloroform extraction the interphase was clear and free of precipitated material. One tenth volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol were added to the aqueous solution, kept for 1 hour at -20°C and then centrifuged (20 minutes, 4000 x g, 4°C). The pellet was washed with 2 ml of 70% ethanol and centrifuged as before. The supernatant was removed completely and the pellet was air dried for 30 minutes. To the semi-dry pellet 0.9 ml TE (1 mM EDTA, 10 mM Tris-HCl, pH 7.5) was added. The tube was incubated in a 37°C water bath overnight to dissolve the DNA. RNase A was then added (final concentration 10 µg ml⁻¹) and incubated for 1 hour at 37°C. The solution was extracted once with 1 volume phenol/chloroform, twice with 1 volume chloroform and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of absolute ethanol for 1 hour at -20°C and centrifuged (15 minutes, 12000 x g, 4°C). The pellet was washed with 1 ml of 70% ethanol, centrifuged (5 minutes, 12000 x g, 4°C) and air-dried for 30 minutes. To the semi-dry pellet 250 µl of 10% TE was added and left in a 37°C water bath overnight. Subsequently the DNA was stored at 4°C.

2.14.3. Small scale preparation of plasmid DNA (minipreps)

The isolation of plasmid DNA was performed using the S.N.A.P. Miniprep Kit (Invitrogen, Netherlands) according to the manufacturer's instructions.
2.14.4. Large scale preparation of plasmid DNA (maxipreps)

The isolation of plasmid DNA was performed using a Plasmid Maxi Kit (Qiagen, Germany) according to the manufacturer’s instructions.

2.15. Preparation of parasite RNA

Parasites were purified as described before. Frozen parasites were thawed on ice, fresh parasites were used directly. The parasite pellet of one mouse was lysed in 2 ml of RNA STAT-60 (BioScience, UK) by repetitive pipetting and kept 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Following this, 400 µl of chloroform was added, the sample shaken vigorously for 15 seconds, incubated at room temperature for 2-3 minutes and centrifuged at 12000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, kept at room temperature for 5-10 minutes and centrifuged at 12000 x g for 10 minutes at 4°C. Following the centrifugation, the supernatant was removed and the RNA pellet washed with 75% ethanol by centrifugation at 7500 x g for 5 minutes at 4°C. Finally, the RNA pellet was briefly air-dried and dissolved in water.

2.16. Restriction enzyme digestion of DNA

The digestion of free genomic DNA and plasmids was performed exactly as directed, in suitable volumes of the buffer specified by the manufacturer of the restriction enzymes.
2.17. DNA and RNA electrophoresis

2.17.1. Electrophoresis of DNA species

DNA species smaller than 20 kilobase pairs (Kbp) were resolved in 1-3% agarose gels in TBE buffer (100 mM Tris-HCl, 100 mM sodium borate, 5 mM EDTA, pH 8.0), using conventional submarine tanks.

2.17.2. Electrophoresis of RNA species

*P. c. chabaudi* AS total RNA was resolved in 1% agarose and 2.2 M formaldehyde in formaldehyde gel-running buffer (20 mM MOPS (pH 7.0), 0.8 mM sodium acetate, 1 mM EDTA (pH 8.0)). RNA samples (10-20 µg in 4.5 µl) were prepared by adding 2.0 µl of 5 X formaldehyde gel-running buffer, 3.5 µl formaldehyde and 10.0 µl of formamide. Samples were incubated for 15 minutes at 65°C, then chilled on ice, centrifuged for 5 seconds and 2 µl of sterile diethyl pyrocarbonate (DEPC) treated formaldehyde gel-loading buffer (50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue, 0.25% xylene cyanol FF) added to each sample. The gel was pre-run for 5 minutes at 5 V cm⁻¹ in formaldehyde gel-running buffer prior to the samples being loaded. Electrophoresis was performed at 3-4 V cm⁻¹ with constant recirculation of the buffer until the bromophenol migrated approximately 8 cm.

2.18. Removal of DNA species from agarose gels

DNA species were removed from agarose gels using a QIAquick gel Extraction Kit (Qiagen, Germany) according to the manufacturer’s instructions.

2.19. cDNA production

2.19.1. Single stranded cDNA

Single stranded DNA was obtained using a cDNA Cycle Kit (Invitrogen, Netherlands) according to the manufacturer’s instructions.
2.19.2. **Double stranded cDNA 3' and 5' RACE analyses**

Double stranded cDNA was obtained from total RNA using a Marathon cDNA Amplification Kit (Clontech, USA) according to the manufacturer’s instructions. The double-stranded cDNA was ligated to RACE adaptors and each RACE (Rapid Amplification of cDNA Ends) reaction was performed by using a primer designed to the adaptor and another primer designed based on DNA sequence of the gene of interest (Figure 2.3, pg 53).

2.20. **Polymerase chain reaction (PCR)**

2.20.1. **PCR with degenerate primers**

Degenerate primers were designed based on conserved regions of the known sequences from the *P. falciparum var* genes. All oligonucleotides were synthesised by the Sequencing and Synthesis Service at NIMR and were provided in 35% ammonia solution. One tenth volume of 3 M sodium acetate and 2.5 volumes of cold absolute ethanol were added, the mixture kept at -20°C for 30 minutes, the oligonucleotide pelleted by centrifugation, washed with 80% ethanol and dissolved in a small volume of distilled water. The oligonucleotide concentration was determined spectrophotometrically by measuring the absorbance at 260 nm (1A₂₆₀ unit of single-stranded DNA = 33 μg ml⁻¹). PCR was typically performed in a 50 μl volume and contained in addition to the template DNA (50 ng): 2.5 mM each dNTP, 2.5 μg of each pair of oligonucleotide primers, 2.5 units of Taq polymerase and 1x enzyme buffer. This buffer was different from reaction to reaction since the Opti-Prime PCR Optimization Kit (Stratagene) was used, in order to select the optimal buffer for each set of primers. In every experiment the PCR cycle conditions were:

1. **DENATURE** 95°C 5 minutes
2. **ANNEAL** X°C 1 minute
3. **AMPLIFY** 72°C 1 minute
4. **DENATURE** 94°C 20 seconds
5. **ANNEAL** X°C 1 minute
### 2.20.3. PCR of long DNA fragments

This PCR was performed with LATaq enzyme and in the presence of approximately 200 ng of intact chromosomal *P. c. chabaudi* AS DNA, 25 pmol of each primer, 20 pmol dNTPs and 150 pmol MgCl$_2$ in a 50 μl reaction volume.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
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<tbody>
<tr>
<td>1. DENATURE</td>
<td>94°C</td>
<td>1 minute</td>
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<tr>
<td>2. DENATURE</td>
<td>98°C</td>
<td>20 seconds</td>
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<tr>
<td>3. ANNEAL</td>
<td>60°C</td>
<td>1 minute</td>
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<tr>
<td>4. AMPLIFY</td>
<td>68°C</td>
<td>5 minute</td>
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30 cycles from step 2 to step 4

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<th>Step</th>
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<tr>
<td>5. EXTENDED EXTENSION</td>
<td>72°C</td>
<td>10 minutes</td>
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### 2.20.3. Reverse transcriptase PCR (rt-PCR)

This PCR was performed using the Advantage cDNA PCR kit (Clontech, USA) according to the manufacturer's instructions.
**Figure 2.3.** Schematic representation of the RACE reactions. Double stranded cDNA was ligated to RACE adaptors and then each reaction was performed by using gene-specific and adaptor-specific primers to amplify 5' and 3' ends.
2.21. Cloning PCR products

PCR bands were extracted as referred to above. The ligation was done with pMOSBlue T-vector (Amersham, Life Sciences) or TA cloning vector (Invitrogen, USA) according to the manufacturer's methodology that eliminates the requirement for additional sequences or restriction sites to be incorporated into PCR primers. The ligation products were transformed into pMOSBlue competent cells (Amersham, Life science) by heat shock or into DH10B cells by electroporation and following the instructions supplied with the cells. The pMOSBlue vector as well as the pCR 2.1 vector allows for blue-white screening, with recombinant colonies appearing white when plated on X-gal and IPTG indicator plates. PCR products greater than 3 Kb were cloned into pCR 2.1-TOPO (Invitrogen, Netherlands) vector and transformed into TOP10 One Shot cells (Invitrogen, Netherlands) by heat shock.

2.22. Southern and Northern blotting

2.22.1. Transfer of resolved DNA species

Genomic DNA was digested and electrophoresed as described above and blotted onto nylon membranes (Qiabrane, Qiagen, USA) using 20X SSC (3 M NaCl, 3.3 M Na_3C_6H_5O_7, pH 7.0) overnight after the DNA had been denatured (30 minutes - 0.5 N NaOH, 1.5 M NaCl) and neutralised (45 minutes - 1 M Tris-HCl (pH 7.4), 1.5 M NaCl). The membranes were then washed in 2X SSC and the DNA cross-linked under UV light.

2.22.2. Transfer of RNA species

RNA was electrophoresed as described before and transferred from the gel to a nylon membrane (Qiabrane, Qiagen, USA) using DEPC-treated 20X SSC overnight after the gel has been rinsed in DEPC-treated water, incubated for 20 minutes in 0.05 N NaOH and incubated for 45 minutes in DEPC-treated 20X SSC. After the overnight transfer, the membranes were washed in 2X SSC and the DNA cross-linked under UV light. Water and SSC were DEPC-treated by adding 0.1% DEPC overnight at room temperature and then autoclaved.
2.22.3. Preparation of radiolabelled DNA species for use as gene probes

All the probes were labelled with [α-32P]dATP using a Prime-It II Random Primer Labeling Kit (Stratagene) as described by the manufacture's instructions.

2.22.4. Hybridization with radiolabelled probes

DNA blots were prehybridized for at least 1 hour at 60°C in Southern hybridization solution (5 X Denhardt's; 5 X SSC; 0.1% SDS (w/v); 100 μg ml⁻¹ denatured nonhomologous DNA) and then the probe was added to the same buffer and hybridized overnight at 60°C. RNA blots were prehybridized for at least 1 hour at 42°C in Northern hybridization solution (50% deionized formamide; 5 X Denhardt's; 5 X SSC; 0.1% SDS; 100 μg ml⁻¹ denatured nonhomologous DNA) and then a labelled probe was added to the same buffer and hybridized overnight at 42°C.

2.23. Sequencing

DNA sequencing was performed by automated sequencing using the ABI 377 as directed by the manufacturer using primers in the vectors or primers flanking the inserts.

2.24. Expression of recombinant proteins

A 300 bp cDNA fragment was cloned into pTrcHis vector C (Invitrogen, USA) at the restriction sites Pst I and Hind III. This construct was transformed into DH10B electrocompetent cells. The fusion tag comprised an N-terminal hexa-histidine (His)₆ followed by the Xpress™ (X) epitope (LYDDDDK). This (approximately 2.5 kDa) fusion polypeptide was N-terminal to the recombinant protein coded by the cDNA clone. Expression was induced when the OD₆₀₀nm of a transformed bacterial culture was 0.6 by adding IPTG to a final concentration of 1 mM followed by incubation at 37°C with vigorous shaking. In a preliminary induction assay, 1 ml samples were collected at one hour intervals for 7 hours to select for the highest level of expression. Optimum
expression of the protein was found to be at 5 hours post-induction. This time was used in all subsequent expression work.

2.25. Fusion protein purification

The recombinant protein contained 6 consecutive histidine residues which allowed the use of a nickel-nitrilotriacetic acid (Ni-NTA, Qiagen) metal-affinity chromatography matrix for selective purification. Bacterial extracts were solubilised in lysis buffer (50 mM Na₂HPO₄/NaH₂PO₄, 0.5 M NaCl, 10 mM imidazole, 8 M Urea, pH 7.4). The samples were then centrifuged at 15000 x g at 4°C for 1 hour, filtered through a 0.45 μm filter and applied to the Ni-NTA column that had been pre-equilibrated with lysis buffer. Following this, the Ni-NTA column was washed with lysis buffer (2 volumes). The recombinant (His)₆-protein was eluted with 200 mM imidazole in lysis buffer. Protein elution was monitored using a UV light detector set at 280 nm. A second step of purification using gel filtration on a Sephadex G50-SF column was performed to further purify the recombinant (His)₆-protein.

2.26. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were solubilised by boiling in SDS sample buffer (0.125 M Tris-HCl (pH 6.8); 20% glycerol (v/v); 4.6% SDS (w/v); 0.01% bromophenol blue (w/v)) with or without 0.1 M DTT. Proteins were fractionated on homogeneous polyacrylamide gels by the method of Laemmli (1970). The percentage acrylamide of the gel varied depending on the size of the proteins that were analysed, and is described in the legend of each figure. Electrophoresis was performed using a Bio-Rad dual vertical slab gel electrophoresis cell. Following electrophoresis, proteins were visualised by either staining with Coomassie Brilliant Blue (Sigma, UK) or by Western blotting (see below).
2.27. Protein concentration determination

Protein concentration was estimated using the BCA Protein Assay Kit (Pierce), with bovine serum albumin as the standard protein.

2.28. Immunization of inbred mice with fusion protein

Eight CBA/Ca inbred mice were immunized with 50 μg of purified recombinant protein in Freund's complete adjuvant i.p. The mice were then boost 3 times via the same route with the same amount of protein in Freund's incomplete adjuvant at four weekly intervals and then bled for serum.

2.29. Western Blot

Proteins separated by SDS-PAGE were transferred to nitrocellulose Extra Blotting membranes (Sartorius, U.K.) using a TE Series Tranphor Electrophoresis Unit at either 80 V for 3 hours or 30 V overnight. A transfer unit is defined as a stack of anodal filter paper, membrane, gel and cathodal filter paper. All the elements are immersed in Transfer buffer (48 mM Tris-HCl, pH 8.3, 39 mM glycine, 20% methanol) during the transfer. Following the transfer, the nitrocellulose was carefully removed and incubated in blocking buffer (5% BSA (w/v), 0.05% Tween 20 (w/v) in PBS) for at least 1 hour at room temperature to block free non-specific binding sites. The blot was probed with primary antibody for 1 hour at room temperature. The antibody and the concentration was different from experiment to experiment and is referred to in each legend for the respective figures. The membranes were washed 3 times (15 minutes each) in wash buffer (0.05% Tween 20 (w/v) in PBS) and incubated with the second goat anti-mouse IgG-HRP conjugated antibody for 1 hour at 1/1000 dilution in blocking buffer. The membranes were washed three times as before and once finally with only PBS. The Western immunoblots were visualised using chloronapthol (0.06% chloronapthol (w/v), 20% methanol (v/v), 0.03% H₂O₂ in PBS).
2.30. Immunofluorescence on fixed \textit{P. c. chabaudi} AS parasitized erythrocytes

Smears of \textit{P. c. chabaudi} AS infected blood were prepared and stored at -70°C inside closed plastic bags in the presence of dessicant. When necessary they were removed from -70°C and incubated at room temperature within the closed bag for 20-30 minutes. Slides were fixed in cold acetone (-20°C) for 30 seconds and 10 µl of the first antibody in a pre-determined dilution in PBS-1% BSA was added and the slide incubated in a humidified box at 37°C for 1 hour. The slides were then washed 3 times (5 minutes each) in PBS and 5 µl of goat anti-mouse IgG FITC conjugated antibody diluted 1/100 in PBS with 1% BSA was added. The slides were incubated in a humidified box at 37°C for 30 minutes, washed as before and mounted with Citiflour. The slides were then observed by fluorescence microscopy.
CHAPTER THREE

Cytoadherence of \textit{P. c. chabaudi} AS Infected Erythrocytes: Characterization of an \textit{in vivo} Model for Sequestration of \textit{P. falciparum}-Infected Erythrocytes

3.1. Introduction

Erythrocytes infected with mature stages of \textit{P. falciparum} are capable of adhering to endothelial cells (sequestration), uninfected erythrocytes (rosetting) and infected erythrocytes (agglutination).

The adhesion of infected erythrocytes to the endothelium of deep vascular beds of different organs (sequestration) may contribute to the pathology observed in one of the commonest causes of death in \textit{P. falciparum} infections - cerebral malaria. However, the full range of factors involved in the pathophysiology of cerebral malaria is poorly understood. Although in the past numerous clinical studies and pathological descriptions have been published, the majority were either not quantitative or not sufficiently rigorous about defining the clinical parameters of cerebral malaria (Berendt \textit{et al.}, 1994). More recent studies, have shown an association between massive cerebral sequestration and cerebral malaria (MacPherson \textit{et al.}, 1985; Pongponratn \textit{et al.}, 1991). It is known that PfEMP-1 exposed at the surface of infected erythrocytes mediates adhesion to endothelial cells through interactions with several adhesion molecules displayed on endothelial cells (e.g. CD36, TSP, ICAM-1 and PECAM-1). Many of these are TNF- and/or IFN-\(\gamma\)-inducible molecules and these cytokines are known to be overexpressed during infection. Thus, cerebral malaria may well result from an interaction between the host and parasite in which upregulated levels of adhesion receptors in the brain direct the sequestration of infected erythrocytes to that site (Berendt \textit{et al.}, 1994). However, some authors still argue that sequestration is not essential for cerebral malaria (Clark \textit{et al.}, 1994).
Furthermore, sequestration is thought to be an important mechanism by which the malaria parasite escapes the host immune response by avoiding passage through the spleen at a stage in its life cycle when it would be most susceptible to destruction by immune mechanisms activated in this organ. However, any real advantage of this evasion mechanism has never been formally proven for *P. falciparum*. Furthermore, other *Plasmodium* species infecting man (e.g. *P. vivax*) produce successful infections without sequestering, but how this is achieved remains unresolved.

Some studies of cytoadherence have used non-human primate hosts such as Rhesus monkeys infected with *P. coatney* (Aikawa et al., 1992) or *Saimiri sciureus* infected with *P. falciparum* (Gysin et al., 1992). However, there are many ethical and methodological problems associated with the use of these animals.

Rodent malaria models can be used as experimental systems for the study of sequestration and offer numerous advantages over the use of primate hosts, including the ready availability of congenic animals, the opportunity for precise measurements of pathology in groups of animals at all stages of the disease, and availability of animals with genetic abnormalities. Most *in vivo* studies on parasite cytoadherence and associated pathology in rodent malaria have been made using *P. berghei* in mice and rats, considered by some workers to be a good experimental model for cerebral malaria (Rest, 1982). However, in this model capillaries and venules are obstructed not by erythrocytes infected with mature forms of parasites but by large mononuclear cells and in this way it differs from *P. falciparum* infections. Thus, in spite of the fact that this model has been used to study some of the pathology associated with human cerebral malaria, it is not a suitable model for examining the role of infected erythrocytes in sequestration.

In *P. c. chabaudi* infected mice peripheral withdrawal of schizonts has been noted (Jarra, 1982), and schizont localization was shown to be predominantly in the liver (Cox et al., 1987; Gilks et al., 1990). Antigenic variation and sequestration are also intimately linked in this organism suggesting that a common molecule is involved in the two phenomena (Gilks et al., 1990) as in *P. falciparum* infections. All these features suggest that *P. c. chabaudi* AS may be a good model to study some aspects of sequestration during a malaria infection. However, not much is known about the nature of the interaction between *P. c. chabaudi* infected erythrocytes and/or mouse endothelial cells and whether or not that interaction is in some ways similar to *P. falciparum* in humans.
In the present chapter, aspects of the interaction between *P. c. chabaudi* AS infected erythrocytes and the tissue of different organs in the infected mouse has been characterized by ultrastructural analysis and use of an adhesion assay. This assay may represent an important advance in the discovery of the protein(s) on the surface of the infected erythrocyte which mediate cytoadherence in *P. c. chabaudi* AS infected mice. The results will be discussed in the context of how this model can be used to better understand sequestration in human infections with *P. falciparum*.

**3.2. Results**

**3.2.1. Evidence for sequestration of infected erythrocytes in different organs during *P. c. chabaudi* AS infection of CBA/Ca mice.**

- **Histological analysis.** Histological sections of brain, kidney, liver and spleen were prepared and haematoxylin stained in order to determine the extent of sequestration of schizont-infected erythrocytes *in vivo*. In the liver and the spleen more than 90% of the blood vessels observed (from small capillaries to larger diameter venules) did contain infected erythrocytes in intimate contact with the vessel walls (Figure 3.1C and D). In the brain, this percentage was much lower (50%) but a few infected erythrocytes could be seen very close to the endothelial cells. Furthermore, some small vessels appeared occluded by infected and non-infected erythrocytes (Figure 3.1A). In the spleen, erythrocytes are commonly seen outside the vessels and many infected erythrocytes could be seen in intimate contact with the surface of endothelial cells lining the vessels (Figure 3.1D). In the kidney, by contrast, even when infected erythrocytes were located closely opposed to the vessel walls, a space between the infected cell and that of the vessel wall was always observed (Figure 3.1B).

- **Ultrastructural analysis.** Samples obtained from brain, kidney, liver and spleen from days 7, 10 and 13 post-infection and as parasites were going through schizogony, were analyzed by transmission electron microscopy (TEM). As controls, similar samples from non-infected mice, and infected mice during the ring stage of the parasite’s growth, were used. The ultrastructure of infected erythrocytes in the peripheral circulation was also studied by TEM at the same time points.
Figure 3.1. Photomicrographs of sections from *P. c. chabaudi* AS infected mouse Brain (A), Kidney (B), Spleen (C) and Liver (D) stained with haematoxylin. Mice were killed at a parasitaemia of approximately 45% and at the time of maximal parasite withdrawal from the peripheral blood. Magnification - 630X. (A) Cross-sectional view of occlusion of a small blood vessel in the brain by infected and non-infected erythrocytes (→). The nucleus of an endothelial cell is shown (E). (B) Cross-sectional view of many vessels in the kidney where no sequestration can be seen. (C) and (D) Cross-sectional views of liver and spleen vessels where infected erythrocytes appear to be in close contact with the vessel walls (→).
At day 7 post-infection only a few infected erythrocytes (5% ± 1%) were observed on the transmission electron micrographs (results not shown). By day 10 (peak of parasitaemia) a higher number of infected erythrocytes can be seen (30% ± 3%) by TEM. The parasites inside these erythrocytes at day 10 post-infection seem to be damaged since they did not contain cytoplasm; however, the parasite plasma membrane was usually still intact (Figure 3.2). By day 13, the number of infected erythrocytes had decreased and reticulocytes were seen in the peripheral circulation (results not shown).

**Brain.** At days 7 and 13 post-infection only low numbers of infected erythrocytes were detectable in the blood vessels of brains from infected mice. At day 10 post-infection infected erythrocytes were detectable in significant numbers. Unlike infected erythrocytes in the peripheral circulation, parasites in the brain at this time were healthy in appearance (cytoplasm, membrane and all organelles intact). Like parasites in the peripheral circulation, these parasites were all late trophozoites (Figure 3.3). In many of the large vessels, neither infected or non-infected erythrocytes showed any contact with endothelial cells (Figure 3.3A). However, endothelial processes (resulting from endothelial swelling) were often seen in contact with infected erythrocytes (Figure 3.3B). In capillaries there was intimate contact between endothelial cells and the plasma membrane of infected erythrocytes (Figure 3.3C). This was in contrast to the situation in uninfected mice where, even in the smallest of capillaries, contact between erythrocyte and endothelial cell was not as intimate. Another very common striking finding in brains from infected mice at day 10 post-infection was the presence of extravascular erythrocytes (Figure 3.3D).

**Kidney.** No major differences between infected and non-infected mice were evident at day 7 post-infection. At day 10 post-infection, and as in the brain, parasites in the kidney appeared healthier than parasites in the peripheral circulation (Figure 3.4A). The behavior of infected and non-infected erythrocytes appeared to be similar in glomerular capillaries and also in proximal and distal tubules. Adhesion of infected cells to endothelial cells was not observed (Figure 3.4A and B). Around the brush borders of proximal tubules, phagocytosed deposits (containing haemoglobin) and lysosomes were observed within the cells suggesting a pathological state (Figure 3.4C and D) that had almost completely disappeared by day 13 post-infection.
Figure 3.2. Electron micrographs of *P. c. chabaudi* AS parasitized erythrocytes in the peripheral circulation. Mice were killed at a parasitaemia of approximately 45% and at the time of maximal parasite withdrawal from the peripheral blood. Magnifications: A - 5000 x, B - 12500 x, C - 16500 x and D - 40000 x. Parasites inside erythrocytes presenting signs of damage (★).
Figure 3.3. Electron micrographs of brain from *P. c. chabaudi* AS infected mice. Mice were killed at a parasitaemia of approximately 45% and at the time of maximal parasite withdrawal from the peripheral blood. Magnifications: A - 6500 x, B - 20750 x, C - 16500 x and D - 8250 x.

(A) and (B) cross-sectional view of small vessels in the brain where there is no occlusion but there are some signs of endothelium proliferation and endothelial processes can be seen in contact with infected erythrocytes (→). (C) cross-sectional view of an infected erythrocyte in intimate contact with the walls of a small capillary. (D) section showing infected and uninfected erythrocytes outside of a vessel wall (→)
Figure 3.4. Electron micrographs of kidney from *P. c. chabaudi* AS infected mice. Mice were killed at a parasitaemia of approximately 45% and at the time of maximal parasite withdrawal from the peripheral blood. Magnifications: A - 4000 x, B - 8250 x, C - 5000 x and D - 16500 x.

(A) and (B) cross-sectional views of kidney vessels where infected and uninfected erythrocytes do not show any contact with the endothelial cells.

(C) and (D) cross-sectional view of proximal kidney tubules with the presence of high numbers of lysosomes (→).
Liver. The most common observation was that of infected erythrocytes densely packed together within large sinusoids (Figure III.5A) with the endothelium showing signs of proliferation into the intracellular spaces (Figure III.5C). In other micrographs damaged endothelial cells were evident (Figure III.5B and C). The much higher percentage of infected cells found in liver sections (75% ± 4%) compared with that seen in the peripheral circulation (30% ± 3%) suggests a "positive selection" for these cells in this organ. These parasites appeared undamaged and there were many more schizonts than were apparent in the peripheral circulation. Infected erythrocytes were frequently observed adhering to the endothelial lining of large vessels. The interaction between infected erythrocytes and endothelial cells shows, frequently, points of adhesion where there is a very close association (< 20 nm) in the absence of knobs (Figure III.5D, ➔). As in spleen, but to a lesser extent, macrophages containing haemoglobin were observed. By day 13 post-infection fewer infected erythrocytes were detectable in liver and cells of the endothelia seemed to have returned to normal proportions and appearance.

Spleen. At day 7 post-infection no major differences in cytoadherence features were observed between infected and non-infected mice. By day 10 post-infection, a higher number of infected erythrocytes (50% ± 2%) were detectable in spleen compared with the peripheral circulation (30% ± 3%). These parasites appeared healthy (Figure III.6A and B) and many were in intimate contact with the vessel's endothelial cells (Figure III.6C). Both infected and non-infected erythrocytes were observed to pass through vessel walls and were found embedded in tissue (Figure III.6C and D). Signs of endothelial fragility such as swelling and damaged cells (Figure III.6) was a very common observation. Macrophages containing residues resembling haemoglobin were very common not only at day 10 but also at day 13 post-infection.

3.2.2. Cytoadherence of *P. c. chabaudi* AS infected-erythrocytes to endothelial cells in vitro and the effect of IFN-γ.

In *P. falciparum*, several adhesion molecules act as receptors for infected erythrocytes. In order to identify some of the endothelial cell's receptors involved in the cytoadherence of *P. c. chabaudi* AS infected erythrocyte, a FACScan analysis using anti-ICAM-1 and anti-VCAM-1 monoclonal antibodies was performed.
Figure 3.5. Electron micrographs of liver from *P. c. chabaudi* AS infected mice. Mice were killed at a parasitaemia of approximately 45% and at the time of maximal parasite withdrawal from the peripheral blood. Magnifications: A - 12500 x, B - 25000 x, C - 40000 x and D - 65000 x.

(A) cross-sectional view of liver vessel with a much higher proportion of infected erythrocytes than in the peripheral circulation. (B) very close contact between infected erythrocytes and endothelial cells. (C) and (D) endothelial processes maintaining contact with infected erythrocytes. (E) endothelial proliferation involving several infected erythrocytes. (F) Infected erythrocyte (IE) without knobs in contact with an endothelial cell (EC) showing a point of adhesion (→).
Figure 3.6. Electron micrographs of spleen from *P. c. chabaudi* AS infected mice. Mice were killed at a parasitaemia of approximately 45% and at the time of maximal parasite withdrawal from the peripheral blood. Magnifications: A - 5000 x, B - 12500 x, C - 12500 x and D - 6500 x.

(A) and (B) cross sectional views of splenic vessels where infected and uninfected erythrocytes do not show any adhesion features. Endothelium shows signs of fragility (>).

(C) and (D) cross-sectional views of infected erythrocytes adhering to endothelial cells (>) of a splenetic vessel, migration of an infected erythrocyte through the vessel wall (★) and presence of infected and uninfected erythrocytes free in the surrounding tissue.
Pre-treatment of endothelial cells with IFN-γ induced the upregulation of ICAM-1 and VCAM-1 (Figure 3.7). When the binding of erythrocytes and infected erythrocytes to a mouse endothelial cell line was compared, adhesion of the latter was significantly higher \((P < 0.05)\) and IFN-γ increased the binding of infected erythrocytes but not uninfected erythrocytes (Figure 3.8A). In \(P. c. chabaudi\) AS infections only mature trophozoites and schizonts disappear from the peripheral circulation. Therefore, adhesion assays were performed to compare the binding of the two types of infected erythrocytes. Infected erythrocytes containing mature trophozoites and schizonts bound significantly more readily than erythrocytes containing immature parasite forms. Furthermore, there was no significant difference between the adhesion of uninfected erythrocytes and erythrocytes infected with immature parasites (Figure 3.8B). The percentage of infected cells in the two populations was the same \((38\% \pm 2\%)\) and they were derived from the same parasite stabilate.

3.2.3. APP and HIS reduce the binding of infected-erythrocytes to endothelial cells

In \(P. falciparum\), it was shown that sera from immune individuals inhibit the \textit{in vitro} binding of infected erythrocytes indicating that antibody can interact with the structures of the infected erythrocyte that mediate binding. Thus, an adhesion assay for \(P. c. chabaudi\) AS was carried out in the presence of different sera/plasma. The adhesion of \(P. c. chabaudi\) AS infected erythrocytes (containing mature trophozoites and schizonts) to endothelial cells was significantly decreased by pre-incubation of parasitized erythrocytes with acute phase plasma (APP) or hyper-immune sera (HIS) but not NP (Figure 3.8C).

3.2.4. \(P. c. chabaudi\) AS infected erythrocytes bind specifically to human CD36

An adhesion assay was performed using immobilized human CD36 in plastic tissue culture plates. Infected erythrocytes, but not uninfected erythrocytes, adhered to the protein (Figure 3.8D).
Figure 3.7. Fluorescence-activated cell sorting analysis of the expression of ICAM-1 (A) and VCAM-1 (B) on the surface of rat endothelial cells in the presence (green) or absence (black) of IFN-γ. Negative controls (red) were performed using an unrelated antibody of the same isotype. IFN-γ significantly increased the expression of both molecules.
Figure 3.8. Adhesion of non-infected (E) and P. c. chabaudi AS infected erythrocytes (IE) to rat endothelial cells and human CD36 in vitro.

A, endothelial cells were treated with 100 U ml⁻¹ of IFN-γ or left untreated and then mixed with P. c. chabaudi AS parasitized erythrocytes containing mature parasites (24% parasitaemia).

B, endothelial cells were treated with 100 U ml⁻¹ of IFN-γ and then exposed to parasitized erythrocytes containing mature parasites (25% parasitaemia) or immature parasites (27% parasitaemia).

C, endothelial cells were treated with 100 U ml⁻¹ of IFN-γ. Parasitized erythrocytes containing mature parasites (27% parasitaemia) were incubated with KGS (BLK), NP, APP or HIS and then exposed to the endothelial cell in vitro.

The results (A, B and C) are presented as relative adhesion (number of bound infected and uninfected erythrocytes per 100 target cells) for the different treatments.

D, adhesion of parasitized erythrocytes to purified CD36 and albumin as a control.

All data are expressed as the arithmetic means of three independent experiments ± standard deviation.
3.3. Discussion

There is no doubt that infected erythrocyte sequestration occurs in *P. falciparum* infections. Furthermore, sequestration has been implicated as the main cause for cerebral malaria, one of the most life-threatening pathological consequences of acute *P. falciparum* infections in humans.

*P. falciparum* is the only human malaria parasite which sequesters and the reason(s) why it is the only one still remains unclear. It is believed that sequestration of mature-stage infected erythrocytes prevents their passage through the spleen and can enhance infected erythrocyte survival. The spleen has long been recognized as a site of potent innate and acquired antimalarial immunity. If *P. falciparum* infected erythrocytes were to enter the spleen, mature-stage infected erythrocytes would be unlikely to survive because, unlike immature-stage infected erythrocytes, they express new surface antigens and are significantly less deformable than ring-stage infected erythrocytes (Cranston et al., 1984). However, if the splenic selection and anti-parasitic activity is so potent, the mechanism by which blood stages of the other human malarials survive transit through this organ is still unclear.

For some time, the rodent model of choice to study cerebral malaria/sequestration has been *P. berghei* ANKA in mice (Mackey et al., 1980), and *P. berghei* in hamsters (Rest, 1982). These exhibit some properties of human cerebral malaria. Nervous system dysfunction, including cerebral pathology (vascular plugging and microhemorrhage), is noted at relatively low parasitaemia as in *P. falciparum* cerebral malaria. Furthermore, paralysis, deviation of the head, ataxia, convulsions and eventual death are common features of these infections. However, the mechanism underlying the pathology observed in these models is distinct from those observed in *P. falciparum* infections. Lymphocytes and monocytes instead of infected erythrocytes are the major cell populations sequestered in the venules of rodents.

Infection of mice with the lethal (17XL) strain of *P. yoelii* was shown to cause blockage of brain capillaries and here there is *in vivo* evidence for cytoadherence of infected erythrocytes to the endothelium of postcapillary venules in the brain (Kaul et al., 1994). Because *P. yoelii* 17XL infection results in little, if any, accumulation of monocytes or macrophages in the brain this infection is arguably a more suitable model...
for cerebral malaria than *P. berghei* ANKA. However, 17XL is a lethal strain and cannot be used as a model to study the importance/role of sequestration in the course of a less virulent malaria infection.

In the present study, cytoadherence of infected-erythrocytes during *P. c. chabaudi* AS infection in mice was observed mainly in the liver but also in the brain and in the spleen. Furthermore, the adhesive properties of *P. c. chabaudi* AS infected-erythrocytes appear to share many features with those of *P. falciparum*. The ultrastructural studies of different organs during infection showed a very close interaction between infected erythrocytes and endothelial cells of blood vessels walls. *P. c. chabaudi* does not express knobs but points of adhesion between infected erythrocytes and endothelial cells were observed. Sequestration occurs mainly in the liver but some sequestration was also observed in the brains of *P. c. chabaudi* AS infected mice. In histological sections, it was possible to observe occlusion of some small vessels, although the same kind of observations were not made in the TEM studies. An explanation for these differences should account for the size of sample used in each study. Sequestration of *P. falciparum* has been shown to present differential rates of sequestration within different areas of the brain. Thus, it is possible that (because of the small size of the TEM samples) the TEM observations were not made in the region of the brain where most sequestration occurs. Some leakage of erythrocytes into the extravascular space was evident. Although endothelial rupture was not apparent, clearly the blood brain barrier seems to be compromised in infected mice by day 10 post-infection. Punctiform haemorrhages of erythrocytes surrounding a ruptured cerebral vessel are common in cerebral malaria in *P. falciparum* (Turner, 1997).

Less than 5% of the mice die as a result of *P. c. chabaudi* AS infection, and those that do, do not present any cerebral symptoms such as ataxia, convulsions, etc. Mice infected with the CB line of *P. c. chabaudi* show higher levels of mortality and there has been speculation about the reasons for this. It would be interesting to check if sequestration of infected erythrocytes in the brain of these mice is higher relative to *P. c. chabaudi* AS. In *P. c. chabaudi* AS the *in vitro* adhesion assays show that the similarities with *P. falciparum* are not only at the histological and ultrastructural levels but also at the molecular level. The results show that pre-treatment of endothelial cells with IFN-γ specifically increased the binding of infected-erythrocytes. IFN-γ increased the expression
of (at least some) adhesion molecules on the surface of endothelial cells (ICAM-1 and VCAM-1) and, thus, these results suggest that, as in *P. falciparum*, adhesion molecules might act as receptors for *P. c. chabaudi* AS infected erythrocytes. Furthermore, *P. c. chabaudi* AS infected erythrocytes adhere to CD36, a known receptor for *P. falciparum* infected erythrocytes.

Other workers have proposed *P. c. chabaudi* AS infected mice as a model for sequestration (Cox *et al.*, 1987; Gilks *et al.* 1990). However, these studies did not show the ultrastructural and molecular similarities between this model and *P. falciparum* in humans. It must be emphasized that the proposition here is not that *P. c. chabaudi* AS infection of mice is a rodent model for human cerebral malaria. Instead, *P. c. chabaudi* AS should be used as a model to study the reason why *P. falciparum* sequesters. Thus, there are a number of differences that should be noted. The fact that *P. c. chabaudi* schizonts can be seen in the peripheral circulation of infected mice clearly indicates that these are not completely sequestered as in *P. falciparum* infections. However, in *P. falciparum* the parasitaemia levels reached are much lower than in *P. c. chabaudi* AS. It is not known if this feature is important in relation to the levels of sequestration observed since the number of receptors on endothelial cells (adhesion molecules) is probably limited. Another difference between *P. c. chabaudi* AS and *P. falciparum* is the absence of knobs in the *P. c. chabaudi* AS mature-stage infected erythrocytes. More recently, the disruption of the *P. falciparum* gene encoding KABRP has shown that this protein is essential for knob formation. Furthermore, the results of this study suggested that knobs play a role in strengthening the interaction between the parasite and the endothelial receptor (Crabb *et al.*, 1997). Therefore, the partial sequestration observed in mice infected with *P. c. chabaudi* AS might be explained by the fact that these mice present very high parasitaemias and a weaker interaction between infected erythrocytes and endothelial cells because of the lack of knobs. Mice infected with a non-sequestering *P. c. chabaudi* AS clone do not show any significant difference in parasite clearance (Gilks *et al.*, 1990). This suggests that either sequestration does not have a similar role in *P. c. chabaudi* as it does in *P. falciparum*, or the premise that spleen avoidance is the primary goal of sequestration is not true. It is important to take into account that the CBA/Ca mouse is not the natural host for *P. c. chabaudi*. Natural infection of the rat *Thamnomys rutilans* with this parasite may last for up to two years and the levels of parasitaemia
found in these rats were always very low (Garnham, 1966). The use of an *in vivo* model such as *P. c. chabaudi* AS in mice will be crucial in resolving these issues. If a complete understanding of the nature of the infected erythrocyte/endothelial cell interaction is achieved, then this interactions can be abrogated, the infected erythrocytes labelled and these cells can be followed to check if they are more readily cleared or not in the spleen. Furthermore, with the increase in transfection technology, transgenic *P. c. chabaudi* AS parasites expressing PfKAHRP could be produced. This may or may not result in the presence of knobs on the surface of *P. c. chabaudi* AS infected erythrocytes. A knob\(^*\) parasite could be used not only to access the real role of knobs in sequestration but also to improve *P. c. chabaudi* AS models as a sequestration model.

It is believed that upregulated levels of adhesion receptors in the brain direct sequestration of infected cells to that site. Recently it was shown that in humans infected with *P. falciparum*, widespread endothelial activation is a feature of malaria in non-fatal (Turner *et al.*, 1998) as it is in fatal malaria (Turner *et al.*, 1994). Examination of endothelial cell phenotype and function in individual tissues will be necessary and the *P. c. chabaudi* AS model may prove very useful in determining the relationship of endothelial activation and the levels of sequestration.
CHAPTER FOUR

Antibody from *P. c. chabaudi* Malaria Infection Recognizes Antigens on the Surface of Homologous Parasitized Erythrocytes and Promotes their Phagocytosis by Macrophages *in vitro*

4.1. Introduction

The fact that children in areas endemic for malaria develop naturally acquired immunity to the disease acts as an incentive for the development of a vaccine. An estimated 500 million clinical cases of malaria occur each year but, of these only a relatively small proportion (1-2 million - mostly young children) develop complicated and/or severe malaria and die. Of the remaining cases many will be primary (possibly acute) infections in non-immunes and will be treated, with varying degrees of success, with anti-malarial drugs. Yet others of these individuals must be capable of controlling potentially dangerous levels of parasitemia, in primary infections, in the absence of chemotherapy. Populations of areas endemic for malaria may be almost continuously exposed to infected mosquitoes during the transmission season. Even as they recover from their primary infections many individuals are susceptible to reinfection and become semi-immune during successive infection episodes. It may take many years to establish protective hyper-immunity capable of preventing clinical disease (Jarra *et al*., 1985; Greenwood *et al*., 1987; Baird *et al*., 1993) but the (premunition) immunity is incomplete and seems to require frequent boosting for its maintainence. Malaria parasites demonstrate extensive antigenic diversity and undergo antigenic variation. Immunity to malaria in a range of hosts including man is markedly parasite species-, line- and variant-specific, although a degree of cross-resistance is seen in some cases (Jeffery, 1966; Pazzaglia *et al*., 1982; Snounou *et al*., 1989). These are factors which may partly explain the observed susceptibility to reinfection in man. As such they represent important considerations in the host-parasite interaction in human malaria and
also in the design and application of effective vaccines. Study of the dynamics and relative efficacy of specific and cross-reactive immune responses occurring during primary infection and re-infection is therefore particularly relevant. Analysis of infections with the rodent malaria *Plasmodium chabaudi chabaudi* has allowed sophisticated modeling of this situation under laboratory conditions. Thus, (i) inbred mice infected with the AS cloned line of *P. c. chabaudi* experience acute but self-limiting infections (Jarra *et al.*, 1985), (ii) *P. c. chabaudi* AS is antigenically diverse and undergoes antigenic variation during a single infection (McLean *et al.*, 1982a; Brannan *et al.*, 1994) and (iii) immunity to the parasite has been demonstrated to include variant-, line- and species-specific components (Jarra *et al.*, 1989b; Snounou *et al.*, 1989; McLean *et al.*, 1982a).

Parasite-derived proteins exposed at the surface of infected erythrocytes are, probably, not only important in pathology (see chapter three) but are also important target antigens for antibody because these proteins are prominently exposed. Some of these proteins undergo clonal antigenic variation and, in humans infected with *P. falciparum*, it has been shown that antibodies against variant-specific antigens (anti-PfEMP-1 antibodies) play an important role in the acquisition of immunity to malaria in children and may play a continuing role in the maintenance of immunity throughout life (Bull *et al.* 1998).

It is important to investigate the role (if any) of antibodies against proteins in the surface of infected erythrocytes in the initial control of parasite replication in *P. c. chabaudi* AS infections.

The present work investigates (i) the presence of antibody in the plasma from mice during acute infection (ii) the capacity of antibody binding specifically to the surface of homologous parasitized erythrocytes and (ii) the ability of such antibody to opsonise these cells for phagocytosis since there is strong evidence that mononuclear cell phagocytic activity may play an important role in the clearance of parasites during infection (Taliaferro *et al.*, 1936; Taliaferro *et al.*, 1937; Zuckerman *et al.*, 1973).
4.2. Results

4.2.1. Kinetics of anti-parasitized erythrocyte antibody production during P. c. chabaudi AS infection

The binding activity of antibody in plasma from P. c. chabaudi AS infection was assayed by ELISA against preparations of total parasitized erythrocyte lysate. Total (parasite binding) Ig levels were examined in plasma samples collected between days 4-16 of a P. c. chabaudi AS infection initiated with $5 \times 10^6$ infected erythrocytes (Figure 4.1, Total Ig). By the use of mouse Ig isotype specific second antibodies, it was possible to differentiate the parasite specific antibody binding by Ig isotype (Figure 4.1, IgM, IgG1 and IgG2a). As early as day 4 post-infection antibody binding was detectable and enhanced relative to NP ($P < 0.01$), it increased between days 4 and 11, peaked at day 12 (as the mice went into crisis) and then gradually decreased. By day 16 post-infection (as the parasitemia was almost resolved) antibody levels were still higher than those observed in NP ($P < 0.01$). In the Ig isotype specific ELISA's, IgM reactivity against parasitized erythrocyte antigen increased, initially quickly (significant difference observed between NP and infected mouse plasma on day 6 post-infection - $P < 0.01$) and then more gradually between days 7 and 11, peaked at day 12 and then decreased quickly. The level of IgG1 reactivity against parasitized erythrocyte antigen preparations increased very gradually, with statistically significant differences (compared with NP) observed only by day 8 post-infection ($P < 0.01$), peaked at day 12 post-infection and was followed by a decrease in antibody reactivity. For IgG2a, the pattern observed was very similar to IgG1.

4.2.2. Surface immunofluorescence detects antibody binding to infected erythrocytes

Antibody binding to the surface of infected or non-infected cells was measured by immunofluorescence using erythrocytes (from non-infected mice) or parasitized erythrocytes containing either young trophozoites or mature trophozoites and schizonts. The cells were pre-treated with NP or APP and antibody binding was detected using FITC conjugated anti-mouse antibody and FACS analysis.
Figure 4.1. ELISA analysis of binding properties of antibody present during \textit{P. c. chabaudi} AS infection. CBA/Ca were infected with $5 \times 10^4$ infected erythrocytes, the course of parasitaemia was monitored and is shown (\textcircled{1}). Plasma samples from the days indicated were collected and tested against parasitized erythrocyte antigen preparation, measured by ELISA for total Ig, IgM, IgG1 and IgG2a and expressed as the mean absorbance reading. Antibody levels were also measured in NP. All data are expressed as the arithmetic means of three independent experiments ± standard deviation.
In these assays, as in the phagocytosis assay, parasitized erythrocytes were harvested from heavily infected mice at 2-3 days post-infection. The binding of antibody from APP to the surface of parasitized erythrocytes was higher than that of antibody from NP (Figure 4.2). Thus, 13.9% of mature trophozoite/schizont-infected cells incubated with APP fluoresced (Panel F), compared with 2.1% of these cells incubated with NP (Panel E). The capacity of antibody, present in APP, to bind to immature-stage parasitized erythrocytes (ring forms/immature trophozoites) was lower when compared with parasitized erythrocytes containing mature trophozoites and schizonts - 3.4% and 13.9% respectively (Figure 4.2, panels D and F). The percentage of infected cells in the two populations was the same (40% ± 2%). Only low (2.3%) levels of antibody binding to uninfected cells were detectable (Figure 4.2, panel B).

We used the same system to study antibody binding to the surface of parasitized erythrocytes using plasma samples obtained on sequential days of infection. Although antibody binding levels in these plasma were elevated between days 7 and 16 relative to NP, this difference was only significant between days 11 and 16 (Figure 4.3). The binding to the parasitized erythrocyte surface of different Ig isotypes contained in APP or NP was also measured. When compared with NP antibody, APP-IgM, IgG1, IgG2a and IgG2b isotype binding was higher, but no difference was observed for IgG3 (Figure 4.4).

4.2.3. Antibody binding to the surface of infected erythrocytes is parasite linespecific

Antibody binding to the surface of parasitized erythrocytes or erythrocytes was measured as above using P. c. chabaudi AS parasitized erythrocytes containing mature trophozoites and schizonts. In this experiment cells were pre-treated with NP, homologous APP (from P. c. chabaudi AS infection) or heterologous APP (from P. c. chabaudi CB infection). The binding of antibody from homologous APP to the surface of parasitized erythrocytes was higher than that of antibody from heterologous APP - 9.6% and 2.4% respectively (Figure 4.5, panels D and F). The difference is statistically significant ($P < 0.01$) and is not merely due to reduced antigen expression on P. c. chabaudi CB PE since APP from CB infected mice effectively labels homologous parasitized erythrocytes (results not shown).
Figure 4.2. Immunofluorescence analysis of antibody in APP binding to the surface of *P. c. chabaudi* AS parasitized erythrocytes and erythrocytes (from non-infected mice). Erythrocytes (Panels A and B) or immature-stage parasitized erythrocytes (Panels C and D) or mature-stage parasitized erythrocytes (Panels E and F) were incubated with NP (Panels A, C and E) or APP (Panels B, D, and F) and antibody binding to the cell surface was quantified in duplicate samples by FACS analysis. The proportion of cells within the predetermined window of positive fluorescence is indicated. The window was determined by using erythrocytes or parasitized erythrocytes incubated with KGS alone instead of a plasma sample.
Figure 4.3. The kinetics of antibody activity during a *P. c. chabaudi* AS infection analysed by surface immunofluorescence. Plasma samples from the days indicated were collected and tested against intact live parasitized erythrocytes in triplicate samples. Antibody binding to the parasitized erythrocyte surface was also measured in NP. The results represent the percentage of positive parasitized erythrocytes and were determined by using a window defined by parasitized erythrocyte samples incubated with KGS alone instead of plasma. All data are expressed as the arithmetic means of three independent experiments ± standard deviation.
Chapter Four  _Immunity to antisens in the surface of infected erythrocyte_

**Figure 4.4.** Immunofluorescence analysis of anti- _P. c. chabaudi_ AS antibody subclasses from APP binding specifically to the surface of parasitized erythrocytes. Parasitized erythrocyte samples containing mature parasites were incubated with NP or APP and the antibody isotype binding to the cell surface identified by immunofluorescence and quantified in triplicate samples by FACS analysis. The results represent the percentage of positive cells and were determined by using a window defined by identical samples incubated with KGS alone instead of plasma. All data are expressed as the mean of three independent experiments ± standard deviation.
Figure 4.5. Immunofluorescence analysis of the line-specificity of APP antibody. Erythrocytes (panels A, C and E) or parasitized erythrocytes (Panels B, D and F) were incubated with NP (Panels A and B), homologous anti-\textit{P. c. chabaudi} AS APP (Panels C and D) and heterologous anti-\textit{P. c. chabaudi} CB APP (panels E and F) and antibody binding detected in triplicate samples. The window was determined by using erythrocyte or parasitized erythrocyte samples incubated with KGS alone instead of plasma.
4.2.4. Homologous APP mediated opsonization leads to phagocytosis of P. c. chabaudi AS infected cells by macrophages

FACS analysis of cells incubated in APP indicated the presence of antibody which specifically recognized antigens on the surface of intact parasitized erythrocytes. This result does not define whether antibody has a role in the clearance of the infected cells during crisis. In the in vitro phagocytosis assay incubation of parasitized erythrocytes in homologous APP resulted in significantly enhanced internalization ($P < 0.01$) of infected erythrocytes when compared to incubation in NP - 52% and 24%, respectively or heterologous APP - 52% and 29%, respectively (Figure 4.6). No significant difference was found between internalization of infected erythrocytes incubated with NP and heterologous APP. Neither APP or NP promoted phagocytosis of non-infected erythrocytes either from normal or infected mice and a significant difference ($P < 0.01$) was found in the comparison between erythrocyte (from non-infected mice) and parasitized erythrocyte (from infected mice) samples.

4.2.5. IgG in APP is mediating opsonization of infected cells

Fractionation of plasma on Protein G produced 2 fractions. The fraction which specifically bound to the column contained IgG and unbound material contained IgM (results not shown). IgG binding to the surface of parasitized erythrocytes was then determined after the cells were first treated with NP, APP or the 2 Protein G fractions and then exposed to FITC conjugated anti-mouse IgG. The IgG containing fraction of APP was the only fraction showing significant binding to the surface of parasitized erythrocytes and the percentage of fluorescent cells in this sample was not significantly different from that in the sample treated with unfractionated APP (Figure 4.7). After fractionation of plasma on Protein G only the IgG fraction of APP was capable of promoting phagocytosis of infected erythrocytes (Figure 4.8).
Figure 4.6. Phagocytosis of *P. c. chabaudi* AS infected erythrocytes pre-incubated with homologous or heterologous APP. Parasitized erythrocytes containing mature parasites, and erythrocytes, were incubated with NP, APP or KGS (BLK), and then exposed to macrophages *in vitro*. The results are presented as the Phagocytic Index (% of macrophages with infected erythrocytes inside) for the different treatments and are further broken down by the numbers of cells inside individual macrophages.
Figure 4.7. Immunofluorescence analysis of antibodies from anti-\textit{P. c. chabaudi} AS APP fractions binding to the surface of parasitized erythrocytes. Parasitized erythrocytes containing mature parasites were incubated with NP (Panel A), APP (Panel B), the IgG fraction from NP (Panel C) and from APP (Panel D) or the non-IgG fraction from NP (Panel E) and from APP (Panel F). IgG binding to the cell surface was quantified in duplicate samples by FACS analysis. The window was determined by using PE incubated with KGS alone instead of a plasma sample.
Figure 4.8. Phagocytosis of infected erythrocytes pre-incubated with Protein G fractionated APP fractions. Parasitized erythrocytes containing mature parasites were incubated with NP, APP or with their Protein G fractions and then exposed to macrophages in vitro. The results are presented as the Phagocytic Index (% of macrophages with infected erythrocytes inside) for the different treatments. Analysis by Student's $t$ test showed no significant difference between the APP and the IgG fraction obtained from it.
4.3. Discussion

The surface of the *P. falciparum*-infected erythrocyte is an important site for host/parasite interaction both with regard to pathogenesis (see Chapter III) and to the host immune response. Variant-specific antibody responses appear to dominate in natural infections (Newbold *et al.*, 1992; Bull *et al.*, 1998). Using *P. knowlesi* in rhesus monkeys it was shown that protective immunity was associated with the development of an opsonising antibody response to the infected-erythrocyte surface (Brown *et al.*, 1971). In *P. c. chabaudi* AS, host immunity distinguished between different variants and their expression was linked to the establishment of chronic infections (Gilks *et al.*, 1990). In the present study, therefore, we show that natural antibody induced during acute *P. c. chabaudi* AS infection binds to the surface of the infected erythrocyte in a parasite-line-specific manner and promotes their phagocytosis by macrophages *in vitro*.

The induction of both antibody and cell-mediated responses by infection with the malaria parasite is well documented (Langhorne *et al.*, 1990; Meding *et al.*, 1991; Simon Haarhaus *et al.*, 1991). In some individuals these responses act to control parasitaemia. However, the importance of each of these mechanisms in the dramatic decrease of parasitaemia observed during crisis remains unclear. Antibody mediated opsonization of infected erythrocytes, followed by their internalization and subsequent destruction by macrophages in the spleen and/or liver, might represent one of the most likely mechanisms to explain the resolution of parasitaemia observed during crisis. As antibody mediated targeting is very specific, this activity may explain the observed parasite species- and line-specificity of resolution. Anti-parasite mediators produced by T-cell activated macrophages capable of damaging intra-erythrocytic parasites, but lacking the targeting specificity intrinsic to antibody, would be less likely to produce such specific parasite clearance.

The anti-parasite properties of APP have been analysed and this shows that this activity is, at least partly, antibody dependent. Analysis by ELISA showed that antibody is produced early in the *P. c. chabaudi* AS infection and surface immunofluorescence demonstrated the presence of antibody in APP reacting specifically with the surface of infected cells by day 11 post-infection. In the ELISA
assay antibody binding was not line-specific and cross-reacted between *P. c. chabaudi* AS and CB antigen preparations (results not shown). In contrast antibody binding in the surface immunofluorescence assay was parasite line specific. That immunoassays utilising disrupted or non-intact parasitized erythrocyte antigen do not reflect the specificity intrinsic to the *in vivo* biological activity of antibody in this system has been noted previously (Jarra *et al.*, 1986). Antigen expression/detection at the surface of live intact malaria infected erythrocyte is relatively parasite maturation stage dependent. Although the parasite cell cycle in *P. c. chabaudi* is highly synchronous mature parasite forms are less evident in the peripheral blood due to sequestration. This might explain the relatively low (max. 13.9%) numbers of positive parasitized erythrocyte in the surface immunofluorescence assay compared to the level of parasitemia in the cell population used (40-50%). In addition, the low levels can also be a reflection of the presence of different antigenic variants into the parasitized erythrocyte population.

We also found a correlation between antibody binding to the erythrocyte surface and phagocytosis *in vitro*, using either unfractionated plasma or the IgG fraction obtained from it. The phagocytic activity was not enhanced by the fraction depleted of IgG (but containing IgM). Antibody binding to the surface of a small proportion of non-infected cells was detected by surface immunofluorescence, but these cells were not phagocytosed by macrophages. To be sure that these cells were not phagocytosed and destroyed very quickly, the phagocytosis assay was performed for different periods of time (such as 10, 15, 20, 30 and 45 min). Even under these conditions uninfected erythrocytes from either infected or normal mice were not seen inside macrophages (results not shown).

These studies indicate that infection of mice with *P. c. chabaudi* AS induced antibody against the surface of parasitized erythrocytes and that this antibody is capable of opsonizing these cells for phagocytosis. A role for macrophages and phagocytosis in the host response to malaria was proposed a long time ago (see Taliaferro *et al.*, 1937). Observations (mainly histological) made by Taliaferro and Canon (1936) indicated that, in experimental infections using canaries and monkeys, there was an initial rise in parasitaemia during which parasites were slowly phagocytosed, primarily in the spleen and to a lesser extent in the liver and bone marrow. Similar observations
have been made in the spleens of rats infected with *P. berghei* (A. Zuckerman *et al.*, 1973). The present study provides histological evidence which supports the above findings in *P. c. chabaudi* infected mice.

The antibody subclasses produced in response to infection are of particular relevance, since different antibody isotypes may have distinct biological functions. Macrophage receptors, recognizing the Fc-region of antibody, are involved in a number of cellular functions, of which the ingestion of IgG-coated particles is fundamental in defence against bacteria and parasites (reviewed by Miklos *et al.*, 1993). In the rat IgG1 is most efficient, opsonizing erythrocytes for phagocytosis by macrophages while IgG2a is more efficient in mediating antibody-dependent cytotoxicity (Miklos *et al.*, 1993). In the mouse, however, the situation seems less clear as here different antibody isotypes are involved in the process of phagocytosis (Ralph *et al.*, 1980). In the surface immunofluorescence assay we identified antibody isotypes present in APP which recognize the surface antigens of parasitized erythrocytes. IgM, IgG1, IgG2a and IgG2b binding to parasitized erythrocytes were found in APP at levels above those in NP. However, no difference in the level of IgG3 binding was observed between NP and APP. Using a similar experimental model, Taylor Robinson and collaborators (1993) detected appreciable levels of IgG1 by ELISA only at day 20 post-infection, with a maximal level detectable during the recrudescence of parasitemia. However, our results show that significant levels of IgG1 can be detected as early as day 8 post-infection by this technique.

Many studies have now shown that B cells and both Th1 and Th2 CD4 T cells play an important role in immunity to erythrocytic malaria. The use of immunocompromised or genetically modified mice suggest that control of primary peak parasitaemia is B cell independent and dominated by Th1 cells, while control of the recrudescence is B cell and Th2 cell dependent (Langhorne *et al.*, 1991; Taylor Robinson *et al.*, 1993; van der Heyde *et al.*, 1994). However, exactly when and why there is the switch from predominantly Th1 to predominantly Th2 activity and the exact roles of these activities in crisis and eventual elimination of parasitaemia is still not clear. What does seem clear in the systems described above is that infected mice can, at least partially, control initial parasite replication during crisis in the absence of B cells. The results of this current study provide evidence that, as early as 1-2 days
after peak parasitemia, specific mechanisms (detecting infected cells versus non-infected cells or homologous line versus heterologous line) act, possibly to damage parasites, but certainly to target infected erythrocytes for clearance. The results also show that antibody seems to play a central role in clearance of infected erythrocytes during this period. The apparent discrepancies in all these results/findings might be explained by assuming that, in the absence of one (or several) immune mechanisms, other compensatory responses become more important/effective.

This study has identified potentially protective antibody in the plasma of mice infected with *P. c. chabaudi* AS at the time of crisis, which specifically recognizes antigens in the surface of infected erythrocytes. These results might explain the specific clearance observed in *P. c. chabaudi* AS infections as well as in humans infections with *P. falciparum*. Specific reagents are needed to investigate if this activity demonstrates variant specificity. In this context the presence of erythrocyte membrane protein-1 (EMP-1; Howard *et al.*, 1988) and var gene (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995) homologues in *P. c. chabaudi* AS needs to be investigated.
CHAPTER FIVE

Attempt to identify the homologue of the Pfvar-genes in *P. c. chabaudi* AS and characterization of a gene family

5.1. Introduction

Using a passive serum transfer protection system, *P. c. chabaudi* AS recrudescences have been shown to be antigenically different from the cloned infecting parental population (McLean *et al.*, 1982a). Using the same system, more recent work has shown that at least one in every 80 schizonts switched the expressed variant antigen type every generation (Brannan *et al.*, 1994). Furthermore, antigenic variation has been shown to be linked to a second phenotype of the parasite that may be involved in immune evasion: cytoadherence-sequestration (Gilks *et al.*, 1990). The molecule responsible for both phenotypes is found only on the surface of erythrocytes infected with late trophozoites and schizonts. Thus, *P. c. chabaudi* AS infection in mice represents a model for studying antigenic variation/sequestration as it occurs in vivo. The variant molecule on the surface of erythrocytes infected with *P. c. chabaudi* AS has not been identified and neither has the gene(s) that code for it. However, the similarities between *P. c. chabaudi* AS and *P. falciparum*, suggests the variant molecule and its gene(s) may be homologues of PfEMP-1 and var, respectively.

In *P. falciparum*, each member of the multi-copy var gene family contains two exons (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995). The deduced protein sequences of the var genes are highly diverse, yet all contain certain conserved motifs and common structural features. Within each protein sequence deduced from var, there are one to five Duffy binding-like (DBL) domains which share homology with cysteine-rich domains of certain *Plasmodium* molecules - for example, EBA-175 (erythrocyte binding antigen) from *P. falciparum* or DABP (Duffy antigen-binding proteins) from *P. vivax* and *P. knowlesi*. The first DBL domain near the N-terminus is the most conserved of the DBL
domains and has amino acid signatures that differentiate it from subsequent DBL domains (Su et al., 1995). Between the first and second DBL domains there is another type of conserved domain, a cysteine-rich interdomain region (CIDR) of 300-400 amino acid residues. The DBL and CIDR domains are thought to represent extracellular domains of the molecule since they are followed by a segment of variable length and a hydrophobic region that fits the criterion of a transmembrane region. Furthermore, sequence diversity of the var genes is most evident in the regions in and around the DBL and CIDR coding domains. This is consistent with the need for the parasite to vary extracellular exposed regions of the molecule that are accessible to immune attack. All these regions are coded by the first exon. The second exon encodes a conserved C-terminal sequence (45-55 kDa) named acidic terminal segment (ATS) because of its amino acid composition. The position of this C-terminal sequence downstream of a single transmembrane segment suggests that it has a cytoplasmic location (Su et al., 1995).

PfEMP-1 mediates the binding of infected erythrocytes to the surface of endothelial cells and displays antigenic variation (Baruch et al., 1996; Gardner et al., 1996). It is a high molecular mass protein associated with the plasma membrane of erythrocytes infected with mature-stage parasites; shows specific detergent extraction properties and sensitivity to low levels of trypsin (Leech et al., 1984; Howard et al., 1988).

This chapter describes a large family of P. c. chabaudi AS genes identified in an attempt to obtain the var homologues in this organism.

5.2. Results

5.2.1. Pfvar probes do not recognize P. c. chabaudi AS genomic DNA

In an initial attempt to obtain P. c. chabaudi AS var homologues, two probes were developed derived from the conserved ATS region of Pfvar, and hybridized to P. c. chabaudi AS genomic DNA. P. falciparum genomic DNA was also used as a positive control. The results indicated that the probes failed to hybridize with P. c. chabaudi AS DNA, even when the filters were washed at low stringency (0.5X SSC at 50°C) (Figure 5.1).
Chapter Five

Attempt to identify *P. c. chabaudi* AS var

Figure 5.1. Southern blot of *P. c. chabaudi* AS (Pc) and *P. falciparum* (Pf) genomic DNA probed with 2 probes for *P. falciparum* var genes (Pfvar probe 1 and Pfvar probe 2). The DNA was digested with the restriction enzyme EcoRI, and then the products were separated by gel electrophoresis, transferred to a membrane and hybridized with the radiolabelled probes. The migration of size markers is indicated on the right (in kb).
Thus, our first attempt to find the \textit{var} homologues in \textit{P. c. chabaudi} AS was unsuccessful. Although such a homologue may well exist in \textit{P. c. chabaudi} AS it may not share extensive sequence homology with \textit{Pfvar}. In \textit{P. falciparum}, the large repertoire of \textit{var} genes show enormous sequence variation among themselves (Su \textit{et al.}, 1995).

5.2.2. \textbf{PCR of \textit{P. c. chabaudi} AS DNA with degenerate primers based on \textit{var} sequence}

In an alternate approach, a decision was made to use the polymerase chain reaction (PCR) to find the homologues I was looking for. This approach allows the design of primers to short, highly conserved motifs within \textit{Pfvar}. As no region is absolutely conserved, degenerate primers were developed to anneal to domains in ATS and the first DBL domain. Eight sets of primers were used (\textit{var}1/\textit{var}2; \textit{var}3/\textit{var}6; \textit{var}3/\textit{var}5; \textit{var}4/\textit{var}5; \textit{var}4/\textit{var}6; \textit{DBL}1\textit{A}/\textit{DBL}1\textit{Br}; \textit{DBL}1\textit{B}/\textit{DBL}1\textit{C}; \textit{DBL}1\textit{A}/\textit{DBL}1\textit{C} - Figure 5.2A) along with a PCR optimization kit containing 12 different buffers plus 4 more additives. Thus, each set of primers was used to amplify \textit{P. c. chabaudi} AS genomic DNA using a total of 48 different conditions. Three sets of primers (\textit{var}3/\textit{var}5; \textit{var}4/\textit{var}5; \textit{DBL}1\textit{A}/\textit{DBL}1\textit{Br}) gave discrete PCR products (Figure 5.2B). Each of these products was cloned (there were 15 in all) and used in Southern blot analysis.

5.2.3. \textbf{Identification of two DNA clones that recognize a gene family}

\textit{P. falciparum} \textit{var} represents a large and diverse gene-family. In order to determine if the specific products obtained with the PCR amplification also recognized a gene-family, each of the 15 cloned fragments were hybridized to \textit{P. c. chabaudi} AS genomic DNA. Two clones did not hybridize at all, 11 clones recognized single copy genes and 2 clones (\textit{PcMM}1 and \textit{PcMM}2) hybridized to multiple fragments in complete digests of genomic DNA. The \textit{PcMM}1 DNA clone was obtained by PCR with the set of primers \textit{var}3/\textit{var}5 while the \textit{PcMM}2 clone was obtained with the set of primers \textit{DBL}1\textit{A}/\textit{DBL}1\textit{Br}. Both probes demonstrated similar hybridization patterns at high stringency, especially for fragments larger than 2 kb (Figure 5.3).
Figure 5.2. (A) Schematic diagram of PfEMP-1, the variant molecule encoded by *P. falciparum* var genes. The molecule comprises several Duffy binding-like (DBL) domains, a cysteine-rich interdomain region (CIDR), a transmembrane segment (TM) and an acidic terminal segment (ATS). Primers were designed based on conserved regions of the known sequences for *P. falciparum* var genes. (B) PCR of *P. c. chabaudi* AS genomic DNA with var3/var5 (lane 1), var4/var5 (lane 3) and DBL1A/DBL1Br (lane 5). Lanes 2, 4 and 6 correspond to the same sets of primers but without template. The migration of size markers is indicated on the left (in bp).
Figure 5.3. (A) Schematic diagram of the variant molecule encoded by *P. falciparum* var genes as shown in Figure 5.2A. The PCR amplification of *P. c. chabaudi* AS genomic DNA with the sets of primers DBL1A/DBL1Br and var3/var5 resulted in several products. Two of these products were PcMM1 and PcMM2. (B) Southern blot of *P. c. chabaudi* AS genomic DNA digested with *DdeI* (lanes 1), *EcoRI* (lanes 2), *Hinfl* (lanes 3), *PstI* (lanes 4) or *Sau3AI* (lanes 5) and probed with PcMM1 and PcMM2 clones. The 2 parallel blots were superimposed and the identical bands are indicated (▼). The results were confirmed in a separate experiment by probing a single blot first with PcMM1 and then, after extensive washing, with PcMM2 (results not shown). The migration of size markers is indicated between the two blots (in kb).
5.2.4. Expression of the novel genes

To investigate if any members of the *P. c. chabaudi* AS gene-family recognized by PcMM1 and PcMM2 clones were actually expressed during asexual stages of the parasite life-cycle, Northern analyses were performed. The results (Figure 5.4) indicated that the sequences within the probes are transcribed. In all experiments there were multiple bands that hybridized with each of the two clones. The strongest one was always at ~4.3 kb. There were others at ~1.5 kb, ~2.5 kb and in some blots a band of about 7.5 kb is also detectable.

5.2.5. Attempt to obtain the sequence of a full-length gene

Southern and Northern blot analysis indicated that the PcMM1 and PcMM2 clones obtained by PCR, do belong to a gene family of which one or more members are transcribed during *P. c. chabaudi* AS blood stage infection. Further detailed analysis of this gene family required the sequencing of a representative gene. The PcMM1 and PcMM2 clones were sequenced (Figure 5.5). Blast searches were performed with these DNA sequences and the results are summarised in Figure 5.6.

In an attempt to get a longer contiguous gene segment, 5’ and 3’ RACE (Rapid Amplification of cDNA Ends) analyses were performed. Two sets of specific primers were designed for each clone (PcMM1 and PcMM2), one forward and one reverse (Figure 5.5 and 5.7A). Double stranded cDNA was obtained from total RNA. During cDNA synthesis a control reaction prepared in the absence of reverse transcriptase failed to result in any product, indicating that the RNA obtained was DNA-free. The double-stranded cDNA was then ligated to RACE adaptors and the RACE reactions were performed as detailed in the methods section. Several products were obtained from each RACE reaction. These were blotted onto nylon membranes and PcMM1 or PcMM2 specific probes were used to determine if these products were extensions of the original fragments. None of the products hybridized to both PcMM1 and PcMM2 (Figure 5.7B) indicating that either (1) both clones are derived from separate sections of the same gene but none of the RACE products overlapped or (2) the clones are derived from genes that belong to the same gene family but not from the same gene.
Figure 5.4. Northern blot analysis of *P. c. chabaudi* AS RNA probed with PcMM1 and PcMM2 clones. Total RNA was electrophoresed on a 1% agarose/2.2 M formaldehyde gel, transferred to a membrane and then hybridized to the radiolabelled probes as described in the Materials and Methods section. The migration of size markers is indicated on the left of each blot (in kb).
**PcMM1**

1
TAAATAAAAAGACACTATGGGTATATAATTTTAAAAATATTTTCTCATA
50
51
AAATATTACATTTTTTCATAATTTTATGTTAGTTTTATCATTGCAG
101
150
AGAAGAGAAAAAGAAAGAAAAAACATGAAAAGTTTATAACCTTG
151
200
TTGGTGCAAAAACGACAAAAAGACGTATAAAAACACTCAGACCAAGA
201
250
AAAACACACAAATATTTATATAAAATCTCGTCAAAAAACAGACTAT
251
300
AAAATCTAATAATTCTGTTATTAGGAAAAAAATGCTCATATTAAATATAT
301
350
ACCAACCTATGGCAGGTAGTCTCGTTACTCTGTPACCATTTATAATTTTTTCTG
400
TTAATTTTTTTGGTTAT

**PcMM2**

1
AATATATTACAAACGGGTATTCTGTTCTTTGTCAGAAGGTACAAAA
50
51
AGAAGACAAGAAAAATGCAATTTAGAAATTAGGTTATCATTGAAAAG
101
150
CAAAATGGGATTTTCTAAAAACATGTGATAGTACAGAAGAAGTTATAA
151
200
TTGGTGCAATACAGAAAAGAAAAATGCAATTTAGAATTTAAAGGAG
201
250
AGATGAAGCTAATTTAAAAAATTGCCACATGAAACATCTGAAGATATTT
251
300
ACTGGTCTCACGAAGAAGATATAATTATGTTGTTGCGAACATGCAGAAGA
301
350
AGATGGGATGCTCGAGGACGTTAGGACTTTCTAAATATGGATTAA
351
400
ACAAACATATAATGCAATTTTCAAATACATTTTCAACCACCACACAG
400
AATTAAGTTGTTTGGACAAAATAAGTTTCTAA

**Figure 5.5.** DNA sequence of PcMM1 and PcMM2 clones. Specific primers designed from each clone are shown in color: a (PcMM1 forward primer), b (PcMM1 reverse primer), c (PcMM2 forward primer) and d (PcMM2 reverse primer).
PcMM1

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PcMM2

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**Figure 5.6.** Results of a blast search of PcMM1 and PcMM2 DNA sequences against all DNA databases. PcMM1 shows high homology with *P. berghei* glutamine alanine rich protein.
Figure 5.7. (A) Schematic diagram of the variant molecule encoded by *P. falciparum var* genes as shown in Figure 5.2A. Specific primers to clones PcMM1 and PcMM2 were designed (see Figure 5.6). (B) Southern blot of *P. c. chabaudi AS* RACE products obtained using the four different primers and probed with either the PcMM1 or PcMM2 clones. The migration of size markers is indicated between the two blots.
DNA sequencing of these RACE products identified an open reading frame without stop codons and with a typical *Plasmodium* AT-rich content. The deduced protein sequences extended the previously determined sequence of PcMM2 and revealed a cysteine-rich domain (Figure 5.8). These deduced protein sequences were aligned against different sequence from PfEMP-1 molecules. The only alignment possible was between the cysteine-rich domain coded by the extended PcMM2 fragment and the first DBL domain in PfEMP-1 (Figure 5.9). However, the limited match between these genes (at the deduced amino acid sequence level) does not permit an unequivocal conclusion that the extension PcMM2 is a homologue of Pfvar.

5.2.6. Expression of the cDNAPcMM2 clone in E. coli and purification of recombinant protein

To characterize as well as to localize the protein(s) coded by these new genes in *P. c. chabaudi* AS, recombinant fusion protein of a cDNA clone (cDNAPcMM2 - obtained by PCR of *P. c. chabaudi* AS genomic DNA between primers a and b designed from the PcMM2 clone and cloned into the commercially available pTrcHis vector C) was produced and antibodies against it were raised in inbred mice.

The expression of the recombinant (His)₆-X-cDNAPcMM2 fusion protein was induced with 1 mM IPTG and samples were collected at one hour intervals for 7 hours. Although, the predicted size for this fusion protein was approximately 13-14 kDa, the protein migrated in SDS-PAGE with an apparent size of 19-20 kDa. The optimum level of expression was found to be 5 hours post-induction (Figure 5.10A) and this time point was subsequently used to purify recombinant fusion protein on a Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography matrix (Figure 5.10B and 5.11). Imidazole was used to elute the selectively bound fusion protein. Optimal concentration of imidazole for elution of the recombinant protein from the column was 200 mM (Figure 5.10B). During large scale purifications the major affinity purified product was the (His)₆-X-cDNAPcMM2 fusion protein (Figure 5.11). A second step of purification using gel filtration was performed to further purify the recombinant fusion protein (Figure 5.12).
**Figure 5.8.** Putative protein sequence and alignment of some of the *P. c. chabaudi* AS clones obtained from RACE products within and nearby the region corresponding to the PcMM2 clone. The cysteine residues are highlighted in color (blue). The different clones showed differences in sequences (red boxes) that, although they may represent sequencing artifacts, could represent different members of the gene-family.
Figure 5.9. Alignment between the amino acid sequence deduced from one of the *P. c. chabaudi* AS clones (PcMMRACE4 - containing a cysteine-rich domain) and several translated *P. falciparum* var genes. The alignment was only possible with the first DBL domain of the *Vï* var. Residues conserved between the *P. c. chabaudi* AS sequence and any one of the PfEMP-1 sequences are in red.
Figure 5.10. SDS PAGE analysis of (A) expression of cDNAPcMM2 cloned in E. coli in soluble (S) and insoluble (P) fractions at various time points after induction, and (B) purification of the recombinant protein on a Ni-NTA affinity chromatography matrix. Samples were analysed on a 10% polyacrylamide gel by SDS-PAGE and the proteins detected by staining with comassie blue. The size of the recombinant protein is indicated (→). The mobility of standard molecular mass markers is indicated on the right of each panel (in kDa).
Figure 5.11. (A) Chromatogram of the elution profile and (B) SDS PAGE analysis of recombinant protein purification on a Ni-NTA affinity chromatography matrix using 200 mM imidazole. In panel B, samples were analysed on a 10% polyacrylamide gel by SDS-PAGE and the proteins detected by staining with coomassie blue. The size of the recombinant protein is indicated (→). The mobility of standard molecular mass markers is indicated on the left of panel B (in kDa).
Figure 5.12. (A) Chromatogram of the elution profile pattern and (B) SDS PAGE analysis of a second step purification by gel filtration on a Sephadex G50-SF column. The size of the recombinant protein is indicated (→).
5.2.7. Anti-cDNAPcMM2 recombinant protein antibodies recognize a protein of high molecular mass

PfEMP-1 is a high molecular mass protein which shows size heterogeneity (~220 - 350 kDa) and insolubility in Triton X-100. To identify and characterize the protein(s) coded by the new P. c. chabaudi AS gene family, antibodies were raised against the recombinant fusion protein in eight CBA/Ca mice. Antisera against this protein were tested for immunoreactivity by Western blotting using proteins in an SDS extract of P. c. chabaudi AS infected erythrocytes. Sera from five of the eight immunized mice recognized a high molecular mass antigen (>200 kDa) in both Triton X-100 soluble and insoluble fractions (Figure 5.13). The preimmune sera of these mice did not react with a similar band. Some immune sera also reacted with other lower molecular mass bands which possibly represented degradation products.

5.2.8. Immunofluorescence analysis of fixed P. c. chabaudi AS parasitized erythrocyte preparations

Sera from mice immunized with the recombinant fusion protein were also used to perform cellular localization studies. If this high molecular mass protein is the homologue of PfEMP-1 then it should be detectable, by IFAT, at the surface of erythrocytes infected with mature-stage parasites. The most intense fluorescence surrounds the parasite, and gives a distinctive ring-like appearance suggesting an association with a parasite membrane (e.g. parasitophorous vacuole membrane (PVM) or the parasite plasma membrane (PM)), rather than at the infected erythrocyte surface (Figure 5.14A). In some preparations, the fluorescence was not limited to the parasite but appeared all around the infected erythrocyte (Figure 5.14B). Furthermore, approximately, 30% of infected erythrocytes were not stained by the immune sera.
Figure 5.13. Western blot of extracts of *P. c. chabaudi* AS parasitized erythrocytes solubilised in Triton X-100 (TS) or insoluble in Triton X-100 (TI), and probed with either normal mouse serum (NS) or serum from 5 mice immunized with the (His)$_6$-X-cDNApcMM2 recombinant protein (IS). The lysates were resolved by SDS-PAGE on a 5% polyacrylamide gel and then transferred to a membrane. The position of a 200 kDa size marker is shown on the left.
Figure 5.14. Immunofluorescence (panels B and D) on acetone fixed *P. c. chabaudi* AS parasitized erythrocytes using as first antibody serum from mice immunized with the (His)$_6$-X-cDNAPcMM2 recombinant protein. The panels on the left (A and C) correspond to the DAPI (nuclear) staining of each parasite.
5.3. Discussion

There is much evidence indicating that *P. c. chabaudi* AS should have homologues of Pfvar. First, *P. c. chabaudi* AS undergoes antigenic variation (McLean et al., 1982a; Brannan et al., 1994). Second, the sequestration phenotype is present in *P. c. chabaudi* AS infections (Gilks et al., 1990). Finally, the balance of evidence suggests that binding to endothelium and antigenic variation are functions of the same molecule (Gilks et al., 1990), presumed to be a homologue of PfEMP-1.

In the current study using a PCR-based approach, two DNA clones were obtained belonging to the same gene family in *P. c. chabaudi* AS. These clones were obtained by using primers to two different regions of the Pfvar and yet each recognized a gene family as expected for a var-like family. In addition, the putative protein sequence of the clone obtained from primers designed for the first DBL domain showed the presence of a cysteine-rich domain. These features are compatible or similar to features observed for Pfvar, although they are by no means definitive.

Antibodies were raised against the recombinant protein from a cDNA clone. These antibodies were shown to bind to a protein of high molecular mass (by extrapolation, approximately 250 kDa) by Western blot analysis. Once again, these results were quite encouraging since PfEMP-1 shows size heterogeneity of around 220 - 350 kDa (Howard et al., 1988). However, these results also showed that the protein(s) recognized by these antibodies are partially soluble in Triton X-100 while it is known that PfEMP-1 is insoluble in the same detergent. The insolubility of PfEMP-1 in Triton X-100 suggests that the protein may be anchored to the erythrocyte skeleton (Howard et al., 1988). In *P. falciparum*, EMP-1 is organized on the surface of the erythrocyte by the knob structure while it is known that *P. c. chabaudi* AS does not have knobs. Thus, the putative homologue of PfEMP-1 in *P. c. chabaudi* AS may not be organized in clusters and, probably, the interaction with the erythrocyte skeleton is much weaker. Furthermore, it is only the surface radioiodinated fraction of PfEMP-1 that has been shown to be Triton X-100 insoluble. It is possible that an internal fraction of the same molecule exists (PfEMP-1 that has been synthesized on the ribosomes or is in the process of being exported). Western blot analyses of protein extracted from *P. c. chabaudi* AS infected erythrocytes would be expected to see both fractions.
If the high molecular mass protein(s) recognized by the immune sera in Western blots play a role in antigenic variation and sequestration, one would predict its presence on the surface of the infected erythrocyte. However, immuno-localizations assays showed very strong fluorescence immediately around the parasite (approximately 70% of the parasites were labeled) but internal to the erythrocyte membrane. In some preparations, however, weak fluorescence was also observed at the surface of the infected erythrocyte. PfEMP-1 has been shown to be a minor constituent protein on the surface of infected erythrocyte (Howard et al., 1988). If *P. c. chabaudi* AS does possess a homologue of PfEMP-1 this will not be organized into knobs and so would probably be poly-dispersed throughout the erythrocyte membrane making its detection much more difficult. The fact that not all the parasites seem to be recognized by the antibody could mean that either (i) the protein is made during a narrow window of the erythocytic cycle or (ii) the protein varies and a particular variant is present only in a subset of the parasitized erythrocytes.

Further work needs to be done to establish whether the protein is indeed a homologue of Pfvar. Thus, experiments need to be performed to determine if the protein is exported to the surface of infected erythrocytes (by radioiodination labeling and trypsin sensitivity), whether it is the binding ligand for endothelial cells and/or the variant antigen on the surface of infected erythrocytes. The antibodies raised in mice should be tested in *P. c. chabaudi* AS adhesion assays to determine whether or not these antibodies can block adhesion. Furthermore, the antibodies should be tested in immunoblotting assays against parasite antigens from first and recrudescence peaks as well as parasite antigens from different strains to check if, as for PfEMP-1, the protein demonstrates size heterogeneity and antigenic variability.
CHAPTER SIX

General Discussion and Future Plans

A dynamic balance characterizes the interaction between the malaria parasite and its human host. In the human populations of endemic areas, this interaction results in acute parasitaemias in primary infections of younger individuals or non-immune adults. Older individuals (who have been exposed to many infection episodes over extended periods and are clinically immune) can control parasitaemia but remain susceptible to reinfection.

How *Plasmodium* parasites survive for extended periods in an immunologically active host remains unclear but it is generally accepted (although not rigorously demonstrated) that one of the main mechanisms by which the parasite evades the host immune system is through antigenic variation. Thus, the parasite, by expressing a new variant type, is able to escape the host response directed to the previous antigenic variant type. In contrast with other organisms studied so far, *Plasmodium* variant antigens are expressed on the host’s cell surface rather than on the organism itself.

But why should *Plasmodium*, in the first place, expose its own antigens on the surface of the host erythrocyte where its intracellular location means it is hidden from the immune system? One reason may be to acquire the ability to sequester in the microvasculature of a number of host organs and thus avoid splenic clearance. Therefore, in order to benefit from one survival strategy, cytoadherence, the malaria parasite requires a second, namely antigenic variation, to minimize the effects of the host immune response directed against the cytoadherent neoantigens on the erythrocyte surface.

In *P. falciparum*, a single molecule (Erythrocyte Membrane Protein 1; PfEMP-1) expresses variant-specific epitopes and also mediates adhesion to endothelium (Leech *et al.*, 1984; Howard *et al.*, 1988; Howard *et al.*, 1989; Biggs *et al.*, 1991; Biggs *et al.*, 1992; Roberts *et al.*, 1992). The identification of the genes encoding PfEMP-1 have demonstrated that this antigen is an extensive family of antigenically and functionally distinct proteins encoded by the large *var* multigene family (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995).
In addition, PfEMP-1 seems to play a role in the acquisition of immunity to malaria (Bull et al., 1998). These results together with the idea that each particular parasite line presents a repertoire of distinct variants (Gupta et al., 1994) may explain the fact that, although human infections in endemic areas comprise a mixture of parasite lines (or even species), many episodes of infection are required before individuals become clinically immune. How all these factors interact to produce the dynamic host-parasite relationship which exists in human infections is a crucial question in malaria research and it was the starting point of this project.

The absence of a controlled in vivo model where all these host-Plasmodium interactions can be studied in the presence of immune pressure/selection has been a major barrier to understanding the detailed nature of malaria in man.

Rodent malaria infections of inbred experimental mice represent one way in which a detailed and critical analysis of this interaction can be achieved. Like Plasmodium parasites infecting man, rodent malaria parasites also comprise four species and a number of well characterized lines. \textit{P. c. chabaudi} infection of mice demonstrate a pattern of infection similar to that seen with \textit{P. falciparum} in humans, i.e., an initial acute phase followed by resolution and then chronic infection with recrudescences (Brown 1976; Jarra et al., 1985). In this infection, evidence for antigenic variation has been obtained (McLean et al., 1982a). Parasite sequestration does occur and there is strong evidence that sequestration and antigenic variation are functions of the same molecule (Gilks et al., 1990). Furthermore, inbred mice infected with cloned lines of rodent malaria parasites have already been used to investigate some aspects of these host-parasite interactions. These studies have revealed that recovery from primary infection renders mice relatively resistant to reinfection with the same parasite line. These animals rapidly control and clear a homologous challenge infection (then remain aparasitemic) but are vulnerable to reinfection with heterologous parasites (Jarra et al., 1985; Jarra et al., 1989b; Snounou et al., 1989; Snounou et al., 1992). This latter finding is also true of mice rendered hyperimmune by multiple infections with homologous parasites (Jarra, personal communication).

In this wider attempt to analyse this complex interaction between the malaria parasite and its host the present work had three main aims: (i) to characterize the cytoadherence features presented by \textit{P. c.chabaudi} AS infected erythrocytes; (ii) to
characterize the specificity of the immune response produced against parasite encoded antigens expressed on the surface of *P. c. chabaudi* AS infected erythrocytes and (iii) to find the homologue of the Pfvar in *P. c. chabaudi* AS.

In the first part of this project I have confirmed that *P. c. chabaudi* AS sequesters mainly in the liver and spleen by binding to endothelial cells. However, I also show, for the first time, that cytoadherence between infected erythrocytes and endothelial cells is also a feature in the brains of mice infected with this parasite. Furthermore, the results of an *in vitro* adhesion assay demonstrate that this binding involves, as in *P. falciparum*, adhesion molecules on the surface of the endothelial cells. These observations suggest this model may be used to study some aspects of sequestration as it occurs in *P. falciparum* in man, such as: (i) the role of the level of expression of adhesion molecules in the choice of the site of sequestration by infected erythrocytes and (ii) the role of sequestration in immune evasion. In this context, the premise that spleen avoidance is the primary goal of sequestration should be questioned since many *Plasmodium* spp. do not sequester and do have successful infections. Recently, it was suggested that agonistic CD36-ligands mediate an anti-inflammatory effect (Voll *et al.*, 1997). Thus, it should be investigated whether or not the binding of infected erythrocytes to CD36 in the microvascular endothelial cells mediates the same effect.

In humans exposed to *P. falciparum* it has been suggested that antibodies against variant-specific antigens (on the surface of infected erythrocytes) play an important role in immunity to malaria. Thus, in the second part of this project I studied the antibody response during acute *P. c. chabaudi* AS infections against antigens in the surface of infected erythrocytes. This study has identified potentially protective antibody during acute *P. c. chabaudi* AS infections, which specifically recognizes parasite-line specific antigens on the surface of infected erythrocytes. These results might explain the specific clearance observed in *P. c. chabaudi* AS infections as well as in human infections with *P. falciparum*. Moreover, the results also show that this antibody blocks the binding of erythrocytes to endothelial cells. All these findings may have important implications in the rationale behind malaria vaccine design.

PfEMP-1 has a central role in evasion of antimalarial immunity and pathogenesis as it mediates antigenic variation, sequestration and, probably, is important in acquired immunity during *P. falciparum* infections. *P. c. chabaudi* infections do undergo antigenic
variation and, in the present work, I have expanded upon previous observations (Cox et al., 1987; Gilks et al., 1990) and showed that \textit{P. c. chabaudi} AS infected erythrocytes present very similar cytoadherence features to the ones observed with \textit{P. falciparum}. Furthermore, parasite-line specific proteins on the surface of erythrocytes infected with \textit{P. c. chabaudi} AS seem to have a role, not only in cytoadherence but also, in acquired immunity. Thus, all of this evidence indicates that \textit{P. c. chabaudi} AS should have a functional homologue of PfEMP-1 on the surface of their infected erythrocytes. In an attempt to find the homologue of Pfvar in \textit{P. c. chabaudi} AS, a gene family was identified. Some features of these new \textit{P. c. chabaudi} AS genes, and the protein(s) encoded by them, such as size and cysteine-content in the N-terminal region, do seem to suggest that these genes might be the homologues of Pfvar. Furthermore, some indication was obtained that the protein(s) coded by these genes is, partially, Triton X-100 insoluble. However, it has been very difficult to show by immunofluorescence that this protein is, at some point of the parasite development, exposed on the surface of the infected erythrocyte. The fact that \textit{P. c. chabaudi} AS infected erythrocytes do not present knobs suggests that a putative homologue of PfEMP-1 in this parasite would not be organized in clusters and, as such, may be more difficult to detect.

Antigenic variation is seen as a mechanism of immune evasion that allows the parasite to maintain a chronic infection and that may have evolved to ensure that the parasite survives between transmission seasons. Recent work suggests that there are approximately 40 var-genes in the \textit{P. falciparum} genome (Newbold, personal communication) and that the switch rate \textit{in vitro} is 2\% per generation (Roberts et al., 1992). If this switch is random and reflects in any way what occurs \textit{in vivo}, then in a few generations the human immune system would have been exposed to the entire var-repertoire and it would be difficult to imagine recrudescence being a consequence of alteration in var-gene expression. This problem might be partially circumvented by the parasite due to the fact that people in endemic areas are exposed throughout their life to many different lines and that these different parasite lines may present different variant genes. However, in some endemic areas the transmission season is only for three months of the year. It is difficult to envisage that the parasite can be maintained for such a long period in an immune host under such conditions. Yet, there is no question that parasites do persist in these areas. Several central questions therefore arise such as: (i) is var-
expression truly responsible for the chronicity of malaria, (ii) are var-switches ordered, enabling the parasite to limit the number of variants seen by the host at any particular time, and (iii) is the var-repertoire naturally extendible, through recombination and/or segmental gene conversion? In the future these questions will be addressed in part through use of a controlled in vivo system where single and multiple infections can be performed with cloned lines. The number of P. chabaudi var will be determined. By sequencing a few, the variable regions of the molecule will be established. Cloning and expression of the variable region of the genes in the P. chabaudi var family will enable the production of antibodies against these regions. By passive transfer of antibodies to the variable regions we will be able to determine the true significance of the anti-EMP-1 immune response in the course of homologous and heterologous infections. This project has identified potentially protective antibody in the plasma of mice acutely infected with P. c. chabaudi AS that has an opsonic activity and is parasite line-specific. If this antibody is variant-specific, then the epitopes that this antibody recognizes should be mapped, and the importance of this antibody during acute and chronic infections assessed. Cloning of the variable regions will also allow us to design specific probes and determine whether or not the individual var-genes are expressed in a sequential order or in a random manner. Furthermore, the var genes for different rodent lines should be sequenced to establish whether or not these present different genotypes as in P. falciparum. If that can be established then a series of genotypic and phenotypic markers can be produced and a detailed analysis of multiple infections can be performed.

P. c. chabaudi AS in mice is, probably, the best available rodent model to study these aspects of host/parasite interactions. Furthermore, results from this project suggest it is a good model to study antigenic variation/sequestration/acquired immunity since these phenomena seem somehow to be intimately linked, and with very similar features to P. falciparum infections in humans. Although at this point, the results seem to suggest that the P. c. chabaudi AS genes identified in this project might be the homologues of Pfvar, further experiments are needed to establish whether or not the molecule(s) coded for by these genes is involved in P. c. chabaudi AS antigenic variation, sequestration or both. If that can be accomplished, then P. c. chabaudi AS will prove to be a practical and powerful model to study Plasmodium infections in an in vivo context, where a new set of
questions about antigenic variation/sequestration/acquired immunity can and should be addressed.
APPENDIX

A. During the last week of writing this manuscript, a blast search of the *P. falciparum* genome database revealed the identity of some short sequences, with a very high degree of similarities (approximately 70%) to the putative protein sequence of PcMMRACE4 clone. These sequences of *P. falciparum* were obtained from chromosome 14. Further analysis need to be performed to determine whether these *P. falciparum* sequences belong to any known *P. falciparum* gene family, including Pfvar.

B. Primers for detection of Pfvar homologues in *P. c. chabaudi* AS genomic DNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
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<tr>
<td>var1</td>
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</tr>
<tr>
<td>var2</td>
<td>CAA AAA TCT TCA/T GGC CAT TCA/T TCA AAC CA</td>
</tr>
<tr>
<td>var3</td>
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</tr>
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<tr>
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</tr>
<tr>
<td>DBL1C</td>
<td>TTT CTA CAA AAA TCT TCT/A GCC CAT TC</td>
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Acute Plasmodium chabaudi chabaudi Malaria Infection Induces Antibodies Which Bind to the Surfaces of Parasitized Erythrocytes and Promote Their Phagocytosis by Macrophages In Vitro

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CBA/Ca mice infected with 5 × 10⁴ Plasmodium chabaudi chabaudi AS-parasitized erythrocytes experience acute but self-limiting infections of relatively short duration. Parasitemia peaks (~40% infected erythrocytes) on day 10 or 11 and is then partially resolved over the ensuing 5 to 6 days, a period referred to as crisis. How humoral and cellular immune mechanisms contribute to parasite killing and/or clearance during crisis is controversial. Humoral immunity might be parasite variant, line, or species specific, while cellular immune responses would be relatively less specific. For P. c. chabaudi AS, parasite clearance is largely species and line specific during this time, which suggests a primary role for antibody activity. Accordingly, acute-phase plasma (APP; taken from P. c. chabaudi AS-infected mice at day 11 or 12 postinfection) was examined for the presence of parasite-specific antibody activity by enzyme-linked immunosorbent assay. Antibody binding to the surface of intact, live parasitized erythrocytes, particularly those containing mature (trophozoite and schizont) parasites, was demonstrated by immunofluorescence in APP and the immunoglobulin G (IgG)-containing fraction thereof. Unfractionated APP (from P. c. chabaudi AS-infected mice), as well as its IgG fraction, specifically mediated the opsonization and internalization of P. c. chabaudi AS-parasitized erythrocytes by macrophages in vitro. APP from another parasite line (P. c. chabaudi CB) did not mediate the same effect against P. c. chabaudi AS-parasitized erythrocytes. These results, which may represent one mechanism of parasite removal during crisis, are discussed in relation to the parasite variant, line, and species specificity of parasite clearance during this time.

An estimated 500 million clinical cases of malaria occur each year. Of these, only 1 million to 2 million, mostly young children, develop complicated and/or severe malaria and die. Of the remaining cases, many will be primary (possibly acute) infections in nonimmune individuals and will be treated, with various degrees of success, with antimalarial drugs. Yet others of these individuals must be capable of controlling potentially dangerous levels of parasitemia, in primary infections, in the absence of chemotherapy. Populations of areas endemic for malaria may be almost continuously exposed to infected mosquitoes during the transmission seasons. Even as they recover from their primary infections, many individuals are susceptible to reinfection and become semi-immune during successive infection episodes. It may take many years to establish protective immunity. How this is achieved is central in any analysis of immunity to malaria.

Malaria parasites demonstrate extensive antigenic diversity and undergo antigenic variation. Immunity to malaria in a range of hosts, including humans, is markedly parasite species, line, and variant specific, although a degree of cross-resistance is seen in some cases (10, 16, 20). These are factors which may partly explain the observed susceptibility to reinfection in humans. As such, they represent important considerations in the host-parasite interaction in human malaria and also in the design and application of effective vaccines. Study of the dynamics and relative efficacy of specific and cross-reactive immune responses occurring during primary infection and reinfection is therefore particularly relevant. Analysis of infections with the rodent malaria Plasmodium chabaudi chabaudi has allowed sophisticated modeling of this situation under laboratory conditions. Thus, (i) inbred mice infected with the cloned line of P. c. chabaudi experience acute but self-limiting infections (6), (ii) P. c. chabaudi AS is antigenically diverse and undergoes antigenic variation during a single infection (3, 13), and (iii) immunity to the parasite has been demonstrated to include variant-, line-, and species-specific components (7, 20). Immunity to malaria in various experimental animal hosts has been shown clearly to be both B- and T-cell dependent (14, 28). More recent studies of Plasmodium chabaudi infections in mice with genetic or experimentally induced lesions of their immune system suggested that parasite clearance after first peak parasitemia (crisis) is B-cell independent (25–27). These authors proposed that T-cell-activated macrophages secrete mediators which are directly cytotoxic to intraerythrocytic parasites. Such activity might well be parasite variant, line, or species specific at the T-cell (induction) level but would be relatively nonspecific at the macrophage (effector) level. Studies of P. c. chabaudi AS-infected (immunologically intact) mice, when animals were superinfected with homologous or heterologous parasites 1 or 2 days into crisis, clearly showed line or species specificity of parasite clearance (7, 20). These results suggested that the mediators of crisis were specific in nature and that nonspecific cell-mediated mechanisms cannot (alone) account for the massive parasite removal that occurs at this time. Later work identified a potent antiparasitic activity in plasma taken from P. c.
chabaudi AS-infected mice during early crisis. Thus, when P. c. chabaudi AS-parasitized erythrocytes (PE) were preincubated with such plasma in vitro and then injected back into reporter mice, the inoculum demonstrated markedly reduced infectivity in a parasite line-specific manner (8). In this report, we (i) demonstrate that plasma from mice undergoing crisis contains antibody which specifically binds to the surface of homologous PE and (ii) investigate the ability of such antibody to opsonize these cells for phagocytosis, since there is strong evidence that mononuclear cell phagocytic activity may play an important role in the clearance of parasitized during infection (22, 23, 29).

MATERIALS AND METHODS

Parasites and mice. CBA/Ca mice and P. c. chabaudi AS and CB parasites were maintained and prepared as previously described (6). The parasites were originally obtained as cloned lines from the WHO Registry of Standard Malaria Parasites, University of Edinburgh.

Preparation of APP and NP. A group of mice were infected intraperitoneally with 5 x 10^7 parasites and their parasitemia was monitored by light microscopy of tail blood films stained with Giemsa’s reagent. A control group was infected intraperitoneally with Krebs saline containing glucose (KGS) (6). On days 11 to 12 postinfection (approximately 1 to 2 days after peak parasitemia), mice from both the infected (infected) and uninfected (control) groups were bled into 200 µl of KGS containing 25 U of heparin per ml at 4°C. The blood was then centrifuged (2,000 x g for 4 to 5 min) at 4°C, and the plasma was removed and snap frozen in liquid nitrogen (APP) and stored at -70°C. APP (always) was always obtained on days 11 to 12 postinfection, and normal plasma (NP) was obtained from the sham-infected mice. In some experiments, plasma samples obtained on sequential days of infection were used; in the figures for these experiments, the relevant axis is labeled with the number of days of infection when plasma samples were taken.

Plasma fractionation. A protein G-Sepharose 4 Fast Flow column was washed at 4°C with 300 ml of phosphate-buffered saline (PBS) followed by 30 ml of binding buffer (0.2 M sodium phosphate [pH 7.0]), 6 ml of NP or APP was loaded onto the column, followed by 30 ml of binding buffer. The unbound IgG fraction (IgG fraction) was collected, and the IgG-rich protein retained on the column was then eluted with 30 ml of 0. M glycine-HCl (pH 2.7). Both fractions were dialyzed against PBS and concentrated to the initial volume of plasma, using Centricon 3 concentrators (Amicon).

Enzyme-linked immunosorbent assay (ELISA). P. c. chabaudi AS-infected CBA/Ca mice (40% parasitemia) were bled into KGS-heparin at 4°C to provide PE. The blood was passed through a CF11 cellulose powder (Whatman, Maidstone, United Kingdom) column to remove leukocytes and then washed three times with KGS with centrifugation at 750 x g for 4 min at 15°C. The final cell pellet was resuspended to 5 ml in KGS, and approximately 3 µl of 10% (wt/vol) heparin was added to the erythrocyte membranes. After centrifugation at 18,000 x g for 5 min at 4°C, the supernatant was removed, the pellets were resuspended with 3 to 4 volumes of a detergent buffer (1% Triton X-100, 5 mM EDTA, 100 mM Tris-HCl [pH 8.3]), and centrifuged at 20,000 x g for 5 min at 4°C. The supernatant was removed, and each well of a 96-well microtiter plate was coated with 50 µl of an appropriate predetermined dilution of this PE antigen in coating buffer (100 mM Tris-HCl [pH 8.0]). After overnight incubation at 4°C, the plates were washed with Tris-buffered saline-0.05% Tween 20 and blocked with 1% BLOTTO (Tris-buffered saline, 0.05% Tween 20, 0.05% dextrin milk) for 2 h at room temperature. Serial dilutions in BLOTTO of NP and plasma from different days of a P. c. chabaudi AS infection were then added. After incubation at room temperature for 2 h, the plates were washed three times as described above. A mouse IgG affinity-purified biotin-conjugated antibody (The Binding Site Co., Birmingham, United Kingdom) was added in (different experiments, conjugated antibodies directed against different mouse Ig isotypes were also used). The plates were incubated for 40 min at 37°C, washed six times, and exposed to streptavidin-conjugated alkaline phosphatase (Sigma) for 40 min at 37°C. The plates were then washed as described above, incubated for 15 min with substrate buffer (10 mM diethanolamine [pH 9.5], 0.5 mM Mg Cl2), exposed to the substrate p-nitrophenyl phosphate (Sigma), and read in a Titer-Tech Multiskan MCC/340 reader with a 405-nm filter. All samples were tested in triplicate, and background values were obtained by using 96-well microtiter plates where only coating buffer (without antigen) was added and the binding of antibody from each sample was assayed as described above. These values were subtracted from the respective values obtained for anti-PE binding.

Surface immunofluorescence antibody assay. PE and nonparasitized erythrocytes (NP) from infected and noninfected CBA/Ca mice, respectively, were washed three times by centrifugation in KGS containing 1% (wt/vol) bovine serum albumin (KGS-B), and 1 µl of the final cell pellet was a 0.5-ml tube. Ten microliters of homologous APP (from a P. c. chabaudi AS infection), heterologous APP (from a P. c. chabaudi CB infection), or NP diluted in KGS-B (1:4 to 1:10) was added, and the cells were gently resuspended before incubation at 37°C for 30 to 60 min. Anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma) was added to a final concentration of 25 µg/ml, and incubation was continued for 15 to 30 min at 37°C. Between each addition and following the final incubation, the cells were washed twice with ice-cold KGS-B. The final pellet was resuspended in 1 ml of KGS-B, and each sample was then analyzed in a Beckton Dickinson FACStar Plus fluorescence-activated cell sorter (FACS), using an Innova 90 argon ion laser at 488 nm. Using predetermined counting parameters for forward scatter (cell size for mouse E) and fluorescence intensity, a total of 10,000 events were recorded. The data were analyzed using FACSplot analysis software.

Phagocytosis assay. Macrophages were obtained from each of the CBA/Ca mice by peritoneal lavage with 3 to 4 ml of ice-cold RPMI 1640 medium supplemented with 5 U of heparin per ml. An erythrocyte-free leukocyte population of 1 x 10^7 to 2 x 10^7 cells/ml was usually obtained. One milliliter of this suspension was added to Leighton tissue culture tubes (Wheaton 385231) containing coverslips, gassed with 7% CO2-5% O2-80% N2, and incubated for 1 h at 37°C. Cells nonadherent to the coverslips were removed by washing the coverslips in situ with RPMI 1640 medium. One milliliter of RPMI 1640 containing 10% (vol/vol) fetal calf serum (FCS) was added to the adherent cells; the tubes were gassed as described above and incubated at 37°C for 2 h. During this period, aliquots of P. c. chabaudi AS PE (10^7 PE/ml containing mature trophozoites or schizonts at 40 to 50% parasitemia) or E in RPMI 1640-10% FCS were incubated with homologous APP, heterologous APP, NP, KGS, or homologous APP fraction at 37°C for 1 h at 70 to 80 rpm (G24 environmental incubator shaker; New Brunswick Scientific Co., Edison, N.J.). The cells were then pelleted by centrifugation, washed three times with RPMI 1640-10% FCS, and resuspended in the same medium. Each well was added to each Leighton tube. After a further incubation for 1 h, nonadherent or noningested PE and E were removed by gentle aspiration, and the coverslips were washed three times with 1 ml of PBS. Noningested but adherent PE and E were then lysed by a brief (20 s) treatment with cold distilled water, followed by an additional wash with 1 ml of PBS. This treatment had no detrimental effects on the integrity of the macrophages. The adherent cells were then fixed with methanol and stained with Giemsa’s reagent, and the numbers of internalized PE and E were assessed by light microscopy and quantitated as described in the legend to Fig. 6.

Statistical analyses. Statistical analyses were performed by using Student’s t-test, with P < 0.05 considered to be significant.

RESULTS

Kinetics of anti-PE antibody production during P. c. chabaudi AS infection. The binding activity of antibody in plasma during P. c. chabaudi AS infection was assayed by ELISA against preparations of total PE lysate. Total (parasite binding) Ig levels were examined in plasma samples collected between days 4 and 16 of a P. c. chabaudi AS infection initiated with 5 x 10^7 PE (Fig. 1, Total Ig). By the use of mouse Ig isotype-specific second antibodies, it was possible to differentiate the parasite-specific antibody binding by Ig isotype (Fig. 1, IgM, IgG1, and IgG2a). As early as day 4 postinfection, antibody binding was detectable and enhanced relative to NP (P < 0.01), increased between days 4 and 6 (P < 0.01), and peaked at day 7 (the mice went into crisis), and then gradually decreased. By day 16 postinfection (the parasitemia was almost resolved), antibody levels were still very high compared to those observed in NP (P < 0.01). In the IgG isotype-specific ELISAs, IgM reactivity against PE increased, initially quickly (significant difference observed between NP and infected mouse plasma on day 6 postinfection [P < 0.01]) and then more gradually, between days 7 and 11, peaked at day 12, and then decreased quickly. The level of IgG1 reactivity against PE antigen preparations increased very gradually, with significant differences observed only by day 8 postinfection (P < 0.01), peaked at day 12 postinfection, and then decreased. For IgG2a, the pattern observed was very similar to that for IgG1.

Surface immunofluorescence detects antibody binding to PE. Antibody binding to the surface of infected or noninfected cells was measured by immunofluorescence using E or PE containing either young trophozoites or mature trophozoites and schizonts. The cells were treated with NP or APP, and antibody binding was detected by using FITC-conjugated anti-mouse antibody and FACS analysis. In these assays, in the phagocytosis assay, PE were harvested from heavily infected mice at 2 to 3 days postinfection. The binding of antibody from APP to the surface of PE was higher than that of antibody from
The induction of both antibody and cell-mediated responses by infection with the malaria parasite is well documented (12, 14, 18). In some individuals, these responses act to control
parasitemia. However, the importance of each of these mechanisms in the dramatic decrease of parasitemia observed during crisis remains unclear. Antibody-mediated opsonization of PE, followed by their internalization and subsequent destruction by macrophages in the spleen and/or liver, might represent one of the most likely mechanisms to explain the resolution of parasitemia observed during crisis. As antibody-mediated targeting is very specific, this activity may explain the observed parasite species and line specificity of resolution. Antiparasite mediators produced by T-cell-activated macrophages capable of activating phagocytes to ingest parasitized red blood cells (PRBCs) may contribute to the rapid decline of parasitemia observed during crisis.

**FIG. 2.** Immunofluorescence analysis of antibody in APP binding to the surface of PE and E. E. (A and B) or PE containing either young parasites (C and D) or mature parasites (E and F) were incubated with NP (A, C, and E) or APP (B, D, and F), and antibody binding to the cell surface was quantified in duplicate samples by FACS analysis. The proportion of cells within the predetermined window of positive fluorescence is indicated. The window was determined by using E or PE incubated with KGS alone instead of a plasma sample.
of damaging intraerythrocytic parasites, but lacking the targeting specificity intrinsic to antibody, would be less likely to produce such specific parasite clearance.

We have analyzed the antiparasite properties of APP in P. c. chabaudi AS-infected mice and show that this activity is, at least partly, antibody dependent. ELISA showed that antibody is produced early in the P. c. chabaudi AS infection, and surface immunofluorescence demonstrated the presence of antibody in APP reacting specifically with the surface of infected cells by day 11 postinfection. In the ELISA, antibody binding was not line specific and cross-reacted between P. c. chabaudi AS and CB antigen preparations (results not shown). In contrast, antibody binding in the surface immunofluorescence assay was parasite line specific. That immunosays using disrupted or noninfect PE antigen do not reflect the specificity intrinsic to the in vivo biological activity of antibody in this system has been noted previously (9). Antigen expression or detection at the surface of live intact malaria-infected E is relatively parasite maturation stage dependent. Although the parasitic cell cycle in P. c. chabaudi is highly synchronous, mature parasite forms are less evident in the peripheral blood due to sequestration. This might explain the relatively low (maximum of 13.9%) numbers of positive PE in the surface immunofluorescence assay compared to the level of parasitemia in the cell population used (40 to 50%).

We also found a correlation between antibody binding to the erythrocyte surface and phagocytosis in vitro, using either unfractinated plasma or the IgG fraction obtained from it. The phagocytic activity was not stimulated by the fraction depleted of IgG (but containing IgM). Antibody binding to the surface of a small proportion of noninfected cells was detected by surface immunofluorescence, but these cells were not phagocytosed by macrophages. To be sure that these cells were not phagocytosed and destroyed very quickly, the phagocytosis assay was performed for different periods of time (such as 10, 15, 20, 30, and 45 min). Even under these conditions, uninfected E from either infected or normal mice were not seen inside macrophages (results not shown).

These studies indicate that infection of mice with P. c. chabaudi AS induced antibody against the surface of PE and that this antibody is capable of opsonizing these cells for phagocytosis. A role for macrophages and phagocytosis in the host response to malaria was proposed over 100 years ago (23). Observations (mainly histological) made by Taliaferro and Cannon (22) indicated that in experimental infections using canaries and monkeys, there was an initial rise in parasitemia during which parasites were slowly phagocytosed, primarily in the spleen and to a lesser extent in the liver and bone marrow. Similar observations have been made in spleens of rats infected with P. berghei (29). We have histological evidence which supports the above findings for P. c. chabaudi-infected mice (results not shown).

The antibody subclasses produced in response to infection are of particular relevance, since different antibody isotypes may have distinct biological functions. Macrophage receptors, recognizing the Fc region of antibody, are involved in a number of cellular functions, of which the ingestion of IgG-coated particles is fundamental in defense against bacteria and parasites (reviewed in reference 15). In the rat, IgG1 is most efficient for opsonization of erythrocytes for phagocytosis by macrophages, while IgG2a is more efficient in mediating antibody-dependent cytotoxicity (15). In the mouse, however, the situation seems not to be so clear, as here different isotypes of antibody are involved in the process of phagocytosis (17). In the surface immunofluorescence assay, we identified antibody isotypes present in APP which recognize the surface antigens of PE. IgM, IgG1, IgG2a, and IgG2b binding to PE were found in APP at levels above those in NP. However, no difference in the level of IgG3 binding was observed between NP and APP. Using a similar experimental model, Taylor Robinson (24) detected appreciable levels of IgG1 by ELISA only at day 20 postinfection, with a maximal level detectable during the recrudescence of parasitemia. However, our results show that significant levels of IgG1 as early as day 8 postinfection can be detected by ELISA.

Many studies have now shown that B cells and both Th1 and Th2 CD4 T cells play an important role in immunity to erythrocytic malaria. Chabaudi AS-infected mice are protected from death by a Th1 response, with induction of IFN-γ and TNF-α, and a Th2 response, with IgG1 and IgG3 production (27-29). We have histological evidence which supports the above findings for P. c. chabaudi-infected mice (results not shown).

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Phagocytic malaria. The results of many studies, particularly those using immunocompromised or genetically modified mice, suggest that control of primary peak parasitemia is B-cell independent and dominated by Th1 cells, while control of the recrudescence is B-cell and Th2 cell dependent (11, 24, 25). However, exactly when and why there is the switch from predominantly Th1 to predominantly Th2 activity and the exact roles of these activities in crisis and eventual elimination of parasitemia are still not clear. What does seem clear in the systems described above is that infected mice can, at least
partially, control initial parasite replication during crisis in the absence of B cells. Our results provide evidence that as early as 1 to 2 days after peak parasitemia, specific mechanisms (detecting infected cells versus noninfected cells or homologous line versus heterologous line) act, possibly to damage parasites but certainly to target PE for clearance. We also show that antibody seems to play a central role in the PE clearance during this period. The apparent discrepancies in all these findings might be explained by assuming that in the absence of one (or several) immune mechanisms, other compensatory responses become more important.

This study has identified potentially protective antibody in the plasma of mice infected with *P. c. chabaudi* AS at the time of crisis. Future experiments will be designed to investigate this activity with respect to (i) whether it demonstrates variant specificity, (ii) the in vivo mechanism of specific parasite clearance observed as the primary peak parasitemia is resolved, (iii) the selection or induction of new parasite variants which may occur during crisis (3), and (iv) the nature of the antigens on the parasitized cell surface. In this context, the presence of erythrocyte membrane protein 1 (5) and var gene (2, 19, 21) homologs in *P. c. chabaudi* AS needs to be investigated. If these goals can be accomplished, then the *P. c. chabaudi* AS model will be a powerful system in which many more aspects of the host-parasite interaction in malaria can be analyzed in detail.

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