Chapter 1

REVIEW OF LITERATURE
1. Review of Literature

1.1 History of malaria

It is speculated that malaria originated in Africa and accompanied human migration to the Mediterranean shores, India and South East Asia. The origin of the name malaria stems from the archaic association between the disease and the bad air of marshy areas around Rome, "malaria" in Italian meaning "bad air" (Bruce-Chwatt, 1985). Writings from the vedic period (1500 to 800 B.C.) describe the enlargement of the spleen in persons having autumnal fever, suggesting that at that time malaria existed in India (Sherman, 1998). Since there is no mention in any of the Hippocratic writings about severe, malignant tertian fever, it is assumed that at that time *P. falciparum* infections were rare or non existent.

By the 12th century, malaria has spread reaching European countries like Spain, Poland and Russia. There are no records of malaria in the New World before European explorers landed on its shores, so it is assumed that they brought *P. malarie* and *P. vivax* to the Americas, *P. falciparum* was introduced later by the importation of African slaves (Bruce-Chwatt, 1988). Thus, by early 1800’s malaria was worldwide in its geographical distribution.

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 England, as "Jesuits powder," so that those suffering from "agues" might benefit from the chemical substance quinine, which it contained (Nobel Prize Foundation, 2006).

The discoveries of Alphonse Laveran in 1880 when he identified the causative agent for human malaria to be a blood parasite, and Sir Ronald Ross in 1897 when he demonstrated that mosquitoes were the vectors of malaria, were two Nobel prize discoveries in malaria, which marked the beginning of a new era for the control of this deadly disease (Gibson, 1998). In 1885, Golgi identified the asexual development and reproduction by multiple fission of *P. vivax* and *P. malariae* and showed the correlation between the beginning of the fever and rupture of the infected erythrocytes.
Today malaria is known as an infection transmitted by mosquitoes of the genus Anopheles. There are four species of Plasmodium, which can cause malaria in humans i.e. P. falciparum, P. vivax, P. malariae, and P. ovale, which belong to the phylum Protozoa. The spectrum of clinical illness ranges from cyclic fevers with rigors and chills, to anemia, hypoglycemia, convulsions, hepatic dysfunctions and bleeding abnormalities (White, 1998).

1.2 Current situation of malaria in the world

Malaria is considered the world’s most important tropical parasitic disease. Approximately 300 – 500 million clinical cases are recorded every year in the world. Each year malaria kills 1-million people worldwide, 80% of which are living in Sub-Saharan Africa. The large majority of the victims are children under the age of 5, as indicated in the first joint World Malaria Report 2005 of the WHO (World Health Organization) and UNICEF (United Nations Children’s Fund). Around 107 countries or territories in the world are affected by malaria, with almost half of them located in Africa south of the Sahara and around 3.2 billion people at risk of acquiring the infection. It has been estimated that malaria kills 3.000 children every day only in Africa (Phillips, 2001).

Many factors contribute to the successful transmission of the infection in endemic areas. The host (human) immunity, the specificities of each parasite species, the anopheline longevity and its avidity for human are intrinsic factors that have the greatest impact on the malaria burden. In addition, the increasing development of resistance of Plasmodium parasites to the available drugs, and of the mosquitos to the insecticides, makes the task of controlling malaria in actual times much more challenging. Among the extrinsic factors, climate changes (mainly rainfall), economic conditions (poverty), political commitment and effectiveness of prevention efforts are the most important determinants.
The economic cost of malaria is very high in the affected regions. Annual economic growth in countries with high malaria transmission has historically been lower than in countries without malaria. Economists believe that malaria is responsible for a 'growth penalty' of up to 1.3% per year in some African countries (Roll Back Malaria, 2005). In areas of low transmission like South East Asia, Central and South America; the number of days of work lost every year because of malaria has been calculated between 1.5 and 14.3 days per person (Trigg et al., 1998). Malaria and poverty are very intimately associated. Malaria is most problematic for countries in Africa, the poorest continent in the world; poverty may promote malaria transmission and in turn malaria may cause poverty.

In areas where transmission is high, such as tropical Africa and Papua New Guinea, children under the age of 5 and pregnant women are most vulnerable to the disease. On the other hand, all age groups are at risk in areas of low transmission; epidemics may be common due to the low level of acquired immunity in the population (Trigg et al., 1998). Human activities like wars, forced migrations and tourism have increased the number of population at risk of suffering malaria in the world. (Center for Disease Control and Prevention, 2006). Figure 1.1 depicts the worldwide occurrence of malaria.
Effective control of malaria depends on three essential elements. The reduction of the population of Anopheline mosquitoes that can transmit the parasite, the early diagnosis and prompt treatment of malarial disease in all areas where people are at risk and an adequate epidemiologic surveillance, which allows forecasting of epidemics in specific regions. The development of effective drugs and/or a vaccine against human malaria parasites, as well as, the implementation of selective vector control strategies are the actual priorities of governments and institutions around the world. At present, no vaccine is in use against malaria, however some of the experimental vaccines are under different stages of trials using parasite proteins as protective antigens (Portfolio Malaria Vaccine Candidates, 2005). In recent years, several international agencies have been created to initiate new programs to combat malaria. Roll Back Malaria was started in 1998, mainly to control malaria in children and pregnant women in Africa. In 1999 two new international programs were started i.e. MMV- Medicines for Malaria Venture and MVI- Malaria Vaccine Initiative. These two programs are funding several research projects involving the discovery of new drugs and the development of an effective vaccine.

1.3 Life cycle of malaria parasites

The life cycle of *Plasmodium* is complex, involving human and mosquito hosts, and many developmental stages (Figure 1.2). Sporozoites, thought to be less than 100 on each occasion, are transmitted to humans by the bite of infected female *Anopheles* mosquitoes. Within 30 to 45 minutes these sporozoites enter the host’s hepatocytes, where they develop into exo-erythrocytic schizonts during the next 6 to 15 days, depending on the species. At the end of the pre-erythrocytic cycle, 30,000 to 40,000 merozoites are released into the blood circulation and invade erythrocytes in a process that takes approximately 30 seconds (Fujioka and Aikawa, 2002). The complete process of invasion can be observed in Figure 1.3

*Plasmodium vivax, Plasmodium ovale* and *Plasmodium cynomolgi* have a dormant stage, named hypnozoite (Krotoski et al., 1982) that may remain in the liver for weeks to many years before the development of preerythrocytic schizogony. This results in relapses of malaria infection.
Within the erythrocyte, the parasite develops over a period of 2 (P. falciparum, P. vivax and P. ovale) or 3 days (P. malariae). Within the parasitophorous vacuole, the parasite modifies its host cell in several ways to enhance its survival. The parasite matures from an initial ring form to a trophozoite, which, following mitotic division, develops into the schizont stage containing up to 32 merozoites. Following rupture of the cell, the merozoites are released which then immediately invade additional erythrocytes.

After invading red blood cells, eventually some of the merozoites differentiate into sexual forms: the male and female gametocytes. When a female Anopheles mosquito takes a blood meal from an infected host, the gametocytes that are ingested with the blood meal mature to male and female gametes in the mosquito mid-gut. After fertilization, the resulting zygote matures within 24 hours into the motile ookinete, which burrows through the midgut wall to encyst on the basal lamina, the extracellular matrix layer separating the haemocoel from the midgut. Within the developing oocyst, there are many mitotic divisions resulting in oocysts full of sporozoites. Between 7 and 15 days post infection, depending on the Plasmodium species and the ambient temperature, a single oocyst forms more than 10,000 sporozoites. These motile sporozoites migrate into the salivary glands and accumulate in the acinar cells of the salivary glands. When an infected mosquito bites a susceptible vertebrate host, the Plasmodium life cycle begins again, with the inoculation of sporozoite stages into the blood stream of the infected host.

1.4 Structure of the merozoite

The erythrocytic merozoite is an ovoid cell and measures approximately 1.5 um in length and 1 µm in width. The apical end of the merozoite is a truncated cone-shaped projection demarcated by the polar rings. Three types of membrane-bound organelles, namely, rhoptries, micronemes and dense granules, are located at the anterior end of the merozoite. The contents of these organelles play a role in the binding and entry of the merozoite into the host cells. The merozoite is surrounded by a trilaminar pellicle that is composed of a plasma membrane and two closely aligned inner membranes (Torii, 1998). There are subpellicular microtubules, which originate from the polar ring of the apical end; they have been suggested to function as cytoskeleton and may be
involved in invasion (Bannister and Mitchell, 1995). The nucleus and mitochondrion lie within the posterior end, an additional structure, referred to as a spherical body, has been identified (Torii, 1998). Another organelle, the apicoplast, has become the focus of research in the field of drug development in recent years, since the discovery that is essential for parasite survival in the erythrocyte and its evolutionary relation with the chloroplast from plants (Fichera and Roos, 1997). This organelle is involved in fatty acid and isoprenoid metabolism and can be targeted with drugs that act as herbicides and do not affect the mammalian host (Waller and McFadden, 2005).

The outer membrane of the extracellular merozoite is surrounded by a surface coat of about 20 nm in thickness; this coat appears to play an important role in the initial steps of the erythrocyte invasion. A series of merozoite surface proteins (MSP), including MSP-9, have been characterized and localized in this membrane; some of these antigens are being developed as a promising vaccine candidates like MSP-1, MSP-2, MSP-3, etc.
Several evidences support the development of a vaccine against erythrocytic stages of malaria parasites; 1) Following repeated attacks of malaria, a majority of infected individuals in endemic areas are able to control the parasite replication to levels that do not result in clinical disease, 2) hyperimmune globulin prepared from the sera of individuals chronically infected with malaria can eliminate circulating parasites from *P. falciparum*-infected individuals, 3) maternal antibodies passively transfer to the fetus may provided a window of protection against clinical malaria (Ballou et al., 2004; Good, 2005)

In contrast to the requirement of vaccines for smallpox, polio or many of the other infectious diseases for which vaccines are available, the requirement for a vaccine against malaria is not universal. One of the main target groups comprises infants, children and pregnant women resident in malaria endemic areas. Another target group is individuals who have never been, or are infrequently exposed to malaria (non-immunes). These non-immunes include tourists, business, and military personnel from countries without malaria who travel to countries where malaria is transmitted; in addition, there is increasing number of residents from malaria endemic countries, living in malaria-free areas, who should be considered as non-immune individuals (Hoffman and Miller, 1996).

A malaria vaccine based on asexual blood-stage antigens is expected to control the magnitude of the asexual parasitaemia and also to decrease the incidence of severe disease. Such “anti-morbidity” vaccine would be of great benefit for the communities living in highly endemic areas, in particular, pregnant women and infants of these communities (Richie and Saul, 2002). It is believed that the principal immune mechanism induced by these vaccines is the generation of protective memory B-cell response that, at the time of infection, will generate antibodies that will neutralize important merozoite antigens, preventing in this way the process of invasion of erythrocytes. However, there is growing evidence from murine models of malaria that cell-mediated mechanisms may be critical to the acquisition of acquired immunity to malaria (Ballou et al., 2004).
1.6 Merozoite Surface Proteins

The proteins located on the surface of the merozoite are considered potential targets of the protective immune response; for that reason their identification and characterization is important for the development of an effective malaria vaccine. The first protein of this kind was identified in 1981 (Holder and Freeman, 1981) and since that time, a total of 10 proteins have been categorized as Merozoite Surface Proteins (MSPs); numbered according to the order in which they have been characterized. In recent times, the information generated from the complete genome sequences of *P. falciparum* and *P. yoelii* (Carlton et al., 2002; Gardner et al., 2002), as well as the development of transfection techniques has facilitate the characterization of new MSPs, not only for their potential as a vaccine candidates but also to better understand their role in the biology of *Plasmodium* parasites.

1.6.1 Merozoite surface protein-9 (MSP-9)

The first description of MSP-9 was done by Stahl et al. (1986), who reported the isolation of a group of *E. coli* clones expressing *P. falciparum* antigens that reacted with human immune sera. One of the antigens that they identified was described as a protein of 102 kDa, predominantly present in schizonts, which partial DNA sequence revealed blocks of hydrophilic aminoacids at the C-terminal. For this reason, the new protein was designated as acidic basic repeat antigen (ABRA) (Recently named PfMSP-9). Simultaneusly, Lyon et al. (1986), identified an antigen of 101 kDa molecular weight (p101) along with 14 more antigens from *P. falciparum* blood-stages, that were isolated from immune clusters of merozoites (ICM), formed in the presence of inhibitory human immune sera. The authors considered this method as a useful approach to identify exposed targets of protective immunity against malaria.
Figure 1.3  (A) Schematic representation of the merozoite structure. (B) Electron microscopy photographs showing the process of invasion of erythrocytes, principal target of vaccines against merozoite antigens. (1) Apical end of a P. knowlesi merozoite attaches to an erythrocyte(E). (2) Further advanced stage of erythrocyte entry by a P. knowlesi merozoite. No surface coat is visible on the portion of the merozoite surface, which has invaginated the erythrocyte membrane, while the surface coat is present behind the junction (arrow) site. (3) Erythrocyte entry by a P. knowlesi merozoite is almost complete. (4) A trophozoite (ring form) stage of P. falciparum is surrounded by the parasitophorous vacuole membrane (PVM). R: Rhotry; D: dense granules; Mn: micronemes; E: erythrocyte; N: nucleus. Bars: 0.5 um. (Photographs are reproduced from Fujioka and Aikawa, 2002).
Subsequently, Chulay et al. (1987) characterized the monoclonal antibody 3D5 (3D5 mAb) that was produced from mice immunized with the ICM, which specifically recognized the antigen of 101 kDa reported by Lyon et al. (1986). With the use of 3D5 mAb it was possible to localize p101 antigen at the surface of schizonts and free merozoites as well as in the parasitophorous vacuole. This antigen was detected as secreted in the media when schizonts rupture occurred in normal culture media but not when the rupture occurred in the presence of immune sera. In addition, the affinity-purified p101 antigen was recognized in western blot by 3D5 antibodies dissociated from the ICM. Using $[^3]$H]lle-labeled parasites, they also showed that p101 antigen was synthesized late in the parasite cycle, when mature throphozoites and early schizonts predominated.

In 1988, Weber et al. (1988) reported the complete DNA sequence of ABRA and described the full-length protein as having 743 amino acids, which included two tandemly repeated regions, one near the amino terminus containing eight hexapeptide repeats of sequence TVNDEDED, and the second near the carboxyl terminus containing primarily KE and KEE repeats. The protein was defined as hydrophilic and highly charged with a calculated isoelectric point of 5.6. Nine potential sites for N-glycosylation were identified in the sequence of ABRA, however no experimental evidence was obtained. In addition, there were no evidences of hydrophobic motifs that could suggest that ABRA was a structural membrane protein.

The immunogenic properties of ABRA has been partially studied. Sharma et al. (1998) used synthetic peptides (12 – 18 residues) covering the most hydrophilic regions of ABRA as well as the repetitive sequences, to study the presence of B and T-cell epitopes in the sequence of this protein. They found that all the peptides were recognized by specific antibodies present in the sera of 50 individuals with acute P. falciparum malaria, with peptide AB-3 (395 - 409 aa) being the most frequently recognized. In addition, high lymphoproliferative response was observed against AB-1(19 – 30 aa) and AB-3 peptides after in vitro stimulation of PBMC from 11 of these individuals. Six of the peptides induced a strong antibody response in rabbits when they were used as immunogen in the absence of any carrier, indicating the presence of both B-cell determinants and T-helper-cell epitopes in these six constructs. The antibodies
generated against peptides AB-1 and AB-5 (518 – 531 aa) inhibited the merozoite invasion of human erythrocytes in vitro up to approximately 90%.

The presence of B and T cell epitopes in ABRA was further confirmed by Kushwaha et al. (2001), who used recombinant proteins representing different fragments of ABRA expressed in Escherichia coli, and purified by affinity chromatography. Immunogenicity studies in mice and rabbits showed that the N-terminal portion was the most immunogenic region of ABRA, IgG1 predominance was found in mice immunized with the constructs designated ABRA(P) (aa 24 – 507) and ABRA (N) (aa 24 – 369). In this study, the antibodies against the middle and C-terminal region of ABRA were able to prevent the parasite growth in 82% and 93%, respectively. The recombinant proteins were also recognized by the sera from P. falciparum-infected patients, with ABRA(P) showing the highest frequency of reactivity (73%). T-cell proliferation experiments in mice immunized with these constructs revealed that the T-cell epitopes were localized in the middle portion of the protein. Pimtanothai et al. (2000) using computer algorithms, predicted T-cell epitopes in the sequence of ABRA and showed that the synthetic peptide ABRA 14 (487 – 506 aa) induced significant T-cell proliferation in splenocytes from immunized mice and Th-1 associated cytokine production.

A study by Bonnefoy et al. (1994) described the used of a P. falciparum protein fraction, containing ABRA and other blood-stage antigens in the range of 90 – 110 kDa, to immunize S. sciureus monkeys that were subsequently challenged with P. falciparum Uganda Palo Alto FUP/SP strain. Three monkeys out of five resisted the dose of virulent parasites, however no correlation was found between the antibody response to ABRA and the state of protection in these monkeys.

The identification of the homologues of ABRA from P. vivax, P. cynomolgi and P. knowlesi (Barnwell et al., 1999; Vargas-Serrato et al., 2002), allowed the delineation of features common to this group of proteins and the definition of a new family named Merozoite Surface Protein – 9. Recently the MSP-9 homologue from P. coatneyi was also described (Vargas-Serrato et al., 2003). One of the main features of this family is the conservation of the N-terminal region, including four cysteine residues in all these proteins and the presence of tandem repeats at the C-terminal region. However the sequence of these tandem repeats is not conserved among the MSP-9 proteins and only
PfMSP-9 (ABRA) contain the repeats KE/KEE that confer the character of acidic-basic repeat antigen. In addition, *P. vivax* MSP-9 and the simian homologues do not contain the block of repeat sequences present in the middle region of PfMSP-9. *P. vivax* MSP-9 is the largest protein of this family, composed of 979 aa, followed by *P. cynomolgi* (818 aa), *P. coatneyi* (764 aa), *P. falciparum* (743 aa) and *P. knowlesi* (707 aa). These proteins are predicted to assume an alpha-helical conformation at the N-terminus and to have a combination of alpha helix and random coils at the C-terminus. There is no indication of GPI lipid anchor associated with these proteins. The level of identity between PfMSP-9 and *P. vivax*, *P. cynomolgi* and *P. knowlesi* MSP-9 proteins is estimated in 33-34%; antisera against the recombinant N-terminal fragment of *P. cynomolgi* MSP-9 and *P. knowlesi* MSP-9 cross-react with the native *P. vivax* MSP-9 but not with *P. falciparum* MSP-9 native protein. In a similar way, antisera against recombinant *P. vivax* MSP-9 N-terminal fragment recognize *P. cynomolgi* and *P. knowlesi* homologues but not *P. falciparum* MSP-9.

Oliveira-Ferreira et al. (2004) have shown that the N-terminal region of PvMSP-9 as well as the second block of tandem repeats are immunogenic in mice, inducing high levels of antibody titers, with predominance of IgG1, IgG2a and IgG2b isotypes. Moreover the immunization with N-terminal PvMSP-9 induced IFNγ and IL-5 by splenocytes re-estimated in vitro.

Another important characteristic of this family of proteins is the presence of species-specific repeat sequences in the C-terminal region. Alignment of all the seven members of this family shows that there is a direct correlation between the length of the repeats and the total number of aa for each protein; for example *P. vivax* and *P. cynomolgi* being the biggest proteins of these family (979 aa and 818 aa respectively), contain two regions with repeat sequences as long as 14 aa which are repeated 4 to 6 times, while in *P. yoelii* (678 aa), the repeat sequences are maximum 9 aa and are repeated only three times. Although the structure of the repeats is different for each MSP-9 homolog, all these sequences are characterized by a high content of glutamic acid that gives a total percentage for the whole protein ranging from 11% to 18%. The function of these repeats in MSP-9 proteins is not known but is possible that they are merely related with the mechanisms of evolution of these proteins.
1.6.1.1 Predicted functions for MSP-9 proteins in Plasmodium parasites

1.6.1.1.1 Binding to erythrocytes

Being located in the surface of merozoites, is logical to think that MSP-9 proteins may interact with erythrocyte proteins at the time of invasion. There are examples of other surface antigens interacting with structural proteins of erythrocytes like MSP-1, ring-infected erythrocyte surface antigen (RESA), etc. (Foley et al., 1991; Herrera et al., 1993). In addition, it has been demonstrated that the erythrocyte protein Band 3 is subjected to proteolytic cleavage in parasite infected erythrocytes, which induced conformational changes that probably leads to cytoadherence (Winograd and Sherman, 1989). It is also possible that parasite proteases are involved in this process.

The hypothesis that PfMSP-9 binds to human erythrocyte proteins was addressed by Kushwaha et al. (2002). Using E. coli expressed recombinant fragments of PfMSP-9, they demonstrate that the cysteine-rich N-proximal region of this protein binds in a specific dose-dependant manner to Band 3 protein. The binding of purified native PfMSP-9 to band 3 protein, confirm the observations with the recombinant proteins. They also observed the conservation of 528 bp region containing the four cysteines in the N-terminal region among fifteen field isolates of P. falciparum, confirming the importance of this region for the function of MSP-9 proteins. Simultaneously, Curtidor et al. (2001) screened the complete sequence of PfMSP-9 to identified specific motifs that bind to human erythrocytes. They were able to define a region in PfMSP-9 encompassed between residues 121 and 240 with high binding specific activity to erythrocyte membranes. They concluded that the specific binding was independent of the charge of the peptides and mainly dependent on hydrophobic interactions, since 30% of the critical residues were hydrophobic. In addition, peptides 2148 (121-140 aa) and 2149 (141-160 aa) were able inhibit the erythrocyte invasion by merozoites, suggesting that they were able to compete with native ABRA for the binding and that this event is important for erythrocyte invasion.

Recently, the interaction of PfMSP-9 with Band 3 protein has been studied in more detail by Li et al. (2004). They demonstrate that the N-terminal portion of PfMSP-9 interacts specifically with a domain denominated 5ABC, present in a non-glycosylated exofacial
region of Band 3 protein, which they previously reported as an important host receptor for the sialic acid-independent invasion pathway. They also showed that 5ABC domain interacts with MSP-142 protein through its 19 kDa C-terminal domain (Goel et al., 2003). They were able to detect the interaction PfMSP-9/5ABC through yeast-two hybrid system, using recombinant constructs representing multiple regions of PfMSP-9 as well as Band 3 protein. The interaction was confirmed with a binding assay in solution, using purified GST fusion proteins expressed in E. coli. As suggested by the results of Curtidor et al (Curtidor et al., 2001), this study demonstrates the importance of PfMSP-9/Band 3 interaction for the erythrocyte invasion. Because 5ABC domain has been shown to bind MSP-142 also (Goel et al., 2003), they studied the interaction of MSP-9 and MSP-142, finding that, indeed, MSP-9 recombinant proteins were able to co-precipitate native MSP-142 from parasite culture supernatant and viceversa. These results allowed them to propose a model for the simultaneous interaction of P. falciparum MSP-9 and MSP-1 with the erythrocyte Band 3 protein (Figure 1.4), in which the co-ligand complex MSP-142 – MSP-9 interacts with the 5ABC domain of the same Band 3 molecule or with different 5ABC domains within the Band 3 dimer/tetramer in the erythrocyte membrane. The existence of MSP-142/MSP-9 in a complex is consistent with the previous evidences of MSP-1 processing fragments associated with other proteins like MSP-636 and MSP-722 in the surface of the merozoites (Pachebat et al., 2001; Trucco et al., 2001). A recent study by Kariuki et al. (2005) confirms these results.

Recently, a network of protein interactions from P. falciparum blood-stages have been described by LaCount (2005), using a high-throughput yeast two-hybrid assay. The interactions were defined by computer analyses of the network connectivity, presence of common protein domains, the coexpression of the genes encoding interacting proteins and Gene Ontology annotations. One of the identified cluster is defined by the direct interaction of MSP-1 and MSP-9 as a core, linking 19 uncharacterized proteins and 16 other proteins that are involved in the invasion of host cells or are localized to the merozoite surface.
1.6.1.1.2 Protease activity

Since the initial reports of Nwagwu et al. (1992) and Weber et al. (1988), it has been observed that the native or recombinant PfMSP-9 is an unstable protein, that degrades in several smaller fragments after its purification. It was also observed by Weber et al. (1988) that this "autoproteolysis" could be prevented by chymostatin. Moreover, Nwagwu et al. (1992) reported that PfMSP-9 (p101-ABRA at that time) was one of several *Plasmodium falciparum* antigens that bound to a chymostatin-polyacrylamide affinity gel, which suggested its behavior as chymotryptic like proteinase.

The experiments of Nwagwu et al. (1992) were the first demonstration that native PfMSP-9 possessed chymotripsin-like activity. Using the monoclonal antibody 3D5, (Chulay et al., 1987) coupled to beads, they were able to pull-down native PfMSP-9 from parasite cell extract and showed its proteolytic activity against the fluorogenic substrate MeOSuc-KLF-AFC. In another approach, they observed protease activity against Suc-LLVY-AFC fluorogenic substrate, associated with the fractions ~200 kDa and ~90 kDa from schizonts extracts fractionated by ion-exchange and gel filtration chromatography; monoclonal antibody 3D5 was able to remove 74 to 96% of the chymotrypsin-like activity from these fractions. In addition, the protease activity associated with PfMSP-9 was sensitive maximally to chymostatin (a chymotrypsin inhibitor) and leupeptin (serine
protease inhibitor but not active against chymotrypsin), but seemed unaffected by treatment with PMSF or EDTA.

The protease activity of recombinant PfMSP-9 has been reported by Kushwaha et al. (2000). In this study, they demonstrated that the protease activity of PfMSP-9 resides in the N-terminal region of the protein. Recombinant fragments representing different regions of PfMSP-9 were expressed in *E. coli* with a His tag or in fusion with MBP; the purified proteins were used for protease assay with fluorogenic substrates. Only the fragment named ABRA-(P) (aa 24 – 507) exhibited chymotrypsin-like activity against the substrates Nsuc-LLVY-AMC and Nsuc-AAPF-AMC, but not against Nsuc-AFK-AMC and NCbz-FR-AMC substrates. As shown for the native PfMSP-9, ABRA-(P) activity was inhibited by chymostatin, and in contrast to the results of Nwagwu et al. (Nwagwu et al., 1992) the activity of ABRA-(P) was inhibited by PMSF while leupeptin did not have any effect.

Inspite of the findings described above, there is no significant homology of PfMSP-9 with any known protease. Nwagwu et al. (1992) proposed that PfMSP-9 could be a serine protease, based on the homology with the sequence SGG of a chymotrypsin enzyme from rat. This sequence is predicted to contain the putative catalytic serine, present in most of the serine proteases and associated with the typical catalytic triad (Ser-195, Asp-102, His-57). There is only one report by Garber et al. (1993) describing partial homology PfMSP-9 with a 60 kDa protease from *Trichomonas vaginalis*; coincidently this protein also showed degradation during its purification. On the other hand, the serine present in the SGG sequence is not conserved in rest of the PfMSP-9 homologues reported until today (Vargas-Serrato et al., 2002); instead *P. vivax*, *P. cynomolgi*, *P. knowlesi* and *P. coatneyi* MSP-9 contain an EGG motif. Vargas-Serrato et al. (2002) has suggested that two His and Asn residues, 12-24 residues apart, which are conserved in *P. vivax*, *P. cynomolgi*, *P. knowlesi* and *P. falciparum* match with the configuration of the catalytic active site of papain-like cysteine enzymes.
1.6.1.1.3 Other predicted functions

Along with the ability to inhibit parasite invasion, the peptide 2149 (aa 141 – 160), described by Curtidor et al (2001) also showed high homology with a human cytosolic phospholipase A2. Moreover, this peptide also exhibited haemolytic and anti-microbial activity \textit{in vitro}. These findings have raised the hypothesis that PfMSP-9 might be acting as a phospholipase in the parasite. Interestingly, Roggwiller et al. (1998) has described the presence of a stage-specific haemolytic activity detected in \textit{P. falciparum} schizonts, that resembles a cytosolic group IV phospholipase A$_2$. HELLs (Haloenol Lactone Suicide Substrate), a specific inhibitor of cytosolic calcium-independent phospholipase A$_2$ activities, inhibited 50% of the detected haemolytic activity as well as the erythrocyte reinvasion by 40 to 50%.

The need of phospholipase activity for the merozoites to alter the erythrocyte membrane, in order to invade is highly probable. It has been reported that \textit{P. falciparum} infection drastically reduced the phospholipid unsaturation index in the erythrocyte membrane (Hsiao et al., 1991). Recently, the characterization of a surface phospholipase from \textit{P. berghei} involved in the migration of sporozoites through cells, has been reported by Bhanot et al. (2005). This new enzyme contains the motif GXSXG characteristic of lipases and a catalytic triad of serine, aspartate and histidine that is present in several phospholipases. By disrupting the open reading frame of this protein, the authors demonstrated that, the absence of this phospholipase activity reduced the infectivity of \textit{P. berghei} sporozoites by 90% and also affected their ability to cross cell membranes \textit{in vitro}. It is interesting to notice that the motif GXSXG showed in the alignment of this protein with other putative lipases of \textit{Plasmodium} (Bhanot et al., 2005), overlaps with a conserved sequence SLGG, which resembles the SGG motif present in PfMSP-9 and associated with its protease activity.

Another study by Hanada et al (Hanada et al., 2002) described the characterization of an enzyme from \textit{P. falciparum} erythrocytic stages (PfNSM), homologous to a bacterial sphingomyelin enzyme and with biochemical properties of a phospholipase C. The addition of the specific inhibitor Scyphostatin inhibited PfNSM recombinant protein activity as well as the intraerythrocytic proliferation of \textit{P. falciparum} in a dose-dependent manner. The morphological changes were observed mainly at the trophozoite stage,
which according to the authors indicates that the inhibited activity is important for the progression from trophozoite to schizont, which coincides with the peak transcription of PfNSM gene.

1.6.2 Other Merozoite Surface Proteins

1.6.2.1 Merozoite surface protein-1 (MSP-1)

MSP-1 was the first merozoite surface protein to be identified in *P. yoelii* as a 230 kDa protein, which induced protection against challenge infection when mice were immunized with the native antigen (Holder and Freeman, 1981). The homologous protein was subsequently identified in all species of malaria parasites studied to date, but much of its functions has been studied in relation to PfMSP-1.

This protein is synthesized as a precursor of large molecular mass (180 – 250 kDa depending on the species) during intra-cellular and hepatic schizogony. It is located over the entire surface of merozoites both in developing schizonts and in mature forms and is bound to the surface of the developing merozoite via a GPI anchor (Holder, 1988).

The protein precursor is processed at least twice by protease enzymes into a number of fragments. At schizont rupture, primary processing occurs, giving rise to 4 major fragments of approximately 83, 30, 38 and 42 kDa, found as a non-covalently associated complex, held together on the free merozoite surface by the C-terminal 42 kDa fragment. This step is strikingly similar in other *Plasmodium* species, suggesting a fundamentally conserved role in invasion of erythrocytes (Carruthers and Blackman, 2005; Holder and Freeman, 1981). A second processing step occurs, which is a pre-requisite for erythrocyte invasion. At the beginning of this process, when the membrane bound fragment of the complex is further cleaved at a single juxtamembrane site (Blackman, 2000). The C-terminal product, a tandem epidermal-growth factor like domain called MSP-119 remains bound to the invading merozoite surface while the rest of the MSP-1 complex (which also includes fragments of MSP-6 and MSP-7) is shed (Blackman, 2000; O'Donnell and Blackman, 2005).
Comparisons of the deduced primary structure of MSP-1 from different *Plasmodium* species have identified two putative epidermal growth factor (EGF)-like domains at the C-terminus (Blackman et al., 1991). Many proteins containing these structural motifs are involved in receptor binding or other cell surface interactions and protein adhesion. Therefore, it has been suggested that MSP-1, and MSP-1\(_{19}\) in particular may be involved in the initial recognition of the red blood cell and have an important role in erythrocyte invasion (Holder et al., 1992). The three-dimensional structures of MSP-1\(_{19}\) from *P. falciparum* (Morgan et al., 1999) and *P. cynomolgi* (Chitarra et al., 1999) have now been elucidated, confirming the double EGF-domain configuration. Transfection experiments using *P. falciparum* and *P. chabaudi* strains in which the MSP-1\(_{19}\) domain with its two EGF-like modules was reciprocally exchanged between the two species (O'Donnell et al., 2000), have demonstrated the conservation of the function of this domain across *Plasmodium* species.

Genes coding for the MSP-1 of *P. falciparum* and *P. vivax* both show extensive antigenic polymorphism with two major allelic forms, which may hamper the development of an effective vaccine based on this molecule (Gibson et al., 1992). However, the 19 kDa fragments of both species are more conserved among strains and show relatively few amino acid substitutions. The N-terminal 83 kDa of PfMSP-1 is antigenic in human populations exposed to malaria (Riley et al., 1993) and it is conceivable that it is advantageous to the parasite to evoke an antibody response to this variable part of MSP-1, to counteract an immune response directed against conserved, functionally essential parts of the molecule. Similarly, shedding of N-terminal processing fragments of MSP-1, including any immune complexes may be another mechanism of immune evasion (Holder and Blackman, 1994).

Early experiments established that antibodies that recognize the C-terminal region of MSP-1 inhibit merozoite invasion *in vitro* (Chappel and Holder, 1993). Subsequently, with the development of recombinant proteins, the 42 kDa and 19 kDa fragments of MSP-1 were extensively studies for their protective efficacies in many pre-clinical immunization trials. At ICGEB, *P. falciparum* MSP-1\(_{42}\) and MSP-1\(_{19}\) these fragments have been produced in *E. coli*, along with the corresponding fragments from *P. vivax* MSP-1. Studies are going on to fully characterized each of these antigens for an inclusion in a cocktail malaria vaccine, and a combination of EBA-175/PfMSP-1\(_{19}\) will be
evaluated in a phase I study (Sachdeva et al., 2004; Sachdeva et al., 2006), (Portfolio Malaria Vaccine Candidates, 2005).

Immunization with *E. coli* expressed recombinant polypeptides corresponding to the C-terminus of *P. yoelii* MSP-1p19 also resulted in very effective protection against this parasite (Ling et al., 1994). Tian et al. (1996) demonstrated that inbred mouse strains are indeed protected against *P. yoelii* by immunization with *P. yoelii* MSP-1p19 linked to GST (GST-MSP-1p19) with CFA, but are differently protected in an H-2 dependent manner; H-2b mice are better protected than H-2k mice and H-2k mice. GST-fusion proteins of the 33 kDa N-terminal fragment of MSP-1p42 (GST-MSP-1p33) in CFA could not mediate a protective effect (Ahlborg et al., 2002).

Since immune response in human populations to MSP-1,9 is relatively low and short-lived compared with the immunogenicity observed in rodents immunized with this antigen, recently it has been proposed that the disulfide bonds present in the 19 kDa C-terminal region, which are critical to stabilized the structure of this antigen, may affect the antigenic processing and therefore the generation of protective CD4+ T (Hensmann et al., 2004).

MSP-1 is one of the most studied malaria vaccine candidate. Immunization in monkeys with recombinant MSP-1,42 and MSP-1,9 has been shown to elicit various degrees of protection against *P. falciparum* challenge. Research groups are carrying out preliminary clinical trials in humans with different preparations of MSP-1 antigens. One of them, Falciparum Merozoite Protein-1 (FMP-1) has been assessed in a phase I clinical trial in malaria-naïve individuals, demonstrating excellent immunogenicity and no safety concerns. However, a phase 2a challenge showed no evidence of protection from infection. Vaccine candidates based on the 19-kDa C-terminal fragment of MSP-1 are being developed independently as clinical candidates by teams at the University of Hawaii and the Institute Pasteur. The University of Heidelberg in collaboration with the USAID is developing a full-length MSP-1 vaccine expressed in *E. coli*; clinical trials are being carry out involving alum and AS02A as adjuvants (Ballou et al., 2004)
It was identified in *P. falciparum* as a 45-54 kDa protein. Unlike MSP-1, this antigen is not processed during parasite maturation but it is also anchored to the merozoite membrane by GPI moiety. The polymorphism in this antigen present in field isolates has been classified in two major families: FC27 and IC-1/3D7 (Smythe et al., 1991).

This antigen has been studied also at ICGEB, BALB/c and C57BL/6 mice were immunized with peptides representing the conserved N-terminal region of MSP-2 protein. Protection was observed only in BALB/c mice that were immunized with the construct containing the B-cell epitope SNTFINNA and challenged with *Plasmodium yoelii yoelii* 265BY parasites; however there was no protection observed upon challenge of immunized mice with lethal *Plasmodium yoelii nigeriensis* strain. Affinity purified rabbit anti-SNTFINNA IgG showed more than 60% inhibition of erythrocyte invasion in *P. falciparum* culture (Lougovskoi et al., 1999).

Immunization and challenge studies have been carried out with this antigen in animal models as well as human volunteers. A study using recombinant vaccinia virus carrying the complete DNA sequence of MSP-2 gene from the FC27 strain, was used to immunized Saimiri monkeys, along with constructs encoding RESA, MSP-1 and AMA-1 antigens. The immunized monkeys did not produce significant antibody titers against MSP-2 after immunization, but they produced significant titers only after challenge. In addition, none of the monkeys were protected against challenge with *P. falciparum* Indochina 1/CDC strain (IC1) (Pye et al., 1991).

The immunogenicity and protective efficacy of a recombinant protein based on a conserved region of MSP-2 was studied in human Swiss volunteers that were challenged with *P. falciparum* sporozoites. The volunteers were immunized with MSP-2 protein (25 kDa) in combination with a circumsporozoite protein construct with a molecular mass of 35 kDa. There was no evidence of protection, as all volunteers develop symptoms of malaria (Sturchler et al., 1995).

The remarkable polymorphism observed in this antigen has been used for epidemiological studies of the parasite populations in endemic areas, in relation with
clinical manifestations of the disease and to discriminate recrudescence from reinfections in treated patients (al-Yaman et al., 1994; Cattamanchi et al., 2003) Also it has been observed that MSP-2 polymorphism is a useful marker to analyze the evolution of parasite populations and the effect of immune response on genetic selection (Ayala and Rich, 2000; Tonon et al., 2004)

A phase 1-2b double-blind, randomized, placebo-controlled trial in children of Papua New Guinea was conducted by Genton et al (Genton et al., 2002). The vaccine named Combination B comprises recombinant *Plasmodium falciparum* ring-infected erythrocyte surface antigen, MSP-1 and MSP-2 (3D7 allele). There was a reduction of 62% in the parasite density in the vaccinated children, however the incidence of malaria episodes was higher in those children and it was associated with FC27-type parasites.

At the moment there are two constructs of MSP-2 that are being pursued as vaccine candidates, and the studies are at the preclinical development level: MSP-2 long synthetic peptide (Lausanne) and MSP-2 3D7 (+FC27) *E. coli* expressed (La Trobe) (Portfolio Malaria Vaccine Candidates, 2005).

1.6.2.3 Merozoite Surface Protein - 3 (MSP-3)

This surface protein was identified by Oeuvray et al. (Oeuvray et al., 1994) as the antigen recognized by antibodies from individuals of endemic areas that had reach a state of premunition (a non sterilizing type of immunity, progressively acquired by individuals repeatedly exposed to malaria). These antibodies were also able to mediate the inhibition of parasite growth *in vitro* by blood monocytes through a mechanism called antibody-dependent cellular inhibition (ADCI). The effect of monocytes acting through this mechanism depends on the cytophilic nature of the antibodies, the authors also observed that cytophilic classes (IgG1 and IgG3) were the most abundant in the sera of these individuals that reached a state of protection. In all the cases, the antibodies that were positive by ADCI recognized a doublet protein in parasite extracts of ~48 kDa.

Sequence polymorphism is found only in the N-terminal region, which also contains three blocks of alanine-rich heptad repeats that are conserved and have been predicted to form an intramolecular coiled-coil (McColl and Anders, 1997). Purified human
antibodies against non-overlapping peptides representing the conserved C-terminal region of MSP-3, have shown to inhibit the parasite growth in vitro through ADCI, which allowed to define more precisely a region of 70 residues (184 -252aa) which induce the specific cytophilic antibody response that was correlated with the state of protection from malaria in these subjects (Singh et al., 2004).

MSP-3 does not contain a predicted transmembrane domain, but is associated with the merozoite surface as a peripheral membrane protein (McColl et al., 1994; Oeuvray et al., 1994). In the D10 line of *Plasmodium falciparum*, MSP-3 is detected as a protein of 62kDa molecular weight at the late trophozoite stage but is subsequently processed to a form of 44kDa (McColl et al., 1994). The mature protein contain three blocks of conserved heptad repeats at the N-terminal and a conserved putative leucine zipper sequence at the C-terminus. It has been noticed that the presence of Ser-Glu-Thr at positions P1', P2' and P3' in the cleavage site is common to other merozoite surface proteins like MSP-1, MSP-6, and MSP-7, which suggest that maybe the same protease or closely related proteases are responsible for this processing events (Pachebat et al., 2001). One of the candidates for this role is MSP-9, due to its location at the merozoite surface and its suggested association with the transport of MSP-3 (Mills et al., 2002; Pearce et al., 2004a).

Homologues of MSP-3 have been identified in *P. vivax* (Bruce et al., 1999; Galinski et al., 2001), and it has been suggested that *P. falciparum* and *P. vivax* MSP-3 are members of a family of structurally related proteins (Galinski et al., 2001; Trucco et al., 2001). Recently, two more genes H101 and H103 have been identified as paralogues of *P. falciparum* MSP-3 (Pearce et al., 2005).

The immunogenicity and vaccine potential of MSP-3 have been studied in animal models. A chimeric protein combining GLURP (25-500 aa) and MSP-3 (212-382 aa) produced in Lactococcus lactis have also shown to be immunogenic in BALBc/CF1 mice inducing antibodies that control parasite growth in vitro through ADCI (Theisen et al., 2004).

*Aotus nancymai* monkeys were immunized with the full-length MSP-3 recombinant protein expressed in yeast and they were challenged with *P. falciparum* FVO strain of
the immunization. Protection was observed in the MSP-3 immunized monkeys to the same extent as the protection registered in a control group immunized with MSP-1<sub>42</sub> (Hisaeda et al., 2002). A vaccination study was carried out using *Saimiri sciureus* monkeys that were immunized with MSP-3 (212-380 aa) in AS02 and were challenged with *P. falciparum* (Uganda Palo Alto (FUP-SP) strain) infected erythrocytes. Partial or complete protection was observed in some of the monkeys, and these protection was related to the prechallenge antibody titers (Carvalho et al., 2004).

Recently a phase I study in human healthy volunteers has evaluate the safety and immunogenicity of a long synthetic peptide representing a conserved portion from the C-terminal region of MSP-3. It was found that MSP-3 long peptide was immunogenic and induced a strong cytophilic response, although there was adverse reactions reported in the volunteers immunized with the peptide emulsified in Montanide (Audran et al., 2005).

### 1.6.2.4. Merozoite surface protein 4 and 5 (MSP-4) (MSP-5)

The genes *msp-4* and *msp-5* are clearly related, each of them is composed of 2 exons and they encode proteins of identical length. The proteins contain hydrophobic signal sequences, apparent glycosylphosphatidylinositol (GPI) attachment signals and a single epidermal growth factor-like (EGF-like) domain at their carboxyl termini (Marshall et al., 1998)

Merozoite surface protein 4 (MSP-4) of *Plasmodium falciparum* is a 40-kDa protein (272 residues) that is first synthesized at the late ring stage and transported to the parasite surface, where it is anchored to the merozoite membrane by a GPI moiety. The most significant feature of this protein is the presence of a single EGF-like domain at the C terminus of the protein; it has been observed that all the proteins containing EGF-like domains are extracellular and they facilitate the interactions between other proteins. The recent identification of EGF binding sites on human erythrocytes raises the possibility that MSP-4 and other merozoite surface proteins containing this motif (MSP-1), are involved in directly binding to erythrocytes during the invasion process. The observation that the EGF-like domain of MSP-1 is carried into the erythrocyte during invasion is consistent with this idea (Marshall et al., 1997).
Homologues of *P. falciparum* MSP-4 and MSP-5 have been described in *P. vivax*, in which MSP-5 protein is localized only at the apical end, in contrast with the localization of rest of the merozoite surface proteins (Black et al., 2002). The homologues from the murine parasites *P. berghei*, *P. yoelii* (Black et al., 1999; Kedzierski et al., 2000), and *P. chabaudi* (Black et al., 1999) has been identified, in these species, there is only a single gene, designated MSP4/5 encoding a single EGF-like domain similar to the EGF-like domain in both PfMSP4 and PfMSP5. The amino acid sequence of the EGF-like motif is highly conserved in rodent malaria species and also shows a considerable degree of similarity with the EGF-like domains found in the *P. falciparum* proteins (Kedzierski et al., 2000).

The antigenic characteristics of MSP-4 protein has been studied by Wang et al. (Wang et al., 1999) who has shown that the reactivity of antibodies against recombinant MSP-4 was highly dependent on the protein conformation, as the recognition by antibodies was affected by reduction and alkylation of the protein, this inhibition was also observed in case of human antibodies binding to the EGF-like domain, indicating that natural immune response recognizes conformational epitopes in this protein. Antibody response to MSP-4 has been detected in 94% of the population in an endemic region of Vietnam and were predominantly of IgG3 type (Wang et al., 2001).

Studies in mice have shown that immunization with MSP-4/5 from different isolates of *P. yoelii* and with MSP-4/5 from *P. berghei*, confers heterologous protection against the challenge with the lethal strain *P. yoelii yoelii* YM similar to that induced by immunization with the homologous MSP4/5 protein (Goschnick et al., 2004; Rainczuk et al., 2003). In another study, the authors report variable levels of protection confer by immunization of mice with MSP-4/5 from *P. chabaudi adami*, depending on the DNA vector and the vaccination protocol (Rainczuk et al., 2003).

Kedzierski et al. (Kedzierski et al., 2002) used the a protein mixture of the EGF-like domains from *P. yoelii* MSP1 and MSP4/5, and evaluate the protective efficacy of this combination. They found that the combination dramatically enhanced the protection against lethal malaria challenge compared to either protein administered alone. The authors suggest that the efficacy of multiantigen combinations of different merozoite surface proteins should be evaluated also. In a similar way, the immunization with the
combination MSP-1\textsubscript{19}/MSP-4/5 using the oral route induced similar levels of protection (Wang et al., 2004).

1.6.2.5 Merozoite surface protein-6 (MSP-6)

Merozoite surface protein 6 is a membrane protein whose mature form (MSP-6\textsubscript{36}) is found as part of the MSP-1 complex. MSP-6 is a dimorphic antigen, with high degree of conservation within the sequences of the alleles from each form. The 3D7-type MSP-6 alleles are detected in parasites from all endemic regions of the world, whereas K1-type MSP-6 alleles have only been detected in parasites from mainland Southeast Asia. Cleavage of MSP-6, which produces a fragment of 36 kDa in 3D7-type MSP-6 and associates with MSP1, also occurs in K1-type MSP-6 but at a different site in the protein (Pearce et al., 2004b).

The C-terminus of MSP-6 corresponding to MSP-636 has high degree of similarity with the C-terminal region of \textit{P. falciparum} MSP-3. However the heptad-repeat motif present in MSP-3 is not present in MSP-6. Because of this similarity, the ability of MSP-6 protein to induce protective cytophilic antibodies has been investigated using the same approach as for MSP-3. Using six overlapping peptides, each representing a different region of the C-terminal MSP-6, Singh et al. (2005) demonstrated the presence of specific functional antibodies against all the peptides present in the sera of 30 malaria-protected African adults. The antibodies were predominantly IgG1 and IgG3 and all of them exhibit ADCI \textit{in vitro}.

1.6.2.6 Merozoite surface protein-7 (MSP-7)

Merozoite surface protein 7 from \textit{P. falciparum} consists of 351 amino acids, mainly hydrophilic (33\% charged residues) with a negative charge cluster from residue 94 to 148. As in case of MSP-6, the cleavage products of MSP-7 (MSP-7\textsubscript{22} and MSP-7\textsubscript{19}) are part of the protein complex which also contain MSP-1 fragments. It is a relatively conserved antigen, with only four sites of sequence variation within the MSP7\textsubscript{22} region. The MSP-7 gene is expressed in mature schizonts, at the same time as other merozoite surface protein genes (Pachebat et al., 2001).
P. yoelii MSP-7 homologue (PyMSP-7) has been described (Mello et al., 2002) along with a group of new proteins structurally related to PyMSP-7, denominated as merozoite surface related proteins (MSRPs). The common feature of these proteins is to interact with the amino-terminal portion of the 83 kDa fragment of MSP-1 from Plasmodium yoelii. In Plasmodium falciparum there are six of these related protein molecules, three of these sequences from P. falciparum (MSRP-1 to -3) were localized at the amino-terminal portion of MSP-1 at the surface of trophozoites. The authors suggest that these new proteins may play a role as molecular chaperones for the MSP-1 molecule. A recent study (Mello et al., 2004) has reported the immunization of mice with P. yoelii MSRP-2 and MSP-7 recombinant proteins and subsequent challenge with P. yoelii 17XL strain; only P. yoelii MSRP-2 conferred protection in the immunized mice.

With the idea of studying the role of MSP-7 in erythrocyte invasion and biosynthesis of MSP-1 complex, Tewari et al. (2005) disrupt the msp-7 gene from P. berghei and they founded that the synthesis and surface expression of MSP1 was not altered in the transgenic parasites. These parasites were able to invaded and multiplied within erythrocytes, however, the overall growth rate was delayed compared with the wild-type parasites. This effect was restored by the influx of reticulocytes in the mice, suggesting that the absence of MSP-7 modified to some extent the invasion process.

1.6.2.7 Merozoite surface protein – 8 (MSP-8)

It was identified by a motif search in the Malaria Genome Project databases, as a new protein of Plasmodium falciparum containing EGF-like domain. It is synthesized as a mature 80-kDa protein which is rapidly processed to a C-terminal 17-kDa species that contains the double EGF module, similar to the secondary process of MSP-1. In contrast with other MSPs, this protein is expressed throughout the parasite asexual life cycle and is also isolated from free merozoites. There is very limited diversity in the MSP-8 gene sequences from various P. falciparum laboratory isolates.

Comparison of the protein sequence of P. falciparum MSP-8 with the homologues from P. yoelii (Burns et al., 2000), P. berghei (Drew et al., 2004), P. chabaudi, P. vivax and P. knowlesi reveal common features in this group of proteins like the presence of an N-terminal asparagines rich domain, which varies in length from 222 aa in PfMSP-8 to 35
aa in PcMSP-8 and the presence of double EGF-like domains at the C-terminus followed by a GPI-anchor moe ty.

The need of PfMSP-8 for the erythrocytic parasite cycle has been studied by disruption of the msp-8 gene in the P. falciparum strains D10 and 3D7 (Black et al., 2005; Drew et al., 2005). Two independent studies have reported that the absence of MSP-8 protein in the transgenic parasite lines does not affect the growth in vitro. In addition, the expression levels of other merozoite surface proteins including MSP1-5, 7 and 10 were not affected in the mutated parasites. Drew et al. (2005) proposed that MSP-8 is an intracellular protein that is most probably involved in an early parasitophorous vacuole function, but is not essential to maintain the erythrocytic cycle of Plasmodium.

In an attempt to study the function of the double EGF-like domains present in the C-terminal regions of MSP-1 and MSP-8 proteins, Drew et al (Drew et al., 2004) have used allelic replacement to construct a chimeric P. falciparum parasite line in which the double EGF module of PfMSP-1 was replaced by the corresponding region of PbMSP-8. In spite of only 20% sequence identity, P. berghei EGF motif was able to complement the function of PfMSP-1 EGF domain, suggesting a common function for these and other EGF-like domains. According to the authors, this function could be related with the evasion of the immune response against MSP-119.

Recently, Shi et al. (2005) reported that mice immunized with recombinant PyMSP-8 were protected against the challenge with the lethal strain P. yoelii 17XL but no protection was observed when the mice were challenged with the non-lethal strain P. yoelii 17X. They founded that the expression of msp-8 gene was two-fold lower in the parasites P. yoelii 17XL isolated from protected mice as compared with parasites from control groups; and there was a considerable increase in the expression of MSP-1, MSP-4/5, MSP-7 among other genes. The authors concluded that antibodies generated by PyMSP-8 immunization preferentially suppressed P. yoelii 17XL growth in mature erythrocytes but not in reticulocytes and that the changes in the expression of these antigens may influence host cell tropism and allow blood-stage parasites to evade the protective immune response against MSP-8.
The *P. vivax* MSP-8 homologue has been reported (Perez-Leal et al., 2004) and is still not clear if this protein undergoes proteolytic processing as the *P. falciparum* homologue. On the other hand, Burns et al. (2000) has reported the isolation of a new protein in *P. yoelii* protein designated PypAg-2, which possess high degree of similarity with PfMSP-8 protein and also induced protection against *P. yoelii* challenge in immunized mice.

### 1.6.2.8 Merozoite surface protein-10

As in case of MSP-8, this protein was identified for its EGF-like domains at the C-terminus. The identity of the EGF-like motifs with those present in other MSP proteins ranges between 30% and 40%, and antibodies against the EGF-like domains of MSP-10 does not cross-react with the EGF-like domains of MSP-1, MSP-4, MSP-5 or MSP-8. Another common feature of this protein is the requirement of proteolytic processing, which generates a C-terminal fragment of 36 kDa from the 80 kDa precursor. Expression of MSP-10 is detected in trophozoites, schizonts and in isolated merozoites. Unlike other merozoite surface proteins described until now, MSP-10 seems to have a dual location in the merozoite surface but also in the apical organelles. Human antibodies recognize different regions of MSP-10 protein and there is very limited sequence diversity of this protein in field isolates from Papua New Guinea (Africa) (Black et al., 2003). Recently, a specific binding motif for erythrocytes has been identified in *P. falciparum* MSP-10 that is common to other MSPs having EGF-like domains (PfMSP1, PfMSP8, and PfMSP10) (Puentes et al., 2005).

### 1.7 *Plasmodium berghei* infection model

Of the 13 species and subspecies of rodent *Plasmodium* that exist, mainly *P. berghei*, *P. yoelii* and *P. chabaudi* have been used for studies on immunogenicity and pathogenesis. *P. berghei* is the only mouse species able to induce cerebral malaria (CM) in mice, rats and hamsters and for this reason it is widely used for studies on the pathogenesis of this complication. *P. berghei* was first isolated from an infected *Anopheles dureni* in the Belgian Congo in 1948. It was later shown that *Thamnomys surdaster* tree rats were the natural host of this parasite. Four *P. berghei* species (SP11, ANKA, NK65 and Kyberg 173 (K173)), have been used to study CM.
Most strains of mice are susceptible to this complication (CBA/ca, CBA/J, CBA/HN, C57BL/6J, C57BL/6N, SL/J/J, 129/Ola, Swiss and NMRI). On the other hand, there are contradictory reports on the resistance of certain strains, such as C3H, BALB/c, DBA/2 and 129Sv/ev. Nevertheless, *P. berghei* ANKA in CBA/Ca and C57BL/6 mice and *P. berghei* K173 in C57BL/6 mice have been the most studied mouse/parasite combinations. Susceptible mice infected with *P. berghei* ANKA develop a neurological syndrome characterized by paralysis, deviation of the head, ataxia, convulsions and coma. It has been shown that *P. berghei* ANKA gets sequestrated mainly in the lungs and in the brain the extent of sequestration varies between parasite species. One major difference from the human disease is that mice do not develop high fevers but instead develop a progressive hypothermia in the days prior to death (Engwerda et al., 2005) (Landau and Gautret, 1998).

1.8 Some adjuvants used in immunogenicity studies with *Plasmodium* proteins

Adjuvants have been defined as "agents that act non-specifically to increase the specific immune response to an antigen. Adjuvants are important in the context of vaccines because they can define the type of immune response that is generated against the specific antigen. A wide range of substances have been used as adjuvants, however their mechanisms of action are poorly understood. They may influence the immune response at several levels, including the mobilization of appropriate antigen presenting cells (APCs) to the injected site, enhancing antigen processing and presentation, and influencing cytokines and co-stimulatory signals necessary for optimal immune response. Certain adjuvants influence antigen uptake by formation of an antigen depot, or by formation of microparticles, which facilitate the entry of antigens into APCs. (Cox and Coulter, 1997; Xiao et al., 2002).

Two type of adjuvants widely used in experimental studies are Freund's Adjuvant and Aluminium compounds (Aluminium hydroxide or Aluminium phosphate), referred as Alum (Brewer et al., 1999). Both of them, with entirely different nature and therefore, different effects on the immune system. The Freund's complete adjuvant (CFA) consists of a water-in oil emulsion including dried, heat-killed *Mycobacterium tuberculosis*. Freund's incomplete adjuvant consists of the same emulsion but without
without Mycobacteria. Their mode of action is not well defined but it is believed that they work by creating a deposition of the antigen at the inoculation site and generating local inflammation that allows accumulation of mononuclear cells that will activate the specific immune response. Freund's adjuvant typically induce cellular immune response of the Th-1 type, characterized by high levels of IFNγ (Miller et al., 2005). CFA produces intense pathologic reactions that can lead to the formation of granuloma and abscess, for this reason its use in humans is not recommended.

Alum compounds have been used over the last 70 years and their use is accepted in humans. It has been observed that these compounds induce basically a Th-2 type response, with low levels of IFNγ and high levels of IL-5, for this reason they are the choice for vaccines where protection is depending upon the generation of neutralizing antibodies. It have been demonstrated that Alum can induce potent Th1 responses in IL-4-deficient mice, suggesting that IL-4 production induced by alum mediates the inhibition of Th1 responses in intact mice (Brewer et al., 1999).