Plasmodium falciparum Merozoite Surface Protein 1

: Antigenicity, Immunogenicity and Structure.

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For Dad and Mum

เพื่อพ่อค้าแม่ครัว
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie brilliant blue</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDC</td>
<td>N-ethyl-N’-(dimethylaminopropyl) carbo-diimide hydrochloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>x g</td>
<td>Acceleration due to gravity</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl phosphatidylinositol</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>H</td>
<td>Heavy chain of immunoglobulin G</td>
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<tr>
<td>HBS</td>
<td>Hepes-buffered saline</td>
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<tr>
<td>hr</td>
<td>Hour</td>
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<tr>
<td>HRP</td>
<td>Horse-radish peroxidase</td>
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<tr>
<td>IFA</td>
<td>Indirect immunofluorescence assay</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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Abbreviations

<table>
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<tr>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>L</td>
<td>Light chain of immunoglobulin G</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>MSP-1</td>
<td>Merozoite surface protein-1</td>
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<tr>
<td>MSP-1_{19}</td>
<td>19 kDa processing fragment of merozoite surface protein-1_{42}</td>
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<tr>
<td>MSP-1_{83}</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NP40</td>
<td>Nonidet P40</td>
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<tr>
<td>P. Plasmodium</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pdb</td>
<td>Protein Databank</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
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<tr>
<td>T</td>
<td>Tween-20</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>----------------------------</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>WT</td>
<td>Wild type</td>
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ABSTRACT

The antigenicity, immunogenicity and structure of *Plasmodium falciparum* C-terminal merozoite surface protein 1 (MSP-119), a leading malaria vaccine candidate, were investigated. Specific antibodies to MSP-119 can be either inhibitory antibodies that inhibit erythrocyte invasion and proteolytic processing of MSP-1, or blocking antibodies that block both the inhibition of erythrocyte invasion and inhibition of proteolytic processing mediated by the inhibitory antibodies. In order to identify the residues contributing to the epitopes of different antibodies on MSP-119, a series of single amino acid substitutions has been made by site-directed mutagenesis. The reactivity of the MSP-119 mutant proteins with a panel of monoclonal antibodies (mAbs) was assessed by western blotting and BIAcore analysis. It was found that, of 27 MSP-119 mutant proteins made, 7 of the substitutions each abolished the binding of one or more mAbs. By combining the substitutions that were found to prevent the binding of blocking mAbs, individual proteins with multiple amino acid substitutions were made. It was found that these proteins continued to bind inhibitory, but not the targeted blocking mAbs; suggesting that it is possible to produce an additive effect within a single protein by combining individual point mutations.

In order to investigate the effects of amino acid substitutions on the proteins’ immunogenicity and their ability to induce protective antibodies, selected mutant proteins were used to immunise laboratory animals and raise polyclonal antibodies. Total immunoglobulin G (IgG) was then purified from the serum of animals.
immunised with the proteins and tested for inhibitory activities in the MSP-1 secondary processing-inhibition and the erythrocyte invasion-inhibition assays. We found that both mouse and rabbit IgGs induced by the mutant protein 12+28 (Cys12→Ile and Cys28→Trp substitutions) significantly inhibited the secondary processing of MSP-1 and erythrocyte invasion by the parasite. Rabbit IgG induced by the mutant protein 27+31+43 (Glu27→Tyr, Leu31→Arg and Glu43→Leu substitutions) significantly inhibited erythrocyte invasion by the parasite, but not MSP-1 secondary processing. These results suggest that the mechanism of inhibition of erythrocyte invasion by inhibitory antibodies is not mediated only by the inhibition of the secondary processing of MSP-1, and other mechanisms may be involved.

Using a computational biology approach, the molecular structures and surfaces of the 12+28 and 27+31+43 mutant proteins were studied and compared to those of the wild type protein. We showed that although the overall structures of the mutant proteins and the wild type protein were not different, the molecular surfaces of the mutant proteins at the substituted sites were changed. The changes to the molecular surface of the mutant proteins at these specific sites may contribute to their ability to induce protective antibodies.
Chapter One

General introduction

Malaria is the most important vector-borne human disease that causes major mortality and morbidity in tropical and subtropical areas of the world. The World Health Organisation estimates that four billion people in approximately 90 different countries are at risk of developing the disease and that up to 500 million cases of malaria occur each year. This results in the deaths of around 2 million people, mainly children under five years of age and pregnant women in sub-Saharan Africa. The four species of *Plasmodium* that infect humans are: *P. falciparum*, that causes the most severe form of malaria responsible for much of the morbidity and most of the mortality; *P. vivax*, *P. malariae*, and *P. ovale*.

Despite the enormous number of clinical cases and deaths, up to present new successful and broadly applicable measures of control have not been developed. The malaria problem is even worsening by the widespread and increasing resistance of the parasite to once effective anti-malarial drugs and the development of resistance by *Anopheles* mosquito vectors to commonly used insecticides. This deteriorating situation for malaria control has stimulated a search for new tools to control both transmission of the infection and the impact of the disease on populations. One such tool is vaccination. Currently there is no effective vaccine available. An effective
vaccine against malaria is urgently needed to control this deadly disease. A malaria vaccine can be used in conjunction with effective anti-malarial drugs and the other conventional control measures to control the impact of malaria, and perhaps one day even eradicate the *Plasmodium* parasite responsible for the disease from the world.

### 1.1 The life cycle of *Plasmodium* spp. in human and mosquito hosts

The *Plasmodium* parasite has a complex life cycle in its human and mosquito hosts (Figure 1.1). Infection in the human host is initiated by inoculation of the sporozoite stage parasites present in the salivary glands of a female *Anopheles* mosquito that had previously fed on a person with malaria, into the peripheral bloodstream. Sporozoites spend less than 30 minutes in the blood before migrating into the liver and invading hepatocytes where they differentiate and multiply asexually to form invasive merozoites. After a maturation period which takes 2 to 10 days depending on the *Plasmodium* species (5-6 days for *P. falciparum*), the hepatocytes burst, releasing extracellular merozoites into the bloodstream. Probably within minutes following release from the liver, merozoites rapidly invade erythrocytes initiating the clinical blood stage or erythrocytic phase of the infection. The parasites multiply asexually many times within erythrocytes over the period of 2 days (*P. falciparum, P. vivax, P. ovale*) or 3 days (*P. malariae*) to produce a new generation of merozoites. When infected erythrocytes rupture, merozoites are released into the circulating plasma to enter new erythrocytes. Initial attachment between merozoites and erythrocytes can occur at any point on the merozoite surface and the erythrocyte surface. The merozoite then reorients so that its apical end, which contains organelles (rhoptries and micronemes), comes in contact with the
erythrocyte. The apical organelles discharge their contents into the space between the apical complex and the erythrocyte membrane, which appears to alter the properties of the membrane to permit its rapid distension to enclose the entering parasite in a parasitophorous vacuole. The outer surface coat of the merozoite is shed in the process and the merozoite is internalised within an erythrocyte within 20-30 seconds of initial contact and attachment. This asexual blood stage is repeated many times in a cyclic manner. Following erythrocyte invasion, a proportion of the parasites do not undergo asexual division as described above but instead differentiate and develop into male and female gametocytes in some infected erythrocytes. The blood-feeding Anopheles mosquito ingests gametocytes which will mature to gametes and undergo fertilization in the mosquito gut to produce zygotes. These develop into invasive ookinetes that burrow into the mosquito’s gut wall to form oocysts. The mature cysts burst to release immature sporozoites that then migrate to the mosquito salivary glands where they mature and from where they can be transmitted to a human host during feeding to repeat the entire process.

1.2 Anti-malarial immunity

Unlike many infectious diseases for which we have effective vaccines that provide long-standing protective immunity, in malaria this kind of sterile protective immunity does not exist. However, considerable data from field studies clearly indicate that development of a malaria vaccine is feasible. It has been shown that residents of highly malaria-endemic areas naturally acquire protective immunity against malaria after repeated infection. Depending on the endemic setting and particularly exposure levels, infants and young children under 5 years of age are
susceptible to high parasitaemia, morbidity and mortality (Snow et al., 1994). It is not until later childhood that clinical symptom decline together with parasite levels after an infection (Marsh 1992). Adults rarely have a high parasitaemia and appear to have acquired a degree of non-sterile immunity (McGregor 1986). These observations clearly indicate that repeated infection induces an immune response that reduces parasite load in infected individuals and reduces the morbidity and mortality of malaria. Several early studies have shown that immunoglobulin purified from the blood of adults who lived in malarious areas can passively transfer protection against *P. falciparum* malaria (Cohen et al., 1961; McGregor et al., 1963). Such data indicate that antibodies against antigens expressed by erythrocytic-stage parasites can have a profound effect on clearing the parasites.

A most important finding that supports the feasibility of developing a malaria vaccine was that immunisation of mice, non-human primates and humans with radiation-attenuated sporozoites showed protection against a subsequent challenge with sporozoites (reviewed in Nussenzweig and Nussenzweig 1989). However the protective immunity induced was short live and species specific. Protected individuals had serum antibodies to the surface coat protein of the sporozoite and it seemed that these antibodies were mediating protection. But this approach has severe limitations such as the difficulty of large scale culture of the sporozoite stage parasites and the cost, stability, and safely of the final product which are necessary for mass vaccination. Therefore, an alternative approach is the construction of parasite subunit vaccines that can be produced in a large amount and the safety of the products can be controlled.
1.3 Strategies for the development of malaria vaccine

Malaria parasites have complex life cycles with different developmental stages. Characterization of the parasite's protective antigens and the effector immune mechanisms involved in the elimination of different parasite stages are important for vaccine development against the disease. A vaccine directed against the pre-erythrocytic stages, namely sporozoites and liver stages, is expected to block the first step of malaria infection: the invasion of hepatocytes by the sporozoites introduced into human body during the mosquito bite and the subsequent development in the liver (Hoffman et al., 1991). Antibodies to proteins on the parasite surface can neutralise sporozoites and prevent subsequent development of liver stages. This vaccine should be useful for uninfected newcomers to endemic areas. However if a few parasites escape the immune response induced by a pre-erythrocytic vaccine, they would eventually multiply and cause a blood-stage infection. Vaccines directed against erythrocytic or asexual blood-stages should decrease the level of parasitaemia and thus reduce clinical symptoms that cause disease severity during blood infection (Pasloske and Howard 1994; Good et al., 1998). The vaccines should raise antibodies to inhibit erythrocyte invasion by the invasive merozoites, inhibit the sequestration of the infected erythrocytes or destroy the infected erythrocytes themselves. Finally, vaccines targeted against sexual blood-stage or transmission-blocking vaccines do not protect the person vaccinated, but instead interrupt the sexual reproduction of the parasite in the cycle of transmission, thus favoring the community. The antibodies raised by these vaccines would interfere with fertilization or prevent the development of the parasite within the mosquito.
However each vaccine has its limitations. For example, parasite proteins show stage specificity and some of them show a high degree of antigenic variation. It seems that the immunity induced is species and stage specific. For example, antibodies induced against the surface protein of sporozoites do not recognise parasites from the erythrocytic stage of the life cycle and *vice versa*. In addition, although an asexual blood-stage vaccine may reduce the parasitaemia or abolish the clinical symptoms, it may not have effects on the development of the sexual stages and transmission of the parasites. A sexual stage vaccine would not protect the individual but may reduce the subsequent infection of the others. Therefore in practice, an optimal vaccine would require a mixture of several proteins from different stages that would have ability to elicit protective immunity that blocks infection as well as prevents pathology and interrupts transmission of the parasites. A successful malaria vaccine would most likely to be a combination vaccine composed of subunits from different parasite stages.

### 1.4 Transmission blocking vaccine candidates

The primary goal of anti-sexual stage vaccines is to block parasite transmission. Blocking transmission could reduce the mortality/morbidity associated with malaria by decreasing the number of infected mosquitoes or reducing mosquito’s parasite load. Transmission-blocking vaccines may have limited use on their own in endemic areas since they do not improve an individual patient’s condition. However, these vaccines might be an important component of multistage vaccines as part of a global approach against malaria infection, and might avoid the spread of escape mutants from other protective components in the multistage vaccines. When
combined with anti-malarial drug treatment, the transmission-blocking vaccine might prolong the use of the drugs by preventing the spread of drug-resistant parasites. Furthermore, a transmission-blocking vaccine might prevent the re-introduction of malaria parasites into areas where malaria has been eradicated.

Early studies have shown that antibodies induced by vaccination of animals with sexual-stage parasites can disrupt development of the parasite within the mosquito midgut (Carter and Chen 1976; Gwadz 1976). A number of target antigens for transmission-blocking vaccines have been identified on the surface of gametes, the extracellular sexual stages of the parasites released from erythrocytes after ingestion by a mosquito, and on the surface of zygotes and ookinete,

1.4.1 Pfs25 and Pfs28

Pfs25, a 25-kDa surface antigen of zygotes and ookinetes (Kaslow et al., 1988), and Pfs28, a 28-kDa surface antigen of late ookinetes (Duffy and Kaslow 1997), are two of the leading transmission-blocking vaccine candidates. Both proteins
are cysteine-rich GPI-anchored proteins containing four EGF-like domains. Kaslow et al. have shown that recombinant Pfs25, expressed in vaccinia virus, induces transmission-blocking antibodies when used to immunise mice (Kaslow et al., 1991). Furthermore, immunisation with a polypeptide analogue of Pfs25 secreted from yeast cells induced, in mice as well as monkeys, antibodies that completely blocked transmission (Barr et al., 1991a; Kaslow et al., 1994). Polyclonal sera to Pgs28, the avian malaria parasite \(P. \text{gallinaceum}\) analogue of Pfs28, appeared to block transmission by inhibiting the transformation of zygotes to ookinetes \textit{in vitro} and suppressing the development of ookinetes to oocysts \textit{in vivo} (Duffy et al., 1993). It has been shown later that antisera against Pfs28 expressed in yeast, also blocked infectivity of the parasites to mosquitoes in the membrane feeding assay (Duffy and Kaslow 1997). Because antibodies to these two antigens appeared to block infectivity by attacking different proteins in the same developmental stage, Pfs25 and Pfs28 were produced as a single recombinant fusion protein and tested as to whether or not they can act synergistically to block parasite infectivity. Gozar et al. have shown that fusion protein of Pfs25 and Pfs28 can induce potent transmission-blocking antibodies in mice (Gozar et al., 1998). Complete inhibition of oocyst development in the mosquito midgut was achieved with fewer vaccinations, at lower dose, and for longer duration than immunisation using either Pfs25 or Pfs28 recombinant protein alone or Pfs25 and Pfs28 mixed together in a vaccine cocktail. However in a recent report of the same author and colleagues, clinical-grade Pfs25 (TBV25H) was shown to elicit more potent transmission-blocking antibodies in rabbits, than did clinical-grade Pfs25-28 fusion protein (TBV25-28) (Gozar et al., 2001). The reformulation of TBV25-28 to enhance its immunogenicity is being investigated.
1.4.2 Pfs230

Pfs230 is expressed intracellularly on the gametocyte surface as a 360-kDa precursor and is processed, by two different proteases, to a 307 or 300 kDa form of surface protein on extracellular gametes (Williamson et al., 1993; 1996; Brooks and Williamson 2000). Many studies have shown that Pfs230 is a target of transmission-blocking antibodies. Quakyi et al. found that two monoclonal antibodies to Pfs230 could independently block transmission but only in the presence of complement (Quakyi et al., 1987). Studies from Graves et al. and Healer et al. have shown that the presence of anti-Pfs230 antibodies in sera collected from humans in malaria endemic areas was strongly associated with transmission-blocking activity (Graves et al., 1988; Healer et al., 1999). It has been shown that a recombinant bacterial-produced protein encoding portions of 2 of the multiple 7-cysteine motifs of Pfs230 can induce antibodies that significantly reduced parasite infectivity to mosquitoes (Williamson et al., 1995). The complement-mediated lysis of gametes is shown to be a significant mechanism of natural transmission-blocking immunity (Healer et al., 1997). Since Pfs230 is recognised by antibodies induced during a natural infection, the response induced by a Pfs230 vaccine may be boosted during a natural infection and maintain a long-lasting transmission-blocking immunity. However, the large size of Pfs230 and the finding that its cysteine domains conformation depends on proper disulphide bond formation have made it a challenge to develop as a vaccine candidate.

1.4.3 Pfs48/45

Pfs48/45 is a doublet of a 48- and a 45-kDa surface protein of *P. falciparum* gametocytes detectable from day 2 of gametocytogenesis through gametogenesis and
fertilization. (Vermeulen et al., 1986; Kocken et al., 1993). Pfs48/45 forms a noncovalent but stable GPI-anchored complex with Pfs230, though these two proteins are encoded by separate genes. Pfs48/45 is one of the prime transmission-blocking vaccine candidates as it has been shown that Pfs48/45 knockout parasites had a reduced capacity to produce oocysts in mosquitoes due to greatly reduced zygote formation (van Dijk et al., 2001); and monoclonal antibodies to Pfs48/45 can block transmission of the parasite to mosquitoes in the absence of complement, although they must be present before fertilization (Rener et al., 1983; Vermeulen et al., 1985; Carter et al., 1990). Pfs48/45 specific antibodies have been detected in a high percentage of sera from people living in endemic areas (Ong et al., 1990; Riley et al., 1990a; Roeffen et al., 1995) which suggests that, like Pfs230, the immune response induced by a Pfs48/45 vaccine can be boosted by natural infection. Furthermore, studies by Roeffen et al. have shown strong correlation between Pfs48/45 specific antibodies and transmission-blocking immunity (Roefen et al., 1996). The limited antigenic and genetic diversity of Pfs48/45 also supports Pfs48/45 as a promising transmission-blocking vaccine candidate (Foo et al., 1991; Kocken et al., 1995). However, the recombinant Pfs48/45 proteins expressed in E.coli and in recombinant vaccinia virus-infected cells can induce Pfs48/45-specific antibodies, but the antibodies failed to block parasite transmission in a standard mosquito membrane-feeding assay (Milek et al., 1998a and b). Like Pfs230, the production of correctly folded cysteine domains of Pfs48/45 has obstructed the development of Pfs48/45-based transmission-blocking vaccine.
1.5 Pre-erythrocytic vaccine candidates

Pre-erythrocytic vaccine development is aimed to design a vaccine that can induce protective immune responses against the sporozoite stage or the liver stage development of the malaria parasite. Two general types of pre-erythrocytic vaccine have been developed. One is a vaccine that can induce antibodies against invasive sporozoites; thus blocking sporozoite invasion of hepatocytes, and another one is a vaccine that can induce T-cell responses against infected hepatocytes; thus leading to the recognition and killing of the infected cells by cell-mediated mechanisms or the action of cytokines. In both cases, the end result is to prevent infection and reduce malaria transmission. Early studies have shown that immunisation with irradiation-attenuated non-pathogenic sporozoites gives protective immunity in animal models and in human against *P. falciparum* and *P. vivax* (Clyde 1975). In human studies, Ramsey *et al.* have shown that volunteers immunised by repeated bites from irradiated *P. falciparum* infected mosquitoes were completely protected from the subsequent sporozoite challenge (Ramsey *et al.*, 1982). However the protection was short-lived and species- and stage- specific. The practical difficulties of producing a sufficient large number of sporozoites and infected mosquitoes for radiation and the safety of the end products are also the problems of attenuated vaccines.

1.5.1 Circumsporozoite (CS) protein

Circumsporozoite (CS) protein is a protein found on the surface of sporozoites of all species of *Plasmodium*. The protein ranges in size from 44 to 67 kDa depending on the species of the parasite. All *P. falciparum* CS proteins have a major central region of ~40 tandem repeats of a tetrapeptide, ~37 of which are Asn-Ala-Asn-Pro...
(NANP) plus four additional Asn-Val-Asp-Pro (NVDP) (Dame et al., 1984). Many studies in malaria-exposed communities have shown that there is a slow development of antibodies to these repeated units (Del Giudice et al., 1986; 1987). Nardin et al. have shown that monoclonal antibodies against the repeats of *P. falciparum* and *P. vivax* CS proteins inhibited sporozoite infectivity in chimpanzees (Nardin et al., 1982). Studies by Nussenzweig et al. have also shown that antibodies to these repeats mediated protection against sporozoite-induced infection in mice (Nussenzweig and Nussenzweig 1989). The recombinant and synthetic forms of the CS protein-based vaccines with or without these repeats, using many kinds of adjuvants, were tested in humans, but the efficacy was poor (Ballou et al., 1987; Herrington et al., 1987; Hoffman and Franke 1994). However, after reformulating the adjuvant used for vaccine preparation, Stoute et al. have shown that the CS protein-based RTS,S vaccine in a new adjuvant formulation of oil-in-water emulsion plus the immune stimulants 3-deacyl-monophosphoryl lipid A (MPL) and QS21 (altogether called vaccine formulation SBAS2; SmithKline Beecham Adjuvant System 2) repeatedly induced protection of human volunteers following challenge with infected mosquitoes (Stoute et al., 1997). RTS,S is a hybrid recombinant vaccine consisting of two polypeptides; the central tandem repeats and carboxy-terminal region of the *P. falciparum* CS protein fused to the hepatitis B surface antigen (RTS), and the hepatitis B surface antigen (S). The mechanism of protection induced by RTS,S/SBAS2 vaccine is still unclear. However a high antibody response to the repeats alone might not be sufficient for protection, because another RTS,S-based vaccine in a different adjuvant formulation (oil-in-water emulsion without MPL and QS21, called SBAS3) has also been shown to induce high antibody responses against the repeats, but gave no protection, suggesting that cellular immune responses also play an important role
in protection. The detailed characterization of the cellular immune response to RTS,S/SBAS2 and further experimental studies are underway.

1.5.2 SSP2/TRAP

Sporozoite surface protein 2 (SSP2) was first identified in *P. yoelii* as a 140-kDa surface protein (Charoenvit *et al.*, 1987). *P. yoelii* SSP2 (PySSP2) is present in the micronemes of sporozoites, on the sporozoite surface and throughout the liver stage in *P. yoelii*-infected hepatocytes (Aikawa *et al.*, 1990a). The gene encoding *P. falciparum* SSP2 (PfSSP2) has now been identified and characterized (Rogers *et al.*, 1992), and it was shown to be the previously described 90-kDa thrombospondin-related anonymous protein (TRAP) (Robson *et al.*, 1988). PfSSP2/TRAP is present on the surface (Cowan *et al.*, 1992; Rogers *et al.*, 1992), and in the micronemes of sporozoites and is present for the first 4 days of the *P. falciparum* liver stage (Rogers *et al.*, 1992). It has been shown recently that although genetically manipulated malaria parasites lacking TRAP could develop into sporozoites These TRAP (-) sporozoites lacked motility and could not invade mosquito salivary glands, thus failing to become infective sporozoites (Sultan *et al.*, 1997), suggesting PfSSP2/TRAP is essential for the parasite. Immunisation with PySSP2 and PyCSP protected mice against challenge with *P. yoelii* (Khusmith *et al.*, 1991), and adoptive transfer of cytotoxic T lymphocyte (CTL) clones against PySSP2 also protected mice against *P. yoelii* challenge by eliminating infected hepatocytes (Khusmith *et al.*, 1994). It has been shown that murine antibodies against recombinant PfSSP2 recognised sporozoites and infected hepatocytes, and also inhibited sporozoite invasion and development in hepatocytes *in vitro* (Rogers *et al.*, 1992). The identification of specific CTL epitopes
on PfTRAP by malaria exposed individuals (Aidoo et al., 1995), and by irradiated P. falciparum sporozoite immunised volunteers (Wizel et al., 1995), have provided critical data for development of an epitope-based anti-liver stage malaria vaccine.

### 1.5.3 HEP17/EXP-1

Hepatocyte erythrocyte protein 17 (HEP 17) is a 17-kDa P. yoelii protein identified on the parasitophorous vacuole membrane of the P. yoelii infected hepatocytes and erythrocytes, and in the cytoplasm of host cells (Charoenvit et al., 1995). It has been shown that a monoclonal antibody directed against this protein eliminated infected hepatocytes in culture and delayed the onset and density of blood-stage parasitaemia in vivo (Charoenvit et al., 1995). Immunisation with a DNA plasmid expressing HEP17 could induce CD8+ T lymphocyte-dependent protective immunity in mice (Doolan et al., 1996a). Based on their extensive sequence homology, expression pattern and antigenic cross-reactivity, the P. falciparum homologue of PyHEP17 is identified as the protein known as P. falciparum exported protein-1 (PfEXP-1) (Simmons et al., 1987; Sanchez et al., 1994). Identity between PyHEP17 and PfEXP-1 is 37% at the amino acid level and has led to PfEXP-1 being proposed as one of the pre-erythrocytic vaccine candidates (Doolan et al., 1996b).

### 1.5.4 LSA-1

Liver-stage antigen 1 (LSA-1) is a 200-kDa antigen that accumulates as flocculent material in the parasitophorous vacuole of infected hepatocytes (Hollingdale et al., 1990). LSA-1 contains several B- and T-cell epitopes that are immunogenic during the course of natural infection (Fidock et al., 1994), and it
stimulates CTLs and gamma interferon (IFN-\( \gamma \)) in naturally exposed individuals (Hill et al., 1992; Fidock et al., 1994). Since it has been shown that CTLs and IFN-\( \gamma \) can kill liver-stage malaria parasites in animal models (Schofield et al., 1987; Wang et al., 1996), and the interleukin-10 (IL-10) responses to recombinant LSA-1 protein were significantly associated with a delayed rate of reinfection following radical cure with chemotherapy (Kurtis et al., 1999), LSA-1 is proposed to be a candidate for a pre-erythrocytic malaria vaccine.

1.5.5 LSA-3

Liver-stage antigen 3 (LSA-3) is a 200-kDa protein, expressed in both sporozoite and liver stages, and is highly conserved among parasites from various geographical regions (Daubersies et al., 2000). \textit{P. falciparum} LSA3 displayed promising antigenic, immunogenic, and protective properties in Aotus monkeys (Perlaza et al., 1998) and chimpanzees (Benmohamed et al., 1997; 2000; Daubersies et al., 2000). Recently several CTL epitopes were also identified within LSA-3 (Aidoo et al., 2000). Furthermore PfLSA-3-specific human antibodies were shown to exert up to 100% inhibition of \textit{in vitro} invasion of \textit{P. yoelii} sporozoites into mouse hepatocytes (Brahimi et al., 2001). This strong \textit{in vitro} activity was reproduced \textit{in vivo} by passive transfer of LSA-3 antibodies. These results suggest that LSA-3 is another important target for pre-erythrocytic vaccine development.
1.6 Blood-stage vaccine candidates

Since the asexual blood stage of malaria is responsible for the clinical symptoms of the disease, vaccines directed against this parasite stage are aimed largely at either eliminating or reducing the parasite load which will reduce malaria-related morbidity and mortality. Early experiments had demonstrated that protective immunity against the blood stage is at least partly mediated by antibodies, since passive transfer of serum or immune IgG obtained from adults in an endemic area had curative effects in children (Cohen et al., 1961; McGregor et al., 1963). A large number of antigens that are possible targets of protective immunity have been identified. Most of them are proteins that are accessible at the time of schizont rupture, merozoite release and erythrocyte invasion. These include proteins on the surface of merozoite, in the secretory apical complex, and in the parasitophorous vacuole. The proteins on the surface of infected erythrocytes that are involved in sequestration to capillaries and rosette formation with other erythrocytes have also been proposed as vaccine candidates. Some of the major merozoite vaccine candidate proteins are discussed in this section, whereas merozoite surface protein 1 (MSP-1) will be discussed in detail in a later section.

1.6.1 MSP-2

Merozoite surface protein 2 (MSP-2) in *P. falciparum* is a 45- to 52-kDa protein anchored on the surface of the merozoite by a GPI moiety (Smythe et al., 1988; Gerold et al., 1996). MSP-2 consists of highly conserved N- and C-termini flanking a central variable region (Smythe et al., 1991). There is evidence suggesting MSP-2 as a target of host protective immune responses including its exposed location.
on the merozoite surface, and parasite growth inhibition by a specific monoclonal antibody to MSP-2 (Epping et al., 1988). Mice immunised with conserved regions of *P. falciparum* MSP-2 have been protected against challenge with the rodent parasite *P. berghei* and *P. yoelii* (Saul et al., 1992; Lougovskoi et al., 1999), despite the fact that MSP-2 is not present in rodent malaria parasites. Synthetic peptides and purified IgG specific to B cell-epitopes residing in the N-terminal conserved region of MSP-2 showed significant inhibition of merozoite invasion of fresh erythrocytes in *in vitro* culture of *P. falciparum* (Lougovskoi et al., 1999; Ocampo et al., 2000). Furthermore, antibodies to MSP-2 are frequently detected in sera from individuals living in endemic areas (Taylor et al., 1995; Ranford-Cartwright et al., 1996). Based on these results, human phase I trials of a multisubunit vaccine containing MSP-2 have commenced (Saul et al., 1999).

### 1.6.2 RAP-1 and RAP-2

Rhoptry associated protein 1 (RAP-1) is synthesized as an 86-kDa precursor, which is subsequently cleaved to generate an 82-kDa molecule (Ridley et al., 1990b; Howard and Schmidt 1995). In late schizogony, a fraction of 82-kDa protein is further processed to yield a 67-kDa molecule (Howard et al., 1998a). As part of its maturation, the processed RAP-1 forms a complex with the 42-kDa protein RAP-2 and the 37-kDa protein RAP-3 (Howard and Reese 1990) located in the rhoptries of *P. falciparum*. It has been shown that antibodies to RAP-1 are able to block merozoite invasion *in vitro* (Schofield et al., 1986; Harnyuttanakorn et al., 1992; Howard et al., 1998b), suggesting that antibodies to this antigen may reduce the replication of the parasites. Monkeys immunised with RAP-1 and RAP-2 were partially protected
against *P. falciparum* parasite challenge (Perrin *et al.*, 1985; Ridley *et al.*, 1990c; Collins *et al.*, 2000). It has been shown that anti-RAP-1 antibodies, mostly IgG1 to the N-terminal part, were detected in people living in endemic areas (Howard *et al.*, 1993; Jakobsen *et al.*, 1997; Fonjungo *et al.*, 1998). The finding that an association between high levels of IgG antibodies to the N-terminal regions of RAP-1 and protection against high densities of *P. falciparum* parasites in Tanzanian children has suggested a possible role of anti-RAP-1 antibodies in human immunity (Jakobsen *et al.*, 1996). It was shown that disruption of *RAP1* gene in *P. falciparum* parasite resulted in RAP-1 can no longer complex with RAP-2, although this disruption has no effects on invasion and growth of the parasite (Baldi *et al.*, 2000).

### 1.6.3 AMA-1

Apical membrane antigen 1 (AMA-1) is an 83-kDa antigen that is synthesized in the mature stages of the parasite and is initially localized in the neck of the rhoptry organelles (Peterson *et al.*, 1989; Crewther *et al.*, 1990). At about the time of merozoite release, the full-length 83-kDa molecule is localised at the apical pole, and an N-terminally processed form of 66 kDa can be detected distributed around the merozoite surface (Peterson *et al.*, 1989; Narum and Thomas 1994). The biological function of AMA-1 is currently unknown. However, its location and stage specificity suggest that it may be involved in the process of erythrocyte invasion. Active immunisation of monkeys or mice with either native (Deans *et al.*, 1988; Narum *et al.*, 2000a) or recombinant (Collins *et al.*, 1994; Anders *et al.*, 1998) form of AMA-1 has protected these animals against simian and rodent parasites, respectively. Monoclonal antibodies raised against *P. falciparum* AMA-1 and against PK66, the *P. knowlesi*
homologue of AMA-1, inhibited merozoite invasion \textit{in vitro} (Thomas \textit{et al.}, 1984; Kocken \textit{et al.}, 1998). Furthermore, passive immunisation with AMA-1-specific polyclonal antibodies of \textit{P. chabaudi}-infected mice prevented lethal parasitaemias (Anders \textit{et al.}, 1998). It has been reported that antibodies to \textit{P. falciparum} AMA-1 can be detected during \textit{P. falciparum} infection in humans (Thomas \textit{et al.}, 1994). Recently, affinity purified human anti-AMA-1 antibodies from the plasma of adults living in the endemic regions in Papua New Guinea were found to be strongly inhibitory to the invasion of erythrocytes by merozoites from both homologous and heterologous lines of \textit{P. falciparum} (Hodder \textit{et al.}, 2001). Early clinical trials with AMA-1 have commenced.

1.6.4 EBA-175

Erythrocyte binding antigen 175 (EBA-175) is a 175 kDa protein located in the apical micronemes of merozoites (Camus and Hadley 1985; Orlandi \textit{et al.}, 1990; Sim \textit{et al.}, 1990), and appears to mediate parasite invasion of host erythrocytes by a sialic acid-dependent binding to its receptor, glycophorin A (Sim \textit{et al.}, 1994). The erythrocyte binding region of EBA-175 is a 616-amino-acid cysteine-rich region, designated region II, that lies in the N-terminal part of the molecule (Adams \textit{et al.}, 1992; Sim \textit{et al.}, 1994). It has been shown that recombinant EBA-175 polypeptides were recognised by pooled human sera from malaria endemic regions (Daugherty \textit{et al.}, 1997). Antibodies specific for the EBA-175 erythrocyte-binding region II, as well as antibodies specific for a conserved sequence within region III to V, termed EBA peptide 4, were shown to block binding of native EBA-175 to human erythrocytes and inhibit merozoite invasion \textit{in vitro} (Sim \textit{et al.}, 1990; Sim \textit{et al.}, 1994; Narum \textit{et al.}, 1994).
2000b). Recently, it has been shown that immunisation with EBA-175 region II induced a significant antiparasitic immune response in *Aotus* monkeys (Jones *et al*., 2001).

### 1.6.5 Pf155/RESA

Ring-infected erythrocyte surface protein (RESA) or Pf155 is a 155-kDa soluble and heat-stable protein (Cowman *et al*., 1984), localised within dense granules in the apical part of merozoites (Aikawa *et al*., 1990b). Pf155/RESA is translocated to the erythrocyte membrane during or shortly after merozoite invasion (Culvenor *et al*., 1991). The protein contains two regions of repeated amino acid sequences, one in the C-terminal region and the other one in the middle of the polypeptide. Purified human antibodies reacting with the repeat regions (Wahlin *et al*., 1984; Berzins *et al*., 1986) and the non-repeat regions (Siddique *et al*., 1998; Siddique *et al*., 1999) were found to inhibit merozoite invasion and *P. falciparum* parasite growth *in vitro*. Furthermore, partial protection against a *P. falciparum* challenge was obtained in *Aotus* monkeys by immunisation with fusion proteins containing regions of the Pf155/RESA molecule (Collins *et al*., 1986) and by passive immunisation with human antibodies reactive with the C-terminal repeat (Berzins *et al*., 1991). Although correlation of the appearance of anti-Pf155/RESA antibodies in individuals from *P. falciparum* endemic areas with the acquisition of clinical immunity suggested the involvement of Pf155/RESA in the protective immune response (Petersen *et al*., 1990; Riley *et al*., 1991; Astagneau *et al*., 1994), the mechanism of invasion inhibition mediated by its specific antibodies is still unclear.
1.6.6 SERA

Serine repeat antigen (SERA) is a major parasitophorous vacuole protein made during the trophozoite-schizont blood stage of *P. falciparum* and *P. vivax* (Delplace *et al.*, 1987). During merozoite release and invasion, a fraction of SERA is processed into 47-kDa N-terminal, 50-kDa internal, and 18-kDa C-terminal domains (Delplace *et al.*, 1988; Debrabant and Delplace 1989). It has been shown that antibodies that recognise SESA agglutinated merozoites and blocked merozoite dispersal (Lyon *et al.*, 1986; 1989; Perkins and Ziefer 1994). Immunisation of mice with recombinant protein of the SERA N-terminal domain induced an antiserum that was inhibitory to parasite growth *in vitro* (Banyal and Inselburg 1985; Barr *et al.*, 1991b). *Saimiri* monkeys immunised with SESA were partially protected with a relatively low and delayed patent parasitaemia (Delplace *et al.*, 1988). Furthermore, immunisation of *Aotus* monkeys with the recombinant protein SERA-1, the polypeptide containing amino acids 24 to 285 of SERA, showed strong protective response against challenge with *P. falciparum* blood stage (Inselburg *et al.*, 1993). The protection appeared to correlate with the presence of serum antibodies that were able to inhibit parasite growth *in vitro* (Inselburg *et al.*, 1993). Recent studies have shown that antibodies specific to the 47-kDa N-terminal domain facilitated the complement-mediated lysis of schizonts, and inhibited parasite growth by cross-linking SERA molecules (Pang and Horii 1998; Pang *et al.*, 1999).
1.7 Synthetic multi-antigen/multi-stage malaria vaccines

In order to combat the Plasmodium parasite that has a complex life cycle with numerous stage-specific antigens inducing activation of different component of host immune system, a single-stage protein-based malaria vaccine may have a limited use, possibly inducing only a partial protective immune response. Therefore, a multistage malaria vaccine for a global vaccination approach against this disease is being developed. Many synthetic multi-antigen/multi-stage vaccines have been produced and trialed by many investigators worldwide. Some of them are discussed in this section.

1.7.1 SPf66

SPf66 is chimeric polypeptide composed of peptide sequences derived from three asexual blood-stage antigens, 9 amino acids from the conserved N-terminus of P. falciparum MSP-1 and two other peptides of unknown origin, and the NANP motifs from the P. falciparum CS protein central repeats used as a spacer between the other individual peptides. Patarroyo et al. showed that Aotus monkeys vaccinated with SPf66 were protected against blood-stage challenge (Patarroyo et al., 1987). Phase I clinical trials of SPf66 showed that the vaccine was immunogenic and the calculated efficacy was 75% after experimental challenge (Patarroyo et al., 1988). Phase IIb and III trials of the vaccine done in Latin America with volunteers that subjected themselves to natural exposure to malaria showed encouraging results of protection against P. falciparum (Amador et al., 1992; Patarroyo et al., 1992; Valero et al., 1993). Furthermore, clinical trials of SPf66 in Tanzanian children aged 1 to 5 years showed a vaccine efficacy of 31% against malaria (Alonso et al., 1994). However,
studies in the Gambian and in Karen children failed to establish protective effects against clinical episodes of malaria (D'Alessandro et al., 1995; Nosten et al., 1996), results that clearly disagreed with those of the previous studies. Several field studies in endemic areas have shown that an antibody response to SPf66 was induced following immunisation with SPf66, but it was difficult to establish a correlation between the antibody response and protection (Alonso et al., 1998; Haywood et al., 1999; Metzger et al., 1999). The reasons for this may be because these field trials were performed in groups of people of different ages living in different areas. Furthermore, the mechanisms involved in SPf66-mediated protection have not been clarified.

1.7.2 NYVAC-Pf7

NYVAC-Pf7 is a new multistage *P. falciparum* vaccine consisting of an attenuated NYVAC vaccinia virus strain that expresses genes encoding seven stage-specific proteins expressed during the sporozoite (CSP, PfSSP2), liver (LSA-1), blood (MSP-1, SERA, AMA-1) and sexual (Pfs25) stages of the parasite's life cycle (Tine et al., 1996). In preclinical testing in *rhesus* monkeys, NYVAC-Pf7 was safe and well tolerated, and it induced antibodies recognising all antigens of the vaccine (Tine et al., 1996). In a Phase I/IIa trial, this construct generated cellular immune responses in 90% of volunteers, but with a low antibody response. Only one of 35 volunteers challenged by the bites of 5 *P. falciparum*-infected *Anopheles* mosquitoes was completely protected, although all 34 volunteers showed significant delay in the appearance of parasites detected in thin blood smears (Ockenhouse et al., 1998). A prime-boost strategy in primate models of *P. falciparum* malaria using attenuated live
virus vectors (NYVAC and ALVAC) followed by boosting with multiple recombinant proteins or naked DNA vectors to enhance immunogenicity, is under investigation by Ockenhouse et al. and Hill et al. (Degano et al., 1999).

1.7.3 CDC/NII MALVAC-1

CDC/NII MAL VAC-1 is a synthetic gene encoding twelve B cell epitopes, six CD4^+ T cell epitopes and three CD8^+ T cell epitopes derived from 9 stage-specific *P. falciparum* antigens (pre-erythrocytic: CSP, SSP2 and LSA-1; erythrocytic: MSP-1, MSP-2, AMA-1, RAP-1, EBA-175; and sexual stages: Pfs27). The gene was cloned and the recombinant protein was expressed in the Baculovirus Expression Vector System (BEVS). In immunisation studies in rabbits, BEVS-expressed CDC/NII MAL VAC-1 antigen was highly immunogenic, and the induced antibodies inhibited *P. falciparum* sporozoite invasion of hepatoma cells and the growth of blood-stage parasites *in vitro* (Shi et al., 1999). Recent immunisation studies in mice have shown that although the antibody response was low, this antigen could induce T cell proliferative and IFN-γ responses (Shi et al., 2000). The antibody and T cell responses to this antigen in naturally exposed individuals are under investigation.

1.8 MSP-1

Of the molecules on the surface of the merozoite, merozoite surface protein-1 (MSP-1) is one of the best characterised malarial proteins and the most promising current candidates for a vaccine against the blood-stage of the malaria parasite *P. falciparum*. Also known as the Precursor to the Major Merozoite Surface Antigen (PMMSA), gp195 and MSA-1 (Holder et al., 1985), MSP-1 is a high molecular mass
protein that varies in size and amino acid sequence in different parasite lines. It is synthesised by the intracellular schizont as a ~200 kDa precursor protein located on the surface of the parasite (Holder and Freeman 1982), and detectable in all *Plasmodium* species (Holder et al., 1992). During the release of merozoites from erythrocytes and re-invasion of new erythrocytes, MSP-1 undergoes at least two proteolytic processing steps (Figure 1.2). In the first modification, as a result of a process called primary processing which takes place at or just prior to the merozoite release from erythrocyte (Holder et al., 1988), the precursor protein is cleaved to form a complex of 4 polypeptide fragments of 83 kDa (MSP-183), 30 kDa (MSP-130), 38 kDa (MSP-138) and 42 kDa (MSP-142) that remain on the merozoite surface (Holder et al., 1987; McBride and Heidrich 1987). This complex also contains two other polypeptides derived from MSP-6 and MSP-7 (Stafford et al., 1994; Pachebat et al., 2001; Trucco et al., 2001). The complex is maintained by non-covalent interactions between different subunits and is held on the merozoite surface by a glycosyl phosphatidyl inositol (GPI) anchor attached to the C-terminal 42 kDa fragment of MSP-1 and inserted into the plasma membrane of the merozoite. At the time of merozoite invasion into an erythrocyte, the membrane bound C-terminal 42 kDa fragment of the MSP-1 derived complex is further cleaved into two polypeptide fragments of 19 kDa (MSP-19) and 33 kDa (MSP-133) by a second proteolytic cleavage, mediated by a calcium dependent serine protease (Blackman and Holder 1992), in a process called secondary processing. This results in the entire complex of MSP-1 being shed from the merozoite surface except for the GPI-linked C-terminal MSP-19 sub-fragment which still remains and is carried into the newly invaded erythrocyte on the surface of the merozoite (Blackman et al., 1990). This suggests that
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this C-terminal MSP-1\textsubscript{19} fragment might play an essential role during the process of erythrocyte invasion by the merozoite.

Recently, the three-dimensional structure of the C-terminal MSP-1\textsubscript{19} fragment has been determined using nuclear magnetic resonance (NMR) techniques (Morgan et al., 1999) and crystallography techniques (Chitarra et al., 1999). Figure 1.3 shows that the C-terminal MSP-1\textsubscript{19} fragment comprises two epidermal growth factor (EGF)-like domains of which each domain contains six cysteine (Cys) residues to form three disulphide bonds in the order 1-3, 2-4 and 5-6. Unlike other pairs of EGF domain (Brandstetter et al., 1995; Downing et al., 1996), EGF-like domains in the MSP-1\textsubscript{19} fragment are folded against each other so that their N-terminus and C-terminus are relatively close together (Morgan et al., 1999). This suggests that the proteolytic processing site that produces the C-terminal 96 amino acid fragment may be very closed to the GPI membrane attachment site at or near residue 96. This supports the idea that a membrane-bound \textit{Plasmodium} protease is responsible for the secondary processing (Blackman and Holder 1992).

1.9 Immune response to MSP-1

Early immunisation studies have shown that an immune response directed against MSP-1 were protective against blood-stage challenge in primate models (Siddiqui et al., 1987; Holder et al., 1988). Active immunisation with MSP-1\textsubscript{19}, the C-terminal fragment of MSP-1, expressed as a correctly folded recombinant protein has been shown to induce protective immune responses in \textit{P. yoelii} (Daly and Long 1993; Ling et al., 1994), in \textit{P. cynomolgi} (Perera et al., 1998), and in \textit{P. falciparum} (Kumar
et al., 1995; Chang et al., 1996). However, the protection was abolished by reduction and alkylation of the recombinant protein (Ling et al., 1994). Furthermore, passive immunisation with certain monoclonal antibodies or polyclonal antibodies that bind to MSP-119 from the rodent malaria parasite *P. yoelii* protected mice against a blood-stage parasite challenge (Majarian et al., 1984; Daly and Long 1995; Ling et al., 1997; Spencer Valero et al., 1998).

Studies of the human immune response in natural infection have also supported the notion that a protective immune response is induced by MSP-119. Studies in malaria endemic areas have shown that a large percentage of the adult population have antibodies to MSP-119 (Egan et al., 1995; Udhayakumar et al., 1995). It has also been shown that the presence of antibodies, T cell proliferative and cytokine responses to MSP-119 are associated with resistance to episodes of fever with high parasitaemia (Riley et al., 1992; Riley et al., 1993; Egan et al., 1996). These results indicate that the use of recombinant MSP-119 as a vaccine may provide an efficient and protective immune response against the malaria parasite by preventing erythrocyte invasion.

It has been shown by *in vitro* studies that monoclonal and polyclonal antibodies to the *P. falciparum* MSP-119, called inhibitory antibody, can effectively prevent secondary processing of MSP-1 and also erythrocyte invasion (Blackman et al., 1990; 1994; Chang et al., 1992; Chappel and Holder 1993). However, there is another group of anti-MSP-119 monoclonal antibody, called blocking antibody, which cannot inhibit both processes but instead blocks the inhibitory activities of the inhibitory antibodies (Guevara Patino et al., 1997). These results suggest that
polyclonal antibodies induced by MSP-1, containing many types of antibodies that may affect the protective immune response induced by the protein.

1.10 MSP-1 based malaria vaccine candidates

As described in the previous sections, *P. falciparum* MSP-1 is a major blood-stage vaccine candidate. Data from epidemiological studies suggested that MSP-1, is an important target for protective immunity. Recently, recombinant proteins containing portions of the MSP-1 molecule were generated, and immunisation studies were carried out. Three of the C-terminal MSP-1-based candidates (MSP-1, and MSP-142) are briefly discussed in this section.

1.10.1 GST-MSP-1

GST-MSP-1 is a recombinant fusion protein expressed in *E. coli*, and has similar folding and antigenicity to the native MSP-1 (Burghaus and Holder 1994; Burghaus et al., 1996). Immunisation-challenge experiments have shown that *Aotus* monkeys immunised with GST-MSP-1 in liposomes or in alum were not protected against blood-stage challenge (Burghaus et al., 1996). However, one of three *Aotus* monkeys immunised with GST-MSP-1 in complete Freund’s adjuvant (CFA) had delayed pre-patent period after challenge, while the other two monkeys were not protected (Kumar et al., 1995). These results suggest that MSP-1 can induce at least partial protective immunity, delaying the pre-patent period of the disease.
1.10.2 P30P2MSP-1$_{19}$

P30P2MSP-1$_{19}$ is a fusion of recombinant MSP-1$_{19}$ protein with the universal tetanus toxoid P30 and P2 helper T-cell epitopes, expressed in Saccharomyces cerevisiae (Kumar et al., 1995). Vaccination of Aotus monkeys with this molecule have proven to protect them against a lethal challenge of *P. falciparum* (Kumar et al., 1995; 2000; Egan et al., 2000). A strong protective response was achieved only when P30P2MSP-1$_{19}$ was used in combination with CFA, but not with other adjuvants that are suitable for human use (Kumar et al., 2000; Stowers et al., 2001). Interestingly, it was shown that monkeys immunised with P30P2MSP-1$_{19}$ in alum, followed by a parasite challenge, were protected against a second challenge four months later, suggesting that a single challenge may underestimate vaccine efficacy. It was also shown that when a previously infected monkey was vaccinated with P30P2MSP-1$_{19}$ in CFA, it was completely protected against a new challenge. This result suggests that prior exposure to the malaria parasite might prime the animal for production of anti-MSP-1$_{19}$ antibodies which might be boosted thereafter by vaccination with the recombinant protein (Egan et al., 2000).

1.10.3 BVp42

BVp42 is a baculovirus-derived recombinant polypeptide corresponding to the 42-kDa MSP-1 C-terminal processing fragment. Chang et al. have shown that rabbit sera raised against BVp42 inhibited *P. falciparum* parasite growth *in vitro* (Chang et al., 1992). BVp42 in CFA has also been shown to be protective in Aotus monkeys against a lethal *P. falciparum* challenge (Chang et al., 1996). Protected monkeys also produced antibodies, which inhibited parasite growth *in vitro*. Like P30P2MSP-1$_{19}$, to
date, BVp42 is effective only when administered with CFA, where as vaccination in combination with other adjuvants resulted in significant lower antibody titers and no protection.

1.11 Aims of this project

The aims of this project are to identify and analyse the location of epitopes recognised by processing-inhibitory and blocking antibodies on MSP-1\textsubscript{19}; and to investigate the protective immune response induced by modified MSP-1\textsubscript{19} mutant proteins. By using a site-directed mutagenesis approach, a series of single amino acid substitutions will be introduced into the MSP-1\textsubscript{19} sequence. It has been shown that most of the studied monoclonal antibodies bind to the first EGF-like domain of MSP-1\textsubscript{19} (Chappel and Holder, 1993). The initial efforts in this study will be focused on the substitution of amino acid residues in the first domain as well as some residues in the second domain. The binding of the MSP-1\textsubscript{19} mutant proteins to the panel of monoclonal antibodies will then be assessed by western blotting and by a surface plasmon resonance technique using a BIAcore machine. In order to investigate the effects of amino acid substitutions on the proteins' immunogenicity and their ability to induce protective antibodies, selected mutant proteins will be used to immunise laboratory animals and raise polyclonal antibodies. Total immunoglobulin G (IgG) will be purified from the serum of animals immunised with the proteins and tested for inhibitory activities in the MSP-1 secondary processing-inhibition and the erythrocyte invasion-inhibition assays. In the last part of the project, by using a computational biology approach, the molecular structures and surfaces of selected mutant proteins will be studied and compared to those of the wild type protein.
Chapter One
General introduction

Figure 1.1
The life cycle of *Plasmodium falciparum* malaria parasite

*P. falciparum* infection of humans begins when an infected *Anopheles* spp. mosquito takes a blood meal and injects infective sporozoites into the peripheral circulation (1). These sporozoites invade hepatocytes in the liver (2) and undergo asexual multiplication producing tens of thousands of invasive merozoite forms of the parasite (3, 4). When the infected hepatocyte ruptures, merozoites are released into the peripheral circulation (5). The merozoites invade erythrocytes and complete another round of asexual multiplication with the production of 16-20 additional merozoites per erythrocyte (6). The released merozoites invade further erythrocytes and carry on the cycle (7). Some intracellular parasites do not divide, but differentiate into the sexual forms of male (8) and female gametocytes (9). These sexual forms are taken from the bloodstream by a feeding *Anopheles* mosquito, gametes are produced (10, 11), and fertilize in the mosquito midgut to form zygotes (12). These zygotes further differentiate into motile forms, called ookinetes (13), migrate through the mosquito gut wall and form oocysts (14). New sporozoites develop in the oocyst, are released (15), and move to the mosquito’s salivary gland (16), where they await injection into another human host, thus completing the life cycle.
Figure 1.2

Proteolytic processing of MSP-1

The membrane-bound intact MSP-1 precursor is processed to form a complex of 4 small fragments of MSP-183, MSP-130, MSP-138 and MSP-142 which are found on the surface of the free merozoite during release. At or just before erythrocyte invasion a secondary processing step cleaves MSP-142 into MSP-133 and MSP-119. MSP-119 is retained and carried into the newly invaded erythrocyte whereas MSP-133 is shed in a soluble form from the merozoite surface as part of a complex with the other MSP-1-derived polypeptides.
Merozoite release

MSP-1 precursor (200 kDa)

Primary processing

MSP-183
MSP-130
MSP-138
MSP-142

Schizont surface

Merozoite invasion

Merozoite surface

Secondary processing

MSP-133
MSP-119

"Ring" stage surface

Soluble complex
Figure 1.3

Molecular image of MSP-119

Molecular image of MSP-119 shows the backbone Cα trace, antiparallel β-sheet elements and disulphide bridges (in yellow) formed by cysteine residues (in CPK colour).
2.1 Monoclonal antibodies

Anti-MSP-1\textsubscript{19} monoclonal antibodies used in this study were: mouse IgG mAbs 1E1, 1E8, 2F10, 8A12, 9C8 and 12D11, produced at Division of Parasitology, NIMR, London, U.K. (Blackman \textit{et al.}, 1994; Burghaus and Holder 1994); mouse IgG mAbs 111.2, 111.4 and 117.2, produced at Department of Molecular Biology, The Wellcome Research Laboratories, Kent, U.K. (Holder and Freeman 1982; Holder \textit{et al.}, 1985; 1987); mouse IgG mAbs 2.2, 7.5, 12.8 and 12.10, kindly provided by Dr. Jana McBride, Department of Zoology, University of Edinburgh, Edinburgh, U.K. (McBride and Heidrich 1987; Blackman \textit{et al.}, 1990); mouse IgG mAb 5.2, kindly provided by Dr. George Hui, Department of Tropical Medicine and Medical Microbiology, University of Hawaii, Hawaii, U.S.A. (Siddiqui \textit{et al.}, 1987); mouse IgM mAb 5B1, kindly provided by Dr. Margaret Perkins, Rockefeller University, New York, U.S.A. (Pirson and Perkins 1985). Anti-MSP-1\textsubscript{33} monoclonal antibody used was: human IgG mAb X509, produced at Division of Parasitology, NIMR, London, U.K. (Blackman \textit{et al.}, 1991).
2.2 Construction of MSP-1\textsubscript{19} mutant clones

The DNA coding the wild type MSP-1\textsubscript{19} domain of \textit{Plasmodium falciparum} (Wellcome strain) MSP-1 has been cloned in expression vector pGEX-3X (Smith and Johnson 1988) to express MSP-1\textsubscript{19} fused to the carboxy-terminus of the \textit{Schistosoma japonicum} glutathione S-transferase (GST) in \textit{Escherichia coli} (\textit{E. coli}) (Burghaus and Holder 1994). Site-directed mutagenesis of MSP-1\textsubscript{19} was carried out using the QuikChange™ site-directed mutagenesis kit (Stratagene). As shown in Figure 2.1, using pGEX-MSP-1\textsubscript{19} as a template, two complementary synthetic oligonucleotide primers containing the desired point mutation were designed and used to extend on the template by temperature cycling with the enzyme \textit{Pfu} DNA Polymerase. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks was generated. Following temperature cycling, the product was treated with \textit{DpnI} endonuclease which will digest the methylated parental DNA template and leave the mutation-containing synthesized DNA as product. The nicked vector DNA incorporating the desired mutation was then transformed into competent cells of the \textit{E. coli} strains XL1-Blue (Stratagene) or DH5\textalpha{} (Pharmacia Biotech), where the nicks will be repaired. Clones were screened by analysis of restriction enzyme digestions and by PCR screening of the insert gene. The DNA sequence of the selected mutant clones was confirmed using a PerkinElmer Applied Biosystems ABI 377 automatic sequencer according to the manufacturer's instructions.
2.3 Expression of GST-fusion MSP-1\textsubscript{19} proteins

Expression of GST-fusion MSP-1\textsubscript{19} protein (GST-MSP-1\textsubscript{19}) was induced with 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG; Melford Laboratories) for 1 hr in the \textit{E. coli} strain TOPP 1 (Stratagene). The cells were harvested by centrifugation at 1,600 \(\times\) g for 20 min at 4\(^\circ\)C and the cell pellet was resuspended in cell lysis buffer (50 mM Tris-HCl/ 1 mM EDTA pH 8.0 containing 0.2\% [v/v] Nonidet P40 (NP40; BDH)). Phenylmethylsulphonyl fluoride (PMSF; Sigma) in isopropanol was added to a final concentration of 1 mM. The cell suspension was sonicated on ice, using a VibraCell sonicator (Sonics \& Materials) at 50\% duty cycle for 3 min. The cell lysate was centrifuged at 20,000 \(\times\) g for 20 min at 4\(^\circ\)C and again at 61,000 \(\times\) g for 1 hr at 4\(^\circ\)C. Supernatant containing soluble GST-fusion protein was applied to a glutathione-agarose column (Sigma), which was equilibrated with 50 mM Tris-HCl/ 1 mM EDTA pH 8.0 at the rate of 6 ml hr\(^{-1}\), and the GST-fusion protein bound to the column was eluted with 5 mM reduced glutathione at the rate of 20 ml hr\(^{-1}\). The eluted GST-fusion protein was dialysed extensively against phosphate buffered saline (PBS) at 4\(^\circ\)C and kept in aliquots at -70\(^\circ\)C.

2.4 SDS-PAGE and Western blotting

Proteins were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) (Laemmli 1970). Samples were solubilised by boiling in SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8, 20\% [v/v] glycerol, 4.6\% [w/v] sodium dodecyl sulphate (SDS) and 0.01\% [w/v] bromophenol blue) without reducing agents, then fractionated on a homogeneous 12.5\%
polyacrylamide gel. Depending on the requirements of the experiments, prestained low range (24,000-102,000 daltons) or prestained broad range (7,400-205,000 daltons) molecular mass markers from Bio-Rad were used. When required, SDS-PAGE-fractionated polypeptides were either stained with Coomassie Brilliant Blue R-250 (CBB; Sigma) or electrophoretically transferred to Optitran BA-S 83 reinforced nitrocellulose (Schleicher & Schull, 0.2 μm pore size) for analysis by western blotting (Towbin et al., 1979). Blots were blocked with 5% BSA in PBS containing 0.5% Tween 20 (PBS-T) for 1 hr at room temperature, then washed once in PBS-T. Blots were probed with first antibodies for 2 hr at room temperature, washed 3 times in PBS-T, and then incubated in 1/1,000 dilution of horse-radish peroxidase (HRP)-conjugated sheep anti-mouse IgG (H+L) (ICN Immunobiologicals) or in 1/1,000 dilution of HRP-conjugated goat anti-mouse IgM (μ chain) (Sigma) for 1 hr at room temperature. Blots were washed 3 times in PBS-T and developed using SuperSignal® Substrate (Pierce) (Walker 1995) as HRP substrate for 1 min. Blots were then placed in plastic wrap and exposed to X-ray film (BioMax MR-1; Kodak). The films were processed with an Agfa Gevamatic60 film processor (Agfa).

2.5 Immobilisation of GST-MSP-119 to the sensor chip CM5

GST-MSP-119 was coupled to a carboxymethyl dextran hydrogen sensor chip CM5 (Pharmacia Biacore) via its amino groups using N-ethyl-N’-(dimethylaminopropyl) carbodiimide hydrochloride (EDC)/ N-hydroxysuccinimide (NHS) chemistry (Johnsson et al., 1991) with the amine coupling kit (Pharmacia Biacore) to activate the CM5 dextran. The binding was aided by electrostatic attraction between the positively charged protein and the negatively charged carboxyl
groups in the matrix. The carboxyl groups on the CM5 dextran matrix were transformed into reactive N-hydroxysuccinimide esters by injection of 50 μl of 1:1 mixture of 200 mM EDC and 5 mM NHS (Pharmacia Biacore) for 10 min. GST-MSP-1\textsubscript{19} was then coupled to the CM5 sensor surface, using 50 μl of a solution at 100 μg ml\textsuperscript{-1} in coating buffer (0.01 M sodium acetate buffer, pH 4.0) for 10 min. Unreacted carboxyl groups were blocked by adding 50 μl of 1 M ethanolamine, pH 8.5 for 10 min. The flow cells were washed with two 20 μl pulses of 10 mM glycine-HCl pH 2.8 for 8 min in total to remove any non-covalently bound proteins. The immobilisation procedure was carried out at a constant flow rate of 5 μl min\textsuperscript{-1} at 25°C. Measurements were performed on the BIAcore 2000 instrument (Pharmacia Biacore) with a CM5 sensor chip (certified, research grade).

2.6 Binding of monoclonal antibodies to immobilised GST-MSP-1\textsubscript{19}

The binding assays were performed with a constant flow rate of 5 μl min\textsuperscript{-1} at 25°C. Purified mAbs 12.8, 12.10, 1E1 and 2F10 were used at 100 μg ml\textsuperscript{-1} in HBS-EP buffer (10 mM HEPES pH 7.4 containing 150 mM NaCl, 3 mM EDTA and 0.005% [v/v] polysorbate 20); mAbs 5B1 and 111.4 were in cell culture supernatant, and ascitic fluid containing mAbs 2.2 and 7.5 was diluted at 1/10 in HBS-EP buffer. The antibodies were allowed to interact with the immobilised GST-MSP-1\textsubscript{19} for 10 min and then the dissociation was followed for a further 5 min. The residual bound antibody was removed by washing the chip with 10 mM glycine-HCl pH 2.4, or when required, 100 mM glycine-HCl pH 1.8.
2.7 Immunisation with MSP-1\textsubscript{19} mutant proteins

MSP-1\textsubscript{19} mutant proteins were used to immunise rabbits and mice. For rabbit immunisation, a male MurexLops rabbit (Murex Biotech, Kent, U.K.) weighting 2.5-3.0 kg was immunised subcutaneously with 100 μg of protein in Freund's complete adjuvant and the response was boosted by a further 3 injections of 200 μg of protein in Freund's incomplete adjuvant 21, 42 and 63 days later. For mouse immunisation, five 6-week-old female BALB/c mice (Specific Pathogen Free (SPF) Unit, NIMR, London, U.K.) were immunised intraperitoneally with 10 μg of protein in Freund's complete adjuvant and the response was boosted by a further 3 injections of 40 μg of protein in Freund's incomplete adjuvant 21, 42 and 63 days later. Test bleed serum was taken before the first immunisation and 14 days after each boost. In both cases, the animals were killed 21 days after the final immunisation and serum was taken.

2.8 Purification of animal IgG

Animal IgG was purified from serum of animals immunised with MSP-1\textsubscript{19} proteins by chromatography on a Protein G (Protein G Sepharose\textsuperscript{®} 4 Fast Flow; Pharmacia Biotech) column. Sera from animals in each immunisation group were pooled together and then diluted in binding buffer (20 mM sodium phosphate, pH 7.0) before being applied to a Protein G column which had been previously equilibrated with binding buffer at the rate of 6 ml hr\textsuperscript{-1}. The column was then washed extensively with binding buffer, and the bound IgG was eluted in 0.1 M glycine-HCl, pH 2.7 at the rate of 20 ml hr\textsuperscript{-1}. The eluted fractions were immediately neutralised with 100 μl
ml\(^{-1}\) eluate of 1 M Tris-HCl, pH 9.0. The eluted IgG was dialysed extensively against PBS at 4°C and kept in aliquots at -70°C.

2.9 Immunofluorescence assay

A thin blood film of mixed stage *P. falciparum*-infected erythrocytes on a glass-slide was fixed in 1:1 [v/v] methanol-acetone for 2 min. Diluted animal sera or purified IgG were incubated on the slides for 1 hr at 37°C in a humid box. The slides were washed twice in PBS and incubated with 1/100 dilution of FITC-conjugated goat anti-mouse IgG (Sigma) or sheep anti-rabbit IgG (Sigma) for 1 hr at 37°C in a humid box. The slides were then washed twice and stained in 0.05% Evans blue and in 1 μg ml\(^{-1}\) DAPI (4',6-diamidino-2-phenylindole) for 5 sec. The slides were dried and mounted in Citifluor\(^{®}\) (Citifluor UKC Chemical Laboratories). They were then examined using a Zeiss III fluorescent microscope.

2.10 ELISA for detection of MSP-1\(_{19}\)-specific antibodies

An enzyme-linked immunosorbent assay (ELISA) was used to titrate the specific IgG for MSP-1\(_{19}\) protein. Wells of 96-well flat-bottomed ELISA plates (Nunc F96 Certified Maxisorp\(^{®}\)) were coated with 50 μl of 10 μg ml\(^{-1}\) of *Pichia*-expressed recombinant 6-His tagged wild type MSP-1\(_{19}\) protein in PBS (a gift from Dr. Bill Morgan, Division of Molecular Structure, NIMR, London, U.K.). Plates were incubated overnight at room temperature and then washed 3 times with 0.05% PBS-Tween (PBS-T). Plates were then blocked by the addition of 100 μl/well of 1% BSA in PBS and incubated at room temperature for 2 hr. Plates were washed 3 times with
PBS-T prior to addition of 50 μl/well of diluted purified IgG samples in PBS containing 0.1% BSA. After a further 2 hr incubation at room temperature, plates were again washed 3 times with PBS-T. Bound antibody was detected by incubating with 50 μl of 1/1000 dilution of alkaline phosphatase-conjugated anti-mouse IgG or anti-rabbit IgG (Sigma) for 1 hr at room temperature and plates were then washed 3 times. In order to detect alkaline phosphatase, 100 μl of freshly prepared substrate solution (1 mg ml⁻¹ p-nitrophenyl phosphate in 1 M diethanolamine buffer, pH 9.8, containing 0.5 mM MgCl₂) was then added to wells and plates were incubated at room temperature for 5 min. The plates were read for absorbance at 405 nm.

2.11 **In vitro culture of *P. falciparum***

The 3D7 and FCB-1 lines of *P. falciparum* were maintained in human erythrocytes at 1-2% haematocrit in a complete medium of RPMI-1640 supplemented with 25 mM HEPES, 25 mM NaHCO₃, 0.2% [w/v] glucose, 25 μg ml⁻¹ gentamicin, 20 μg ml⁻¹ hypoxanthine and 0.5% Albumax™ (Gibco BRL) for routine cultivation. Cultures were gassed with a mixture containing 7% CO₂, 5% O₂ and 88% N₂, and incubated at 37°C. The medium was changed daily. Cultures were resuspended and transferred to 50 ml screw-cap centrifuge tubes (Corning) and cells pelleted at 570 x g for 5 min on a bench centrifuge (Sigma 4-10). The supernatant was aspirated and the pelleted cells were resuspended and returned to culture using 800 ml Nunclon flasks (Nunc) at 100 ml of culture per flask. Parasites were maintained at a maximum parasitaemia of 10-15% and were monitored by taking smears of culture every day. The smears were dried, fixed with 100% methanol and stained with 10% [v/v] Giemsa’s stain in phosphate buffer pH 7.2.
2.12 Synchronisation of *P. falciparum* culture

A combination of flotation on gelatin (Pasvol *et al.*, 1978) and disruption with sorbitol (Lambros and Vanderberg 1979) methods was used. Parasite cultures were routinely synchronised once a week in order to maintain synchrony. When bulking up cultures for merozoite preparation, the parasites were synchronised at 48-hour intervals until 4 days before merozoite collection, from which point they were simply fed daily. All media and solutions were prewarmed to 37°C before use.

Cultures containing mature, segmented schizonts were pelleted in 50-ml screw-cap tubes at 570 x g for 5 min at room temperature. The supernatant was aspirated and the volume of the pellet was estimated. The pellet volume was then multiplied by 2.4, and warm medium was added to the tube to bring the volume up to the calculated figure. A volume of warm Gelofusine Flexpak (B. Braun Medical) equal to the total volume of cells plus medium was added to the tube. The contents of the tube were carefully, but completely, resuspended and then transferred to a fresh tube. The tube was sealed, without gassing, and placed in a 37°C incubator for 30 min without disturbance. After 30 min incubation, the tube was carefully removed from the incubator. At this stage two phases were visible; the top, brownish layer containing mature forms of the parasite, whereas the lower, red layer contains uninfected erythrocytes and immature forms of the parasites. The upper layer was removed and transferred to a fresh tube. An equal volume of warm complete medium was added, and the cells were pelleted at 570 x g for 5 min. The pellet was carefully resuspended in fresh medium and added to a culture flask containing fresh medium and fresh washed erythrocytes such that the parasitaemia was 2-10% and the final
haematocrit was 20-25%. The flask was gassed and incubated without disturbance at 37°C for 3-4 hr. During this period schizont rupture and erythrocyte re-invasion occur.

Following the incubation step, the culture was treated with sorbitol to remove residual schizonts. The cells were pelleted at 570 x g for 5 min, the supernatant was aspirated and the pellet was resuspended into at least 10 volumes of 10% [w/v] sorbitol in water. The suspension was left for 10 min at 37°C and then pelleted at 570 x g for 5 min, washed once in serum-free medium and recultured. The cultures treated in this way contain only ring forms of the parasites, the most mature of which were only 3-4 hours old.

2.13 Purification of schizonts and naturally released merozoites

Mature schizonts from synchronous cultures were purified on Gelofusine Flexpak as described above. The target age of the schizonts was 40-44 hours old, containing about 8-20 nuclei; the parasitaemia of such enriched preparations was at least 90%. Since the released merozoites adhere strongly to the membrane sieves in the presence of Albumax, the schizonts were recultured in warm complete medium containing 10% human serum and 5 mM EGTA for the collection of merozoites to be used in the secondary processing of MSP-1 assay. Merozoite release was assessed by Giemsa-stained smears of the culture at 1-2 hour intervals.

Cutures were transferred to 50-ml tubes and centrifuged at 570 x g for 5 min at room temperature to pellet the schizonts. The supernatant from the pelleted culture,
which contained free merozoites, was passed under negative pressure through a pre-soaked 3-μm pore size and then a pre-soaked 1.2-μm pore size acrylic-supported membrane filter (Versapor membranes, Gelman Sciences), held in filter units (Sterifil System (47 mm); Millipore). The schizont pellet was resuspended immediately in warm medium and recultured for further merozoite collection. The filtered supernatant was transferred to 50-ml flanged polycarbonate tubes (Beckman) and centrifuged in a Sigma 4K15 swing-out rotor at 5,000 x g for 10 min at 4°C. The supernatant was aspirated from the tubes, and the merozoite pellet was stored in aliquots at -70°C. The purified merozoite preparations were checked by Giemsa-stained smears to ensure the complete absence of contaminating schizonts and erythrocytes.

2.14 Assay for inhibition of secondary processing of MSP-1

The merozoite pellets stored at -70°C were thawed on ice and washed twice with ice-cold calcium and magnesium-free PBS (PBS-CMF) supplemented with 1 mM EGTA. The merozoites were pelleted in a microcentrifuge at 13,000 x g for 10 min at 4°C. Washed merozoites were then resuspended in ice-cold 50 mM Tris-HCl pH 7.5 containing 10 mM CaCl$_2$ and 2 mM MgCl$_2$ (reaction buffer). Aliquots of about 1 x 10$^9$ merozoites were dispensed into 1.5 ml microcentrifuge tubes on ice. The antibodies were then added to the merozoite suspension at a final volume of 20 μl. Merozoites were maintained on ice for 30 min to allow antibody binding, then transferred to a 37°C incubator for 1 hr to allow processing to proceed. Assays always included the following controls; a "positive processing" control sample of merozoites, resuspended in reaction buffer or reaction buffer with PBS; a "negative processing"
control sample of merozoites, resuspended in reaction buffer plus 1 mM PMSF, and a zero time (0 hr) control, in which processing was stopped before the 37°C incubation step by adding 20 µl of SDS-PAGE sample buffer without a reducing agent. All other samples’ reaction was stopped using the same technique after one-hour incubation at 37°C. The samples were boiled for 5 min and clarified by centrifugation for 10 min at 13,000 x g in a microcentrifuge. Eighteen microlitres of each sample was subjected to electrophoresis on 12.5% polyacrylamide gels. Separated proteins were transferred to Optitran BA-S 83 reinforced nitrocellulose. Blots were blocked with 5% BSA in PBS-T for 1 hr at room temperature, then washed once in PBS-T. Blots were probed with biotinylated anti-MSP-133 mAb X509 (a gift from Dr. Simon Wilkins, Division of Parasitology, NIMR, London, U.K.) for 2 hr at room temperature, and washed 3 times in PBS-T. Bound antibody was detected using 1/2000 dilution of HRP-conjugated streptavidin (Vector Laboratories) for 1 hr at room temperature. Blots were then washed 3 times in PBS-T and developed using SuperSignal® Substrate as HRP substrate for 1 min. Blots were then placed in plastic wrap and exposed to BioMax MR-1 X-ray films. The films were processed with an Agfa Gevamatic60 film processor.

2.15 Assay for inhibition of erythrocyte invasion in vitro

Schizonts were purified as described in 2.13. The level of parasitaemia of the parasite suspension enriched for the schizonts was determined by examination of a Giemsa-stained smear and it was >90% in all experiments. It was then adjusted to 1% parasitaemia by adding the enriched schizonts to the stock suspension of uninfected erythrocytes in RPMI-1640 with the final haematocrit at 5%. Eighty microlitres of the
parasitised erythrocytes were placed into the wells of a sterile 96-well flat bottom plate containing 20 µl of test IgG samples. Each IgG sample was tested in duplicate at the final concentration of 1 mg ml\(^{-1}\).

For neutralisation experiments, IgG samples at the final concentration of 2.5 mg ml\(^{-1}\) were allowed to interact with the wild type GST-MSP-1\(_{19}\) protein (Ag) at the final concentration of 0.5 mg ml\(^{-1}\) for 5 min at room temperature. They were then added to the parasite cultures where the final concentration of IgG samples and Ag were 1 mg ml\(^{-1}\) and 0.2 mg ml\(^{-1}\), respectively. As comparison control, the final concentration of 0.2 mg ml\(^{-1}\) of Ag and the final concentration of 1 mg ml\(^{-1}\) of IgG samples alone were also tested in the parasite cultures. The positive-inhibition control wells contained parasitised erythrocytes in EGTA at the final concentration of 10 mM while the negative-inhibition control wells were cultures in the presence of PBS since all IgG samples were dialysed and maintained in PBS. After 24 hr culture at 37°C, a thin blood smear was made from each well, and the final parasitaemia was determined by microscopic examination of the number of 'ring' stage parasites within 2,000 or more red blood cells. Multiple ring stages within a single erythrocyte were recorded as one count. The results were recorded as mean percentage of erythrocyte invasion plus/minus the standard deviation (S.D.) from 4 counts of duplicate cultures of each sample. The statistical significance of differences between samples and control (control set to 100% invasion) were determined using Student’s \(t\)-test.
2.16 Molecular modelling of MSP-1₁₉ proteins

The structural data for *P. falciparum* (1CEJ.pdb) and *P. cynomolgi* (1B9W.pdb) MSP-1₁₉ proteins were obtained from the Brookhaven Protein Databank (PDB) (http://www.rcsb.org/pdb). Structural alignment of the two proteins was performed with the SAP (Structural Alignment of Proteins) algorithm (Taylor 2000). The MODELLER (Sanchez and Sali 2000) and PROMODII (Guex and Peitsch 1997) programs were used to generate models. Model verification and energy minimisation were performed using WHATCHECK (Hooft *et al.*, 1996) and GROMOS96 (van Gunsteren *et al.*, 1993) programs, respectively. The ribbon, molecular surface and electrostatic potential images were then created using Swiss-PdbViewer version 3.7b2 (Guex and Peitsch 1997) and MOLSCRIPT (Kraulis 1991) programs.
Figure 2.1

Protocol for QuikChange™ site-directed mutagenesis of MSP-119

Using pGEX-MSP-119 as a template, two complementary mutagenic primers containing the desired point mutation were used to extend on the template by temperature cycling with *Pfu* DNA Polymerase. Following temperature cycling, the mutated plasmid containing staggered nicks was treated with *DpnI* endonuclease that will digest the methylated parental DNA template and leave the mutation-containing synthesized DNA as product. The nicked vector DNA incorporating the desired mutation is then transformed into *E. coli*, where the nicks will be repaired. Clones were screened by analysis of restriction enzyme digestions and by PCR screening of the insert gene. The DNA sequence of the selected mutant clones was confirmed by DNA sequencing.
Mix denatured plasmid and annealing primers containing desired mutation.

Temperature cycle to extend and incorporate mutation primers resulting in nicked circular strands.

Digest parental DNA template with DpnI.

Transform the resulting annealed double-stranded nicked DNA molecules.

After transformation, E. coli cells repair nicks in plasmid.
Chapter Three

Identification of inhibitory and blocking antibody epitopes on MSP-1

3.1 Introduction

During the blood stage infection, each infected erythrocyte can give rise to about 20 new invasive merozoites. Thus the parasitaemia can increase rapidly, resulting in the symptoms of malaria, severe morbidity and mortality. It has been proposed that protective immune responses against merozoites induced by a vaccine will interrupt the asexual blood stage merozoite multiplication, which would protect against malaria (Holder 1996). It has been shown that some antibodies against MSP-1\textsubscript{19}, a leading malaria vaccine candidate, can inhibit erythrocyte invasion \textit{in vitro} (Blackman \textit{et al.}, 1990) (Figure 3.1). These antibodies that inhibit erythrocyte invasion also inhibit the secondary processing of the 42 kDa fragment, suggesting that specific antibodies to MSP-1\textsubscript{19} could inhibit erythrocyte invasion by a mechanism that involves inhibition of the serine protease activity which is responsible for the secondary processing (Blackman \textit{et al.}, 1994). There are another 2 groups of non-neutralising antibodies that bind to MSP-1\textsubscript{19}, but do not inhibit erythrocyte invasion or inhibit secondary processing of MSP-1. The first group of antibodies is defined as
‘blocking antibodies’ since they compete with the inhibitory antibodies for the binding to MSP-119, and block both the inhibition of erythrocyte invasion and the inhibition of secondary processing of MSP-1 mediated by inhibitory antibodies. This allows MSP-1 processing and invasion to proceed, in the presence of inhibitory antibodies (Guevara Patino et al., 1997). It has also been shown that some of these blocking antibodies bind to epitopes elsewhere in the MSP-1 complex (Guevara Patino et al., 1997). The second group of non-neutralising antibodies is referred to as ‘neutral antibodies’ since they do not show any blocking activities and have no known biological effects. This suggests that polyclonal antibodies induced against MSP-119 might contain inhibitory, blocking and neutral antibodies.

In the presence of blocking antibodies, the processing-inhibitory and erythrocyte invasion-inhibitory activities of inhibitory antibodies are not effective. A vaccine based on MSP-119 should be designed to abolish or minimise the induction of blocking antibodies and maximise the induction of inhibitory antibodies. That means it should contain the sequences that only form epitopes that are targets of, and can induce, inhibitory antibodies, but should not contain the sequences that form epitopes for blocking antibodies. If the polypeptide contains epitopes for both inhibitory and blocking antibodies, then perhaps it can be modified to remove the blocking antibody epitopes without affecting the inhibitory antibody epitopes. A research goal in the development of a vaccine against malaria based on MSP-119 is the identification of a modified protein which would induce inhibitory, but not blocking antibodies when used to immunise an individual.
Previous studies have shown that monoclonal antibodies against MSP-1\textsubscript{19} react with correctly folded conformational epitopes in the first EGF domain or epitopes formed from the two EGF domains (Chappel and Holder 1993), which interact with each other (Morgan \textit{et al.}, 1999). Inhibitory mAbs 12.8 and 5B1 bind to the first domain, whereas mAb 12.10 requires the presence of both EGF domains. Blocking mAbs 1E1, 2.2, 7.5 and 111.4 bind to recombinant MSP-1\textsubscript{19} and block the binding of 12.8; mAbs 1E1 and 7.5 also block the binding of mAb 12.10 (Guevara Patino \textit{et al.}, 1997). These monoclonal antibodies represent, at least in part, the repertoire of inhibitory and blocking antibodies induced during immunisation, and therefore it is important to know more about their binding sites on MSP-1.

In order to identify the residues contributing to the binding sites of different antibodies on MSP-1\textsubscript{19} and to understand the antibody reactions with MSP-1\textsubscript{19}, the effect of engineered point mutations on antibody binding was studied. The amino acids that have been changed were those amino acids which show high conservation in comparisons between several \textit{Plasmodium} species and strains (Figure 3.2), and those residues which are the key residues for structural integrity and for receptor binding found in other EGF-like motifs. The radical changes consisted of, for example, replacing an aliphatic residue with a charged polar residue, replacing a positively charged side chain with a negatively charged side chain, replacing an amino acid with a large side chain with an amino acid with a smaller or no side chain (glycine), replacing a polar amino acid with a charged polar amino acid, replacing a polar amino acid with an aromatic amino acid, replacing a large aromatic amino acid with an amino acid with a smaller side chain, and replacing cysteine residues that are involved in disulphide bonds. The structural models and their physio-chemical
properties of all amino acids occurring naturally in proteins are shown in Figures 3.3 and 3.4, respectively.
Figure 3.1

Schematic showing the action of MSP-1 specific antibodies on secondary processing and erythrocyte invasion

The membrane-bound intact MSP-1 precursor is processed to form a complex of 4 smaller fragments of 83 kDa (MSP-1$_{83}$), 30 kDa (MSP-1$_{30}$), 38 kDa (MSP-1$_{38}$) and 42 kDa (MSP-1$_{42}$) on the surface of the free merozoite during merozoite release (A). At or just before erythrocyte invasion, MSP-1 undergoes secondary processing cleaving MSP-1$_{42}$ to MSP-1$_{33}$ and MSP-1$_{19}$, and invasion proceeds (B). Inhibitory antibodies (Y) bind to MSP-1$_{19}$ and inhibit the secondary processing, thus erythrocyte invasion is interrupted (C). In the presence of blocking antibodies (Y), antibodies that recognise MSP-1$_{19}$ as well as other parts of MSP-1, the functions of the inhibitory antibodies are interfered with, so that invasion proceeds (D).
Figure 3.2

Sequence alignment of MSP-1\textsubscript{19} in *Plasmodium* spp.

All cysteine residues are coloured in red. The specific cysteine pairs that form disulphide bonds between them are numbered in the same colour. Conserved residues are marked with an asterisk; semiconserved residues are marked with double dots and weakly conserved residues are marked with a single dot. Red arrows indicate the hydrophobic residues at the EGF-domain pair interface in *P. falciparum*, and the corresponding conserved residues in the other sequences. Residues coloured in yellow, with cysteine residues positioned in between them, form secondary $\beta$ structure in the molecule. The abbreviations are as follows: Pf: *P. falciparum*, Py: *P. yoelii*, Pb: *P. berghei*, Pc: *P. chabaudi*, Pvin: *P. vinckei*, Pv: *P. vivax* and Pcy: *P. cynomolgi*. 
Figure 3.3

Molecular models of the 20 amino acids naturally occurring in proteins

The model of amino acids occurring at neutral pH is shown. Carbon, oxygen, nitrogen, sulfur and hydrogen atoms are in grey, red, blue, yellow and white, respectively. Below the name of the amino acid are the three-letter and the one-letter abbreviations commonly used.
Figure 3.4

A Venn diagram showing the physio-chemical properties of the 20 amino acids naturally occurring in proteins

The diagram shows the properties of the 20 amino acids relating to size, charge and hydrophobicity. The amino acids are divided into two major sets, one containing amino acids which contain a polar group (Polar) and a set which exhibit a hydrophobic effect (Hydrophobic). A third major set, Small, is defined by size. Other sets include: full-charge (referred to as Charged) which contains the subset Basic (Acidic is defined by implication), and Aromatic for the amino acids that have aromatic ring(s). This diagram is modified from the Venn diagram published by Taylor (Taylor 1986).
3.2 Results

3.2.1 Construction of MSP-1\textsubscript{19} mutant proteins containing a single amino acid substitution

Amino acid substitutions in the sequence of MSP-1\textsubscript{19} have been inserted using the QuikChange\textsuperscript{™} site-directed mutagenesis kit which uses a method based on a 2-mutant primer-polymerase chain reaction as described in the Materials and Methods. The codons used for each mutated position are shown in Table 3.1. The new mutant plasmid inserted into the bacterial expression vector has been screened and checked by DNA sequencing to confirm that only one amino acid substitution has been introduced. The expression of MSP-1\textsubscript{19} mutant proteins containing single amino acid substitution fused to GST was induced using IPTG and the proteins were purified by affinity chromatography using a glutathione-agarose column. Purified mutant proteins were divided in aliquots and kept at -70\textdegree C until use. In this study, 27 MSP-1\textsubscript{19} mutant proteins containing a single amino acid substitution have been created. The summary of these mutant proteins is shown in Table 3.2.

3.2.2 Western blotting analysis of the binding activity of monoclonal antibodies to MSP-1\textsubscript{19} mutant proteins containing a single amino acid substitution

The binding activity of mAbs to MSP-1\textsubscript{19} mutant proteins was detected by western blotting analysis. As summarised in Table 3.2, 27 MSP-1\textsubscript{19} mutant proteins containing a single amino acid substitutions have been tested for their antigenic properties with a panel of mAbs. Of these 27 MSP-1\textsubscript{19} mutant proteins, 7 of the
substitutions each abolished the binding of one or more mAbs. Selected western blots showing the effect of mutation on mAb binding are shown in Figure 3.5. Substitutions that affect the binding of inhibitory antibodies help to define the epitopes recognised by these antibodies. The Glu26→Ile mutation abolished both the binding of a processing-inhibitory mAb 12.8, one of the mAbs that is capable of preventing the secondary proteolytic processing of MSP-1 and erythrocyte invasion in vitro; and the binding of blocking mAb 2.2, one of the mAbs that binds to the native MSP-1 and interferes with the binding of the processing-inhibitory antibodies. This substitution also reduced the binding of the inhibitory mAb 5B1. We found that substitutions of cysteine residues affected the mAb binding. The Cys12→Ile mutation abolished the binding of all inhibitory mAbs 12.8, 12.10, 5B1; and blocking mAb 2.2. Replacement of Cys28 by Trp also abolished the binding of inhibitory mAbs 12.8, 5B1 and blocking mAb 2.2, and reduced the binding of 12.10. Interestingly, not all the substitutions affected the binding of mAbs, for example Leu22→Arg, had no effect on the binding of any of the mAbs tested.

Of particular interests were those amino acid substitutions that prevent the binding of blocking antibodies but have no effect on the binding of inhibitory antibodies. Replacement of Asn15 by Arg abolished the binding of mAb 7.5 and reduced the binding of mAb 111.4. Replacement of Glu27 by Tyr abolished the binding of mAb 2.2. Replacement of Leu31 by Arg abolished the binding of mAb 1E1, although it also reduced the binding of 12.8. Replacement of Glu43 by Leu abolished the binding of mAb 111.4. Glu43→Leu also reduced the binding of mAbs 1E1 and 7.5. An additional interesting mutation was Tyr34→Ser that reduced the binding of all blocking mAbs but did not affect the binding of any inhibitory mAbs.
The locations and effects of amino acid substitutions on inhibitory and blocking antibody binding were identified on the 3-dimensional structure of MSP-1\textsubscript{19} as shown in Figures 3.6A and B, respectively. Residues in EGF-1 and EGF-2 domains are coloured white and grey, respectively. For each antibody, the location of mutations that abolished binding are coloured in red, mutations that reduced binding are coloured in yellow, and locations of those that had no effect upon binding are coloured in blue. For ease of viewing, each structure is shown in four 90° rotations around the γ-axis to provide optimal display of the residues that appear to contribute to each epitope.

3.2.3 BIAcore analysis of the binding affinity of monoclonal antibodies to MSP-1\textsubscript{19} mutant proteins containing a single amino acid substitution

The above western blotting results, showing that some MSP-1\textsubscript{19} mutant proteins do not bind to the blocking antibodies, were confirmed using a quantitative surface plasmon resonance technique performed using the BIAcore2000 machine. The MSP-1\textsubscript{19} mutant proteins were immobilised on the CM5 sensor chip surface and then allowed to interact with the mAbs as described in the Materials and Methods. Figure 3.7 shows the results of the binding of selected mAbs to the immobilised MSP-1\textsubscript{19} mutant proteins with a single amino acid substitutions. The results show the percentage of binding, in resonance units, of the mAbs compared between the MSP-1\textsubscript{19} mutant proteins and the wild type MSP-1\textsubscript{19} protein. It is clear that the results obtained by BIAcore experiments parallel those obtained by western blotting. Antibodies that did not bind to a modified protein on blots also showed reduced binding on the sensor chip. It was confirmed that the mutations of Asn15→Arg,
Glu27→Tyr, Leu31→Arg, Glu43→Leu largely affected the binding of blocking mAbs 7.5, 2.2, 1E1 and 111.4, respectively.

3.2.4 Construction of MSP-119 mutant proteins containing multiple amino acid substitutions

As described above, four individual MSP-119 mutant proteins containing a single amino acid substitution, each of which abolished the binding of 4 individual blocking mAbs, but not inhibitory mAbs, were found. In order to create an individual protein that does not bind any of the blocking mAbs, combinations of amino acid substitutions, that were found to prevent the binding of blocking mAbs but do not affect the binding of inhibitory mAbs, were made using the QuikChange™ site-directed mutagenesis kit, the same technique used to create a MSP-119 mutant protein as described previously. Although substitutions of the individual Cys12 or Cys28 affected the binding of inhibitory mAbs, the double amino acid substitutions of this cysteine pair which form a disulphide bond in the first EGF domain of \textit{P. falciparum} MSP-119, were also made. The new mutant plasmids inserted into the bacterial expression vector were checked by DNA sequencing to confirm that the desired point mutations have been introduced. The MSP-119 mutant proteins containing multiple amino acid substitutions fused to GST were expressed and then purified. The summary of these mutant proteins and their antigenic properties assessed by western blotting with the panel of inhibitory and blocking mAbs is shown in Table 3.3.
3.2.5 Western blotting analysis of the binding activity of monoclonal antibodies to MSP-1\textsubscript{19} mutant proteins containing multiple amino acid substitutions

As shown in Table 3.3, the mutant protein 27+31+43, in which the mutation of Glu27→Tyr, Leu31→Arg and Glu43→Leu were combined, abolished the binding of blocking mAbs except mAb 7.5, but continued to bind all inhibitory mAbs. Addition of either Asn15→Arg, the substitution that abolished the binding of mAb 7.5, or Tyr34→Ser, the substitution that reduced the binding of all blocking mAbs, to the mutant protein 27+31+43 produced the mutant proteins 15+27+31+43 and 27+31+34+43, respectively. These proteins bound none of the four blocking mAbs, but still continued to bind all inhibitory mAbs. For MSP-1\textsubscript{19} mutant proteins with double amino acid substitutions, 12+28, in which the second disulphide bond in the first EGF domain was removed, both 12+28 proteins continued to bind to all monoclonal antibodies except blocking mAb 2.2. Of the two 12+28 proteins, the Cys12→Ile plus Cys28→Trp combination was selected for further investigation.

On incorporation of the 12+28 changes, Cys12→Ile plus Cys28→Trp, in 15+27+31+43, the six amino acid substitution mutant protein 12+15+27+28+31+43 was made. This modified protein continued to bind inhibitory mAbs 12.8 and 5B1 with reduced binding to inhibitory mAb 12.10, but bound none of the blocking mAbs. Interestingly, the five amino acid substitution mutant protein 12+15+27+31+43 which has one free cysteine residue (Cys28), the seven amino acid substitution mutant protein 12+15+27+28+31+43+53, and the eight amino acid substitution mutant
protein 12+15+27+28+31+34+43+53 could not bind to any inhibitory or blocking mAbs.

3.2.6 BIAcore analysis of the binding affinity of monoclonal antibodies to MSP-19 mutant proteins containing multiple amino acid substitutions

As shown in Figure 3.8, it was confirmed that the mutant protein 27+31+43 bind to all inhibitory mAbs, and blocking mAb 7.5. MSP-19 mutant proteins 15+27+31+43 and 27+31+34+43 can bind to inhibitory mAbs, but not to any blocking mAbs. Despite the western blotting results showing that the mutant protein 12+28 bound to all mAbs except mAb 2.2, the BIAcore binding results showed that this protein bound to all mAbs except mAbs 2.2 and 12.8. The BIAcore binding results for the six amino acid substitution mutant protein 12+15+27+28+31+43 also showed that the protein bound none of the mAbs tested except the anti-GST antibody which is a positive control, while the western blotting results showed that the protein bound to inhibitory mAbs 12.8 and 5B1 with reduced binding to mAb 12.10.

3.2.7 Western blotting analysis of the binding activity of neutral monoclonal antibodies to MSP-19 mutant proteins

As summarised in Tables 3.4 and 3.5, neutral mAbs were assessed for their binding to MSP-19 mutant proteins by western blotting analysis. Substitution of only a single cysteine residue at either Cys12 with Ile or Cys28 with Trp abolished the binding of mAbs 111.2, 117.2 and 5.2, with reduced binding of mAb 9C8 to the Cys12→Ile substituted mutant protein. Substitution of Leu31 with Arg abolished the binding of mAbs 111.2 and 9C8, with reduced binding of mAb 8A12. Monoclonal
antibody 2F10 binding to MSP-1 protein was destroyed when Thr48 and Asn53 were replaced with Lys and Arg, respectively. The replacement of Asn53 with Arg also abolished the binding of mAb 12D11 with reduced binding of mAb 117.2. Two more interesting substitutions are Arg20→Glu, which affected the binding of 5 out of 8 neutral mAbs, and Lys40→Ile that affected the binding of 6 out of 8 neutral mAbs tested (Table 3.4).

Table 3.5 shows the binding activity of neutral mAbs to mutant proteins with multiple amino acid substitutions. Both 12+28 mutant proteins abolished the binding of mAb 5.2. Combination of Glu43→Leu with Thr48→Ile produced the 43+48 mutant protein abolishing the binding of mAbs 111.2 and 2F10, and affecting the binding of mAbs 9C8, 12D11, 117.2 and 8A12. Modification of the protein with more than 2 amino acid substitutions completely terminated the binding of mAbs 111.2 and 9C8. Five amino acid substitution mutant protein 12+15+27+31+43 also abolished the binding of mAbs 117.2, 5.2 and 1E8, while six amino acid substitution mutant protein 12+15+27+28+31+43 abolished mAbs 117.2 and 5.2, but not mAb 1E8. Similar to the binding activity with the inhibitory and blocking mAbs, seven amino acid substitution mutant protein 12+15+27+28+31+43+53, and eight amino acid substitution mutant protein 12+15+27+28+31+34+43+53 could not bind to any neutral mAbs tested.

The locations and effects of single amino acid substitutions on neutral antibody binding were identified on the 3-dimensional structure of MSP-1 as shown in Figure 3.9.
3.3 Discussion

As a step towards the development of a MSP-1-based malaria vaccine, it is crucial to understand the function and fine specificity of the antibody response to this protein. In this part of the study, we wished to study MSP-119 epitopes for a range of antibodies that had previously been shown to bind to the protein and function during erythrocyte invasion. For this, a series of amino acid substitutions in MSP-119 has been introduced using the site-directed mutagenesis technique. It was found that single amino acid substitutions were able to affect binding of different antibodies, and some of the changes resulted in proteins that could no longer bind to individual mAbs. However, the fact that most mAbs continued to bind to individual modified proteins suggests that the structures were largely intact. No two mAbs showed identical binding patterns to the panel of modified proteins. By mapping the binding results of each mAb on the 3-dimensional structure of MSP-119 (Chitarra et al., 1999; Morgan et al., 1999), information on the epitopes recognised by each of the mAbs was obtained (Figures 3.6 and 3.9).

From western blotting analysis, it was shown that mAb 12.8 does not bind to the Glu26→Ile mutant and that the mutations Arg20→Glu, Glu24→Lys, Lys29→Ser, Leu31→Arg reduce its binding (Table 3.2). These results suggest that the epitope for mAb 12.8 is located in part along the major antiparallel β-sheet in the first EGF domain because these amino acids are located in this region. It has been shown previously that mAb 12.8 binds to the first EGF domain in the absence of the second domain (Chappel and Holder 1993). Substitutions of individual Cys12 or Cys28
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affected the binding of all inhibitory mAbs and blocking mAb 2.2. This might be the result of changing of protein structure in the first EGF domain caused by a free cysteine residue in these mutants. Although the binding epitope(s) for mAb 5B1 has not been identified, it might locate not very far from the binding epitope(s) for mAb 12.8 in the first EGF domain since the mutations of Cys12→Ile or Cys28→Trp abolished, and Arg20→Glu, Glu26→Ile reduced its binding. Although the binding epitope(s) for mAb 12.10 could not be identified in this study, it was reported that substitution of Tyr84 by Arg abolished its binding (Uthaipibull et al., 2001). Tyr84 is located close to the end of the second domain that is also close to the N-terminus, as shown in the MSP-119 structure (Morgan et al., 1999), where the processing takes place. It also has been shown that mAb 12.10 recognised octapeptides of amino acids comprising antiparallel β-sheet in the first EGF domain, which is also shown in this study to possibly contain the mAb 12.8 binding epitope (Uthaipibull et al., 2001). The binding of mAb 12.10 to MSP-119 is known to depend on both EGF domains (Chappel and Holder 1993). This finding explains the fact that mAbs 12.8 and 12.10 cross compete for binding to MSP-1 (Wilson et al., 1987; Guevara Patino et al., 1997), consistent with them having very close binding sites.

The patterns of reactivity of all blocking antibodies are distinct. The mutations that affected the binding of mAbs 1E1, 7.5 and 111.4 are located in the top half of the molecule whereas mAb 2.2 binding were affected by mutations of amino acids mainly along the major antiparallel β-sheet (Figure 3.6B). The mutations that abolished the binding of mAbs 1E1, 7.5 and 111.4 were Leu31→Arg, Asn15→Arg and Glu43→Leu, respectively. These results supported the findings that mAbs 2.2, 7.5 and 111.4 bind to the first EGF domain of the MSP-119 protein (Chappel and Holder
1993). It was also shown that mAbs 1E1 and 7.5 reacted with octapeptides containing amino acids that were mutated and affected the binding of these mAbs in this study (Uthaipibull et al., 2001). It was interesting that mAb 2.2 binding was abolished by Glu26→Ile, the mutation that also abolished the binding of inhibitory mAb 12.8.

The results from this part of the study suggest that blocking antibodies' epitopes on MSP-1$_{19}$ may be of 3 types. The first one is those epitopes that are formed within MSP-1$_{19}$ by amino acids that are not close within the linear primary sequence to the epitopes that are targets of inhibitory antibodies, for example in the top half of the MSP-1$_{19}$ molecule, but can affect the binding of inhibitory antibodies. The second type is those epitopes that overlap with the epitopes that are targets of inhibitory antibodies, for example as shown here Glu26 is important for the binding of both blocking mAb 2.2 and inhibitory mAb 12.8. The third one is those epitopes that are outside MSP-1$_{19}$. It also has been shown that some blocking antibodies bind to epitopes elsewhere in the MSP-1 complex (Guevara Patino et al., 1997). In order to inhibit the secondary processing of MSP-1, we propose that the inhibitory antibodies may have to bind to their epitopes located near the N-terminus of MSP-1$_{19}$. This idea has been supported by the fact that mAb 12.10 binds to both Tyr84 which is located at the end of second EGF domain, as well as to amino acids within the first EGF domain that are not far from the N-terminus of MSP-1$_{19}$ (Uthaipibull et al., 2001).

In this study, four individual amino acid substitutions, Asn15→Arg, Glu27→Tyr, Leu31→Arg, Glu43→Leu, were found to be able to abolish the binding of four individual blocking mAbs, without affecting the binding of inhibitory mAbs. Another interesting substitution is Tyr34→Ser that reduced the binding of all
blocking mAbs. On incorporation of these amino acid substitutions into a single protein, it was shown that these modified proteins, 15+27+31+43 and 27+31+34+43, continued to bind the inhibitory mAbs but fail to bind to any of the blocking mAbs (Table 3.3 and Figure 3.8). These results showed that it is possible to produce a single protein that, by combining individual point mutations, gives an additive effect. This observation may be critical for the development of vaccines against malaria based on MSP-1.

It has been shown that active immunisation with MSP-19 expressed as a correctly folded recombinant protein can induce a protective immune response in *P. yoelii* (Daly and Long 1993; Ling et al., 1994) and *P. cynomolgi* (Perera et al., 1998). As shown in Figure 3.2, there are only four cysteine residues that form 2 disulphide bonds, instead of 6 cysteine residues that form 3 disulphide bonds in *P. falciparum*, in the first EGF domain of MSP-19 from the rodent, primate and *P. vivax* malaria parasites. In this study, we have replaced this cysteine pair (Cys12 and Cys28) in order to investigate its function in the *P. falciparum* protein. We found that replacement of only one cysteine, either Cys12 or Cys28 alone, will affect the binding of all inhibitory mAbs and blocking mAb 2.2 (Table 3.2). However the binding of inhibitory mAbs was recovered if both cysteine residues were replaced (Table 3.3 and Figure 3.8).

In this study, the binding activity of neutral antibodies to MSP-19 protein was also examined since, in addition to the epitopes of inhibitory and blocking antibodies, the binding sites of other anti-MSP-19 antibodies, such as neutral antibodies, are also important to locate. The information obtained will give a better view of the antibody
binding pattern on the MSP-1\textsubscript{19} protein. In this study, neutral mAbs were tested for their binding activity against all the mutant proteins produced. As shown in Tables 3.4 and 3.5, each antibody gives a distinct binding pattern against mutant proteins. By mapping the information obtained from the western blotting data on the 3-dimensional structure of MSP-1\textsubscript{19}, the areas that are important for the binding of each antibody can be identified (Figure 3.9). It was found that the epitope of mAb 2F10 is located in the area where the two EGF-like domains connected since the substitution of Thr\textsubscript{48} and Asn\textsubscript{53} by Lys and Arg, respectively, abolished its binding. This result is supported by the abolished or reduced binding activity of mAb 2F10 to mutant proteins with multiple amino acid, including Thr\textsubscript{48} or Asn\textsubscript{53}, substitutions (Table 3.5). The binding of mAb 12D11 was also abolished by the substitution of Asn\textsubscript{53} by Arg, with reduced binding to amino acids in the upper part of the first EGF domain. Combining these data with the results from its binding to mutant proteins with multiple amino acid substitutions, it was suggested that the mAb 12D11 binding site(s) is in the second EGF domain. The binding of mAbs 111.2, 117.2 and 5.2 to the protein was shown to require the second disulphide bond in the first EGF domain since their bindings were abolished when either Cys\textsubscript{12} or Cys\textsubscript{28}, or both cysteines for mAb 5.2, were substituted. However there was a report that mAb 111.2 needs both EGF domains in order to bind to the MSP-1\textsubscript{19} protein (Chappel and Holder 1993). Furthermore, the replacement of Leu\textsubscript{31} by Arg also abolished the binding of mAb 9C8. These results suggested that the binding epitope(s) for these mAbs are in the first EGF domain. Although the bindings of mAbs 1E8 and 8A12 were not completely abolished by any single amino acid substitution, the substitutions of amino acids in the bottom part of the first EGF domain largely affected the binding of mAb 1E8 while the amino acids that affected the binding of mAb 8A12 distributed in all parts
of the protein. However, the mutant proteins with seven or eight amino acid substitutions, which included the Asn53→Arg substitution, abolished their binding suggesting that the binding of mAbs 1E8 and 8A12 may need the second EGF domain.

Although neutral antibodies themselves do not inhibit the processing of MSP-1 or the erythrocyte invasion by the merozoite, and also do not interfere with the inhibition activities of inhibitory antibodies, little is known about their ability to compete with blocking antibodies. The investigation of whether or not neutral antibodies can interfere with the activities of blocking antibodies is underway.

It was shown in this study that the five amino acid substitution protein 12+15+27+31+43 which has one free cysteine residue bound to only 3 neutral mAbs. The seven amino acid substitution protein 12+15+27+28+31+43+53 and the eight amino acid substitution protein 12+15+27+28+31+34+43+53 bound to none of the monoclonal antibodies. The six amino acid substitution protein 12+15+27+28+31+43 bound only to inhibitory mAbs 12.8 and 5B1, and another 4 neutral mAbs, with reduced binding to mAb 12.10, as shown by western blotting results. These results suggest that a suitable combination of the mutations is needed to maintain the correct molecular structure of the protein. A vaccine based on MSP-1 mutants to induce primarily inhibitory antibodies and not blocking antibodies may be an effective way to induce immunity to malaria, but the protein also needs to have a correct structure to induce the protective antibodies.
Table 3.1

Summary of the codons used to introduce the amino acid substitutions

The amino acids that have been changed and the codon used for each mutation in the selected position are summarised. The amino acids are shown in a one-letter code, as summarised in Figure 3.3, while the codons coding for the amino acids are shown in brackets. Symbols * and # indicate the positions that were used for single and multiple amino acid substitutions, respectively.
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Figure 3.5

Western blotting analysis of the binding activity of monoclonal antibodies to MSP-1<sub>19</sub> mutant proteins containing a single amino acid substitution

GST-MSP1<sub>19</sub> mutant proteins were expressed from plasmids into which nucleotide substitutions had been introduced to change a single amino acid residue. The proteins were purified from lysates of recombinant *E. coli* using a glutathione agarose column and subjected to SDS-PAGE and western blotting. The inhibitory mAbs 12.8, 12.10 and 5B1 (left-hand lanes) and the blocking mAbs 1E1, 2.2, 7.5 and 111.4 (right-hand lanes) were used to probe MSP-1<sub>19</sub> mutant proteins.
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Table 3.2

Summary of MSP-119 mutant proteins with a single amino acid substitution and their binding to inhibitory and blocking mAbs

Amino acid substitutions in the MSP-119 polypeptide sequence have been introduced using a site-directed mutagenesis technique. The DNA sequence of mutant clones confirmed that only a single amino acid substitution was introduced at the position indicated. The mutant proteins were tested for their binding activity to inhibitory and blocking mAbs, as assessed by western blotting analysis. The antibody binding activity, compared between the wild type (WT) and mutant proteins, is shown as strong binding (2 and blue); binding (1 and yellow); no binding (0 and red).
## Antibody Binding

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<td>K80I</td>
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</table>
Figure 3.6

Location in a representative structure of the 3-dimensional model of MSP-119 of the residues modified by mutation and the effect of the mutation on inhibitory and blocking antibody binding

The amino acids in the first and second EGF domains are coloured white and grey respectively, except for the N-terminal residue Asn1 (coloured magenta), the C-terminal residue Asn96 (coloured black), and the residues that have been modified. The structure is shown for each of the (A) inhibitory mAbs 12.8, 12.10, 5B1, and (B) blocking mAbs 1E1, 2.2, 7.5 and 111.4 with 4 views rotated 90° around the y-axis. The residues that have been changed and then abolished or affected the binding of individual mAbs are numbered. Those that had no effect on the binding of mAbs are shown in blue, those that abolished mAb binding are shown in red, and those that reduced mAb binding are shown in yellow. The specific residue changes are described in Table 3.2.
A. Inhibitory antibodies

12.8

12.10

5B1
B. Blocking antibodies

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</table>
Figure 3.7

BIAscore analysis of the binding of monoclonal antibodies to MSP-1$_{19}$ mutant proteins containing a single amino acid substitution

Purified GST-MSP1$_{19}$ proteins were covalently bound to CM5 sensor chips and antibody solution was flowed over the chip. At binding saturation the increase in resonance units due to antibody binding was recorded. For comparative purposes the binding of antibodies to the mutant proteins (with the changes at positions 12, 15, 26, 27, 28, 31, 34, and 43 identified in Table 3.2, respectively) was compared to the binding of antibodies the wild type MSP-1$_{19}$ protein (binding set at 100%). The results of a representative experiment are shown. The antibodies used were polyclonal antibodies specific for GST; the inhibitory mAbs 12.8, 12.10 and 5B1; the blocking mAbs 1E1, 2.2, 7.5 and 111.4.
Table 3.3

Summary of MSP-1, mutant proteins with multiple amino acid substitutions and their binding to inhibitory and blocking mAbs

The mutant proteins with multiple amino acid substitutions at the positions indicated were tested for their binding activity to inhibitory and blocking mAbs, as assessed by western blotting analysis. The antibody binding activity, compared between the wild type (WT) and mutant proteins, is shown as strong binding (2 and blue); binding (1 and yellow); no binding (0 and red).
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<tr>
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Figure 3.8

BIAcore analysis of the binding of monoclonal antibodies to MSP-119 mutant proteins containing multiple amino acid substitutions

The analysis was carried out as described in the legend to Figure 3.7. For comparative purposes the binding of antibodies to the mutant proteins (with the changes at positions 12 and 28; 27, 31 and 43; 27, 31, 34 and 43; and 15, 27, 31 and 43, respectively) was compared to the binding of antibodies to the wild type MSP-119 protein (binding set at 100%). The results of a representative experiment are shown. The antibodies used were polyclonal antibodies specific for GST; the inhibitory mAbs 12.8, 12.10 and 5B1; the blocking mAbs 1E1, 2.2, 7.5 and 111.4.
Table 3.4

Summary of MSP-119 mutant proteins with single amino acid substitution and their binding activity to neutral mAbs

The mutant proteins with single amino acid substitution at the position indicated were tested for their binding activity to neutral mAbs, as assessed by western blotting analysis. The antibody binding activity, compared between the wild type (WT) and mutant proteins, is shown as strong binding (2 and blue); binding (1 and yellow); no binding (0 and red).
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### Table 3.5

**Summary of MSP-119 mutant proteins with multiple amino acid substitutions and their binding activity to neutral mAbs**

The mutant proteins with multiple amino acid substitutions at the positions indicated were tested for their binding activity to neutral mAbs, as assessed by western blotting analysis. The antibody binding activity, compared between the wild type (WT) and mutant proteins, is shown as strong binding (2 and blue); binding (1 and yellow); no binding (0 and red).
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Figure 3.9

Location in a representative structure of the 3-dimensional model of MSP-1, of the residues modified by mutation and the effect of the mutation on neutral antibody binding

The amino acids in the first and second EGF domains are coloured white and grey respectively, except for the N-terminal residue Asn1 (coloured magenta), the C-terminal residue Asn96 (coloured black), and the residues that have been modified. The structure is shown for each of the neutral mAbs 2F10, 12D11, 9C8 and 111.2 (A) and mAbs 5.2, 117.2, 1E8 and 8A12 (B) with 4 views rotated 90° about the y-axis. The residues that have been changed and then abolished or affected the binding of individual mAbs are numbered. Those that had no effect on the binding of mAbs are shown in blue, those that abolished mAb binding are shown in red, and those that reduced mAb binding are shown in yellow
A. Neutral antibodies (1)

2F10

90°

180°

270°

12D11

9C8

111.2
B. Neutral antibodies (2)

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Chapter Four

Effects of antibodies induced by mutant proteins on MSP-1 secondary processing and erythrocyte invasion

4.1 Introduction

MSP-1 is receiving increasing interest as a candidate antigen for a blood-stage malaria vaccine. Epidemiological studies in some malaria endemic areas have shown that the levels of serum antibodies against MSP-1$_{19}$ are strongly associated with resistance to morbidity caused by falciparum malaria (Riley et al., 1992; al-Yaman et al., 1996; Egan et al., 1996), although some recent studies reported no correlation between them (Dodoo et al., 1999). These studies suggest the important role of MSP-1$_{19}$ in the induction of protective immunity against the parasites. It has been shown that a panel of mAbs, specific for epitopes within MSP-1$_{19}$ has the ability of inhibiting the secondary processing of MSP-1 and interfering with erythrocyte invasion by merozoites. On the basis of these findings, it was proposed that MSP-1 secondary processing is a pre-requisite for erythrocyte invasion by the malaria parasite. It is possible that processing-inhibitory antibodies may perform these inhibitory activities by interfering with the proteolytic activity of the protease responsible for the secondary processing of MSP-1 (Blackman et al., 1994), or by a disruption of the
Chapter Four

Effects of purified antibody on MSP-1 secondary processing and erythrocyte invasion

eythrocyte receptor-parasite ligand interaction which is required during the contact between erythrocyte and the parasite for a successful invasion.

Previous studies have shown that a number of anti-MSP-19 mAbs, which themselves do not inhibit MSP-1 processing and erythrocyte invasion, can block the ability of inhibitory mAbs to interfere with the MSP-1 processing and erythrocyte invasion. That means the balance between inhibitory and blocking antibodies induced by immunisation might be a critical factor in determining whether or not the immune response is effective in preventing invasion. A vaccine based on MSP-19 mutants to induce primarily inhibitory antibodies and not blocking antibodies may be an effective way to induce immunity to malaria. Therefore it is important to investigate the immunogenicity of the modified MSP-19.

This study has previously identified the location in the structure of MSP-19 of the epitopes recognised by inhibitory and blocking antibodies using a site-directed mutagenesis approach. Based on this information, MSP-19 mutant proteins with multiple amino acid substitutions that can prevent the binding of some or all blocking antibodies were made. Selected mutant proteins were used to immunise laboratory animals to raise polyclonal antibodies. We propose that these proteins will induce a polyclonal response that is more inhibitory than that induced by the wild type MSP-19 protein. In this chapter, we investigated the effects of polyclonal antibodies induced by the immunisation of mice and rabbits with MSP-19 mutant proteins in 2 functional assays: MSP-1 secondary processing-inhibition, and erythrocyte invasion-inhibition assay.
4.2 Results

4.2.1 MSP-119 mutant proteins raised specific antibodies in animals

To investigate whether or not the MSP-119 mutant proteins can induce protective antibodies, experimental animals were immunised with proteins as described in Materials and Methods. The serum samples were collected before the first immunisation, 14 days after each boost and after the final bleed. The antigen-specific polyclonal antibodies induced were tested by immunofluorescent assay. It was shown that the specific polyclonal antibodies were raised by immunisation with all mutant proteins in animals as early as 14 days after the first boost (data not shown).

In this study, we also performed immunisations with proteins, in order to see whether or not the antibody repertoire raised by primary immunisation with MSP-119 wild type protein can be selectively induced by boosting immunisation with mutant proteins. Rabbits were primed by immunisation with the wild type protein and the immune response was boosted with subsequent immunisation with mutant proteins 27+31+43 or 15+27+31+43.

4.2.2 Purification of specific IgG

Total IgG was purified from serum of animals immunised with MSP-119 proteins by affinity chromatography. Pooled sera diluted in binding buffer were applied to a Protein G column, and then washed extensively with binding buffer. The
bound IgG was eluted by elution buffer, and the eluted fractions were immediately neutralised in 1 M Tris-HCl, pH 9.0. The eluted IgG was dialysed extensively against PBS at 4°C and kept in aliquots at -70°C. In this study, the purified IgG from immune serum of animals immunised with mutant protein ‘X’ is termed ‘X IgG’, i.e; purified IgG from immune serum of rabbit immunised with the wild type protein is called ‘rabbit wild type IgG’; purified IgG from immune serum of mice immunised with the mutant protein 27+31+43 is called ‘mouse 27+31+43 IgG’.

4.2.3 Purified IgG from immune serum of animals immunised with MSP-119 mutant proteins recognises recombinant wild type MSP-119 protein in ELISA

In order to quantify the level of MSP-119-specific antibodies present in the affinity purified IgGs, an ELISA against the Pichia-expressed 6-His tagged recombinant MSP-119 wild type protein was performed as described in the Materials and Methods. As shown in Figures 4.1A and B, MSP-119-specific IgG was raised by immunisation with the wild type and all mutant proteins in both mouse and rabbit, respectively. The results also showed that antibodies raised to all proteins could bind to the MSP-119 wild type protein with no apparent significant reduction in titre. No detectable binding of normal mouse and rabbit IgGs was detected. Overall the rabbit IgG had a higher titre.

4.2.4 Effects of purified IgG in the MSP-1 secondary processing-inhibition assay

In order to investigate the effects of the purified IgG on the processing of MSP-1, the MSP-1 secondary processing-inhibition assay was used. This assay is used to determine effects of an antibody on MSP-1 processing based on the
measurement of the amount of MSP-1_{33} produced; this protein is a component which is present in the MSP-1 shed complex. Intact merozoites used in this assay were harvested from cultures of mature schizonts in the presence of the chelating agent EGTA, as described in the Materials and Methods. The natural released merozoites still have the MSP-1 complex on their surface. The inhibition of secondary processing by EGTA is reversible by addition of excess calcium (Blackman and Holder 1992). In this study, intact merozoites were washed with ice-cold PBS-CMF containing EGTA before resuspending them in ice-cold reaction buffer containing CaCl$_2$ and MgCl$_2$. The purified IgG samples at a final concentration of 1 mg ml$^{-1}$ were allowed to interact with intact merozoites in suspension on ice for 30 min before transferring the samples to 37°C incubator to let the processing to occur. After the reaction, the merozoite suspension was spun down, and supernatant from the assay was run on SDS-PAGE gels before transferring to nitrocellulose membrane. MSP-1_{42} and MSP-1$_{33}$ were then detected using human anti-MSP-1$_{33}$ mAb X509.

The results of the MSP-1 processing-inhibitory assay using purified mouse and rabbit IgG are shown in Figures 4.2A and B, respectively. As a positive control for inhibition of MSP-1 secondary processing, a serine protease inhibitor, PMSF was used, as this compound has been reported to inhibit the secondary processing of MSP-1 (Blackman and Holder 1992). It was shown that both mouse and rabbit 12+28 IgG could significantly inhibit the secondary processing of MSP-1 as compared to the negative control with no antibody (NoAb), and in the presence of normal and wild type IgG from both animal species. Although the mouse wild type IgG and rabbit WT/27+31+43 also showed some inhibition in the assay based on the amount of
MSP-1_{33} detected, the inhibition was not significant compared to those of 12+28 IgGs.

4.2.5 Effects of purified IgG in the erythrocyte invasion-inhibition assay

The most common experimental method used to determine whether or not an antibody can disrupt the asexual blood stage cycle of *P. falciparum* at invasion is by adding the antibody to *in vitro* culture containing highly synchronised mature schizonts and fresh erythrocytes. Any effect on erythrocyte invasion is determined by counting the numbers of infected erythrocytes at the ‘ring’ stage present in Giemsa-stained thin blood smears from the culture after 24 hours.

Using such approach, the purified IgG from the serum of animals immunised with MSP-1_{19} mutant proteins was added to the culture of *P. falciparum* parasites at a final concentration of 1 mg ml^{-1} to see whether or not the antibodies present were able to interfere with parasite invasion. As a positive control for inhibition, a final concentration of 10 mM EGTA was used, as this has been demonstrated to effectively inhibit parasite invasion *in vitro* (Wasserman *et al.*, 1982; McCallum-Deighton and Holder 1992). The results were recorded as mean percentage, from 4 counts of duplicate cultures of each sample, of erythrocyte invasion plus/minus the standard deviation (S.D.) compared to those of normal IgG of each animal species, which was normalised to 100% invasion.

The morphology of the ring stages appeared normal on Giemsa-stained smears for all the cultures incubated either with or without purified IgG. There was no
evidence of merozoite agglutination or dead/dying intracellular parasites. Some schizonts were observed after 24 hr in the cultures treated with EGTA but not in the cultures treated with purified IgG, indicating that the purified IgG did not inhibit either the development of the schizonts or the release of the merozoites.

As shown in Figures 4.3A and B, as a positive inhibition control, EGTA significantly inhibited erythrocyte invasion by both *P. falciparum* parasite lines, 3D7 (MAD20 type MSP-1) and FCB-1 (Wellcome type MSP-1), respectively. Rabbit 27+31+43 IgG significantly inhibited erythrocyte invasion of both parasite lines by more than 60% as compared to normal rabbit IgG. Rabbit 12+28, WT/27+31+43 and WT/15+27+31+43 IgGs also significantly interfered with erythrocyte invasion by both parasite lines, while rabbit 15+27+31+43 and 27+31+34+43 IgGs interfered with erythrocyte invasion by FCB-1 and 3D7 parasites, respectively. Rabbit wild type IgG also inhibited erythrocyte invasion at a lower level by FCB-1 parasites. Rabbit 12+15+27+28+31+43 IgG did not inhibit erythrocyte invasion of any parasite lines as compared to normal rabbit IgG.

Mouse mutant protein IgGs were not very effective at inhibition of erythrocyte invasion of both parasite lines as compared to the rabbit IgGs. However, mouse 12+28 IgG showed a significant invasion-inhibition effect on FCB-1 parasites as compared to normal mouse IgG. Mouse 12+15+27+28+31+43 IgG also inhibited erythrocyte invasion of both parasite lines at a lower level, as well as mouse 27+31+43 and 27+31+34+43 IgGs that interfered with erythrocyte invasion of FCB-1 and 3D7 parasites, respectively. Mouse wild type and 15+27+31+43 IgGs did not have any inhibitory effects on erythrocyte invasion by both parasite lines.
4.2.6 The inhibition of erythrocyte invasion by purified rabbit IgGs is mediated by their antibody activities

In order to confirm that the erythrocyte invasion-inhibition activity of rabbit 12+28, 27+31+43 and WT/27+31+43 IgGs is mediated by their specific antibody activities, and not by any contamination acquired during IgG purification or by other factors, a neutralisation experiment was designed. It is proposed that the inhibitory antibodies interrupt invasion by binding to MSP-1 present on the surface of merozoites. In order to neutralise the rabbit IgGs, they were allowed to bind the recombinant wild type GST-MSP-1\textsubscript{19} protein (Ag) in solution before adding to the parasite culture for erythrocyte invasion-inhibition assay. As shown in Figures 4.4A (the assay on 3D7 parasites) and 4.4B (the assay on FCB-1 parasites), rabbit 12+28, 27+31+43 and WT/27+31+43 IgGs on their own showed significant invasion-inhibition activity. But this inhibition activity was abolished when the IgGs were allowed to bind to wild type GST-MSP-1\textsubscript{19} protein (Ag) prior to adding to the culture assay.
4.3 Discussion

As basic research toward the development of an MSP-1-based malaria vaccine, we have studied the effects of site-directed mutagenesis on MSP-1\textsubscript{19} protein aiming to improve its immunogenicity for induction of protective immunity. In this part of the study, the modified MSP-1\textsubscript{19} proteins with multiple amino acid substitutions that have been shown, in the previous chapter, to bind inhibitory, but not some or all blocking, antibodies have been studied for their immunogenicity and their ability to induce a protective antibody response in animals. The selected modified proteins have been used to immunised mice and rabbits and it was shown that each modified protein, and the wild type protein, could raise a specific antibody response as early as 14 days after the first boosting injection suggesting that the proteins were immunogenic.

It is known that the results obtained from studies using whole serum rather than purified immunoglobulin fractions are difficult to interpret, and any inhibition can not always be assumed to be due to the antibodies rather than other factors present in the serum. In this study, we aimed to investigate the ability of the modified proteins to induce protective antibodies, which are mainly IgG. The IgG was affinity purified from the serum of animals immunised with the proteins and tested for binding to the wild type MSP-1\textsubscript{19} protein. It was shown by ELISA that purified IgGs from serum of animals immunised with the modified proteins could bind the wild type protein (Figures 4.1A and B). The results suggested that the wild type protein and all modified proteins tested had more or less the same immunogenicity and showed no
great difference in the induction of the total MSP-1\textsubscript{19}-specific IgG. These findings also suggested that the substitution of amino acids, up to six amino acids in this study, in the wild type MSP-1\textsubscript{19} protein did not affect its overall immunogenicity and its ability to induce specific antibodies. In this study, the rabbit IgG showed a higher titre than the mouse IgG. Although the level of the IgG induced by each mutant protein in each animal species was not very different in term of gross binding to the wild type protein, the quality of the IgG is the most important interest in this study. The purified IgGs were then tested for their biological activities using the MSP-1 secondary processing-inhibition and the erythrocyte invasion-inhibition assays. The effects of the purified IgGs in both assays are summarised in Table 4.1.

It has been shown that recombinant \textit{P. yoelii} and \textit{P. cynomolgi} MSP-1\textsubscript{19} protein, which has only 2 disulphide bonds in the first EGF domain, can induce a protective immune response against parasite challenge (Daly and Long 1993; Ling \textit{et al.}, 1994; Perera \textit{et al.}, 1998). In order to investigate whether or not a mutant \textit{P. falciparum} MSP-1\textsubscript{19} protein that does not have the second disulphide bond in the first EGF domain can induce the protective immune response like the homologous protein in \textit{P. yoelii}, the mutant protein 12+28 was made. Cys12 and Cys28 in \textit{P. falciparum} MSP-1\textsubscript{19} were replaced with Ile and Trp, respectively; the amino acids that are present in the sequence of \textit{P. yoelii} (Figure 3.2). As shown in Figures 4.2A and B, mouse and rabbit 12+28 IgGs both significantly inhibited the secondary processing of MSP-1. Rabbit 12+28 IgG also significantly inhibited the erythrocyte invasion by both 3D7 and FCB-1 parasites while mouse 12+28 IgG significantly inhibited only FCB-1, but not 3D7, invasion of erythrocytes (Figures 4.3A and B). These results suggested the importance of the presence of the second disulphide bond in the first EGF domain of
MSP-1. This bond is present only in *P. falciparum* parasites, but not in other species of *Plasmodium*. The exact function of this disulphide bond, except for holding the protein structure together, is not known. It may help the *P. falciparum* parasites in the immune evasion mechanisms. One of these ways may be by forming a region that has binding epitopes for blocking antibodies, such as the blocking mAb 2.2, as discussed in Chapter 3, that upon binding will interrupt the activities of the inhibitory antibodies. Another possibility is that this disulphide bond may hold the protein structure together in order to protect specific epitopes that are important for immune effector cells to recognise the parasites. It has been shown that unfolding of protein by disrupting a disulphide bond can enhance both processing and exposure of immunogenic epitopes to specific T cells (Collins *et al.*, 1991; Egan *et al.*, 1997). Thus reduction of this disulphide bond may disrupt the induction of appropriate immune responses and/or the binding of blocking antibodies to the protein, instead exposing the epitopes for inhibitory antibodies to the immune system. However, not all the disulphide bonds can be reduced, as they are required to hold the correct structure of the protein together. Reduction and alkylation of all cysteines in *P. yoelii* yoelii MSP-1 abolished the protein’s protective activity (Ling *et al.*, 1994).

The more striking results were the significant inhibitory activities of rabbit 27+31+43 IgG on erythrocyte invasion by parasites. As shown in Figures 4.3A and B, rabbit 27+31+43 IgG significantly inhibited erythrocyte invasion by both parasite lines by more than 60% as compared to normal rabbit IgG, although it had no effects on the secondary processing of MSP-1 (Figure 4.2B). The inhibitory activities on erythrocyte invasion by rabbit 27+31+43 IgG was at a higher level than those of rabbit 12+28 IgG. However, mouse 27+31+43 IgG affected only erythrocyte invasion
of FCB-1 parasites, but not of 3D7 parasites, and had no effects on the secondary processing of MSP-1. These inhibitory activities were confirmed by the significant inhibitory activities on erythrocyte invasion of both parasite lines mediated by rabbit WT/27+31+43 IgG, an antibody that was induced by primary immunisation with the wild type protein followed by boosting immunisations with 27+31+43 protein. However, rabbit WT/27+31+43 IgG had no significant effect on the secondary processing of MSP-1.

Immunisation with mutant protein 15+27+31+43 could induce protective IgG since rabbit 15+27+31+43 IgG could inhibit erythrocyte invasion by FCB-1, but not 3D7, parasites. The inhibition of erythrocyte invasion by both parasite lines by rabbit WT/15+27+31+43 IgG confirmed the induction of protective antibody by the mutant protein 15+27+31+43. However, rabbit WT/15+27+31+43 IgG failed to inhibit the secondary processing of MSP-1. These inhibition results by rabbit WT/27+31+43 and WT/15+27+31+43 IgGs showed the possibility of a selective induction of protective antibodies by subsequent boosting immunisations, with candidate proteins, from an antibody repertoire primarily induced by the wild type protein. These findings suggest that the immunisation with a malaria vaccine of individuals already exposed to malaria can still provide a protective immune response against the parasite by a mechanism that selectively induces protective antibodies from already activated antibody-producing cells.

It was shown in this study that rabbit 12+28, 27+31+43 and WT/27+31+43 IgGs had significant erythrocyte invasion-inhibition activities. Although purified IgGs were used in the assay, there were questions of whether the inhibitory activities might
be due to other factors, for example toxic contaminants introduced during the preparation of the IgG. Figures 4.4A and B show the results of the neutralisation experiments that confirmed the inhibitory activities of the IgGs were due to their antigen binding properties. The inhibitory activities were clearly abolished when they were neutralised by preincubation with the MSP-1_{19} protein. It has been shown that the protective immunity induced by recombinant \textit{P. yoelii} MSP-1_{19} protein is antibody mediated (Ling \textit{et al.}, 1994; Daly and Long 1995; Ling \textit{et al.}, 1997).

The findings that a mutant MSP-1_{19} protein could induce protective antibodies that inhibited parasite invasion of the erythrocytes, but did not inhibit the secondary processing of MSP-1, suggested that inhibition of parasite invasion by protective antibodies may be mediated by more than one mechanism. It has been proposed by Blackman \textit{et al.} that antibodies to MSP-1_{19} inhibit erythrocyte invasion by a mechanism that involves inhibition of the serine protease activity which is responsible for secondary processing (Blackman \textit{et al.}, 1994). In this study, we provide new evidence suggesting a new MSP-1 secondary processing-\textit{independent} mechanism for the erythrocyte invasion-inhibition by MSP-1_{19} specific antibodies. Therefore the protective antibodies induced, in this study, by mutant proteins may be divided into 2 groups. The first group is those antibodies that can inhibit both MSP-1 secondary processing and erythrocyte invasion, suggesting that they inhibit erythrocyte invasion by interfering with MSP-1 secondary processing. The second group is those antibodies that can inhibit erythrocyte invasion by the parasites, but do not inhibit MSP-1 secondary processing. This group of antibodies acts in a new mechanism to inhibit the invasion of erythrocytes by the parasites. One possibility is by disruption of the erythrocyte receptor-parasite ligand interaction which is required during the
contact between erythrocyte and parasite for successful invasion. Identification of the characteristics and specificity of the second group of antibodies will lead to more understanding about the erythrocyte invasion mechanisms used by the parasite.

To investigate the effect of antibodies in the MSP-1 secondary processing, the western blot-based assay has been used in this study. However this assay is still not perfect due to problems with sensitivity, reproducibility and inability to quantify the MSP-1\textsubscript{33} produced which is the key factor to assess the effect of antibodies in the assay. Two alternative quantitative methodologies have been evaluated: ELISA and surface plasmon resonance analysis (BIAcore). Wells of ELISA plates or a CM5 sensor chip coupled with MSP-1\textsubscript{33}-specific human mAb X509 were used to capture the soluble fragments present in the supernatant of incubated merozoites. The bound MSP-1\textsubscript{33} was then detected, in ELISA, using a second MSP-1\textsubscript{33}-specific mouse mAb G13. A very high background was observed in the ELISA approach, due to the non-specific binding of rabbit IgG samples to the ELISA plate surface and cross-reactivity of mouse IgG samples to the secondary antibody used to detect mouse mAb G13. Further efforts should be focused on the reduction of the background by using more specific reagents such as mAbs against other different fragments of MSP-1. In the BIAcore approach, MSP-1\textsubscript{33} was detectable but not at a significant level making it difficult to distinguish differences between samples. Further improvements should increase the amount of captured antibody in the initial coating and of MSP-1\textsubscript{33} in the test supernatant, in order to amplify the signal detected by the machine.
Chapter Four
Effects of purified antibody on MSP-1 secondary processing and erythrocyte invasion

Figure 4.1
Purified IgG from immune serum of animals immunised with MSP-119 mutant proteins recognises recombinant wild type MSP-119 protein in ELISA

Purified mouse (A) and rabbit (B) IgG binding to the wild type MSP-119 protein was measured by ELISA as described in the Materials and Methods. The absorbance at 405 nm was measured for serial dilutions of the IgGs (in $\mu$g ml$^{-1}$) plotted on a logarithmic scale for clarity.
A. Mouse IgG

B. Rabbit IgG
Figure 4.2

Effects of purified IgG in the MSP-1 secondary processing-inhibition assay

Washed 3D7 merozoites were incubated with purified mouse (A) and rabbit (B) IgGs on ice for 30 min prior to further incubation at 37°C for 1 hr, except the 0 hr sample that was solubilised in SDS after 30 min incubation on ice. All other samples were solubilised in SDS after 37°C incubation. The samples were then subjected to SDS-PAGE prior to western blotting analysis using mAb X509 that is specific for an epitope within MSP-1$^{33}$. The final concentration of purified IgGs was 1 mg ml$^{-1}$, and for PMSF the positive control, it was 1 mM. NoAb indicates the incubation of merozoites in the presence of no antibody (the negative control). Normal indicates incubation of merozoites in the presence of normal mouse/rabbit antibody (the antibody control).
B. Rabbit IgG

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<td>PMSF</td>
</tr>
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27+31+43
15+27+31+43
27+31+34+43
12+15+27+28+31+43
WT / 27+31+43
WT / 15+27+31+43
Figure 4.3

Effects of purified IgG in the erythrocyte invasion-inhibition assay

Panels A and B show the effects of purified IgG on the erythrocyte invasion by *P. falciparum* parasites strain 3D7 and FBC-1, respectively. IgG samples were added to highly synchronous *in vitro* cultures of the parasites at the final concentration of 1 mg ml$^{-1}$. As a negative control, parasites were incubated in the presence of PBS (since all IgG samples were in PBS) as well as in the presence of 10 mM EGTA (EGTA) for a positive control. Normal indicates incubation in the presence of normal mouse/rabbit IgG. Each sample was tested in duplicate and the cultures were grown for 24 hr to allow re-invasion of erythrocytes by the parasites. The number of ‘ring’ stage parasites was determined by microscopic examination of 2,000 or more erythrocytes in methanol-fixed Giemsa-stained smears. The results show the mean percentage invasion by parasites in the presence of IgG sample plus/minus standard deviation (S.D.). Values that differed significantly, as determined by Student’s *t*-test, from the control normal IgG, which was set to 100% invasion, are indicated. *** ; p<0.001, ** ; p<0.01, *; p<0.05.
Percentage erythrocyte invasion
(± S.D.)

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<tr>
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<tr>
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<td>WT15 + 27 + 31 + 43</td>
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Rabbit
Mouse
Percentage erythrocyte invasion
(± S.D.)

PBS
EGTA
Normal
Wild type
12+28
27+31+43
15+27+31+43
27+31+34+43
12+15+27+28+31+43
WT/27+31+43
WT/15+27+31+43
Normal
Wild type
12+28
27+31+43
15+27+31+43
27+31+34+43
12+15+27+28+31+43
Figure 4.4

The inhibition of erythrocyte invasion by purified rabbit IgG preparations is mediated by their specific binding to MSP-1

Panels A and B show the effects of purified rabbit IgG or neutralized IgG on the erythrocyte invasion by *P. falciparum* parasites lines 3D7 and FBC-1, respectively. IgG samples at a final concentration of 2.5 mg ml\(^{-1}\) were allowed to bind the wild type GST-MSP-1\(_{19}\) protein (Ag) at a final concentration of 0.5 mg ml\(^{-1}\) for 5 min prior to addition to *in vitro* cultures of the parasites, with final concentrations of IgG and Ag at 1 mg ml\(^{-1}\) and 0.2 mg ml\(^{-1}\), respectively. A final concentration of 1 mg ml\(^{-1}\) of IgG samples or 0.2 mg ml\(^{-1}\) of Ag alone were also tested as controls in the parasite cultures. As a negative control, parasites were incubated in the presence of PBS (since all IgG samples were in PBS) as well as in the presence of 10 mM EGTA (EGTA) for a positive control. Normal indicates incubation in the presence of normal mouse/rabbit IgG. Each sample was tested in duplicate and the cultures were grown for 24 hr to allow re-invasion of erythrocytes by the parasites. The number of ‘ring’ stage parasites was determined by microscopic examination of 2,000 or more erythrocytes in methanol-fixed Giemsa-stained smears. The results show the mean percentage invasion by parasites in the presence of IgG sample plus/minus standard deviation (S.D.). Values that differed significantly, as determined by Student’s *t*-test, from the control normal IgG, which was set to 100% invasion, are indicated. ***; p<0.001, **; p<0.01, *; p<0.05.
Percentage erythrocyte invasion
(± S.D.)

- PBS
- EGTA
- Ag
- (Wild type)
- (Wild type)+Ag
- (12+28)
- (12+28)+Ag
- (27+31+43)
- (27+31+43)+Ag
- (WT/27+31+43)
- (WT/27+31+43)+Ag
- (WT/15+27+31+43)
- (WT/15+27+31+43)+Ag
Percentage erythrocyte invasion (± S.D.)

- PBS
- EGTA
- Ag
- (Wild type)
- (Wild type)+Ag
- (12+28)
- (12+28)+Ag
- (27+31+43)
- (27+31+43)+Ag
- (WT/27+31+43)
- (WT/27+31+43)+Ag
- (WT/15+27+31+43)
- (WT/15+27+31+43)+Ag
Table 4.1

Summary of the effects of purified IgG on MSP-1 secondary processing and erythrocyte invasion

The effects of purified IgG induced by mutant proteins in both animal species on MSP-1 secondary processing and erythrocyte invasion assays are summarised. + ; inhibition, - ; no inhibition. Significantly different from control; *** ; p<0.001, ** ; p<0.01, * ; p<0.05.
<table>
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<tr>
<th>IgG samples</th>
<th>Animal species</th>
<th>Inhibition of MSP-1 secondary processing</th>
<th>Inhibition of erythrocyte invasion</th>
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<tr>
<td></td>
<td></td>
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<tr>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>Mouse</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>Mouse</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>+</td>
<td>**</td>
</tr>
<tr>
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<td>Mouse</td>
<td>-</td>
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<tr>
<td></td>
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Chapter Five

Studies on molecular structure of MSP-1\textsubscript{19} mutant proteins

5.1 Introduction

MSP-1\textsubscript{19} is currently the leading candidate for the development of a vaccine against the blood-stage of the malaria parasite. Recently, the 3-dimensional structure of \textit{P. falciparum} and \textit{P. cynomolgi} MSP-1\textsubscript{19} has been determined by NMR (Morgan \textit{et al.}, 1999), and crystallography techniques (Chitarra \textit{et al.}, 1999), respectively. Both studies have shown the MSP-1\textsubscript{19} molecule to be composed of 2 epidermal growth factor (EGF)-like domains. EGF-like domains are present in a large spectrum of extracellular proteins with widely varying roles in biological processes such as cell development and growth, cell adhesion, blood coagulation and fibrinolysis (Campbell and Bork 1993). An EGF domain usually consists of 30-60 amino acids with four to eight cysteine residues. The topology is described as a two-stranded $\beta$-sheet followed by a loop to a short C-terminal two-stranded $\beta$-sheet. Large numbers of proteins contain one or more copies of the EGF domain (Campbell and Bork 1993). Some EGF-like domains are calcium-binders, for example the epidermal growth factor molecule, while others such as MSP-1\textsubscript{19} do not bind calcium (Morgan \textit{et al.}, 1999).
The functional significance of these domains across unrelated proteins is not clear and their classification is difficult because of the existence of different domain sub-types (SCOP, CATH and Dr. Delmiro Fernandez-Reyes, Division of Mathematical Biology, NIMR, London, U.K.; personal communication) (Figure 5.1). The Structural Classification of Proteins (SCOP; http://scop.mrc-lmb.cam.ac.uk/scop/) (Murzin et al., 1995; Hubbard et al., 1999) classifies MSP119 (1cej [P. falciparum] and 1b9w [P. cynomolgi]) into the Class of small proteins, knottins Fold (beta-hairpin with two adjacent disulphides), EGF/Laminin Superfamily and Family MSP-1 (Figure 5.2). The CATH (Class Architecture Topology Homology Superfamily proteins structure classification; http://www.biochem.ucl.ac.uk/bsm/cath) (Orengo et al., 1997) classifies MSP-119 under the Class of preliminary single domain assignments with partially classified Architecture. These differences between these two structural classification methods show the difficulties of defining a homogeneous structural class for this kind of domain. This is partly due to the noise generated by the assignment of the EGF-like suffix to a large number of protein sequences based on undefined sequence similarity thresholds (Dr. Delmiro Fernandez-Reyes, personal communication).

Each EGF domain of P. falciparum MSP-119 has 6 cysteine residues forming 3 disulphide bonds in the order 1-3, 2-4 and 5-6 which correspond to pdb residues 7-18, 12-28 and 30-41 in domain 1 and 49-62, 56-76 and 78-92 in domain 2 (Figure 3.2). The cysteines 4 and 5 are separated by only one residue, while the spacings between the other cysteine residues are longer, usually from 3-13 residues. The 2 and 4 cysteines are centrally located within the EGF domain, while the 1-3 and 5-6 are at the N- and C-terminal ends of the domain, respectively. Three-dimensional analysis
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Studies on molecular structure of MSP-1\textsubscript{19} mutant proteins

has shown a striking feature of MSP-1\textsubscript{19} that its 2 EGF domain interactions form a U-shaped structure resulting in its C- and N-termini being relatively close together, which contrasts with structures observed in other pairs of EGF domains (Brandstetter \textit{et al.}, 1995; Downing \textit{et al.}, 1996). This finding indicates that the proximity of the C- and N-terminal positions may be significant, since it suggests that the proteolytic processing site at which a protease cleaves MSP-1\textsubscript{19} from MSP-1\textsubscript{42} may be very close to the GPI attachment site at or near residue 96.

Conservation of the overall sequence with 50-70\% similarity (Blosum62 matrix) and of key domain interface residues of the MSP-1\textsubscript{19} across \textit{Plasmodium} species (Figure 3.2) suggest that this region of the molecule may have a similar EGF domain pair arrangement. Extensive homology modelling using \textit{P. falciparum} and \textit{P. cynomolgi} MSP-1\textsubscript{19} as structural templates have been performed and confirmed this prediction (Dr. Delmiro Fernandez-Reyes, personal communication). The cysteine residues that would form the 2-4 disulphide bond in a normal EGF domain are not present in the first domain of MSP-1\textsubscript{19} for all species except \textit{P. falciparum} (Figure 3.2). Thus, the existence of an extra disulphide bond in the first domain of \textit{P. falciparum} MSP-1\textsubscript{19}, as compared to other species, may have significance for the human parasite.

In previous chapters of this study, we have demonstrated that the binding of both inhibitory and blocking antibodies to recombinant MSP-1\textsubscript{19} proteins can be profoundly affected by the substitution of single and multiple amino acids in the protein. Some of the MSP-1\textsubscript{19} mutant proteins containing multiple amino acid substitutions have been shown to induce protective antibodies in animals. These
antibodies could inhibit the MSP-1 secondary processing and erythrocyte invasion \textit{in vitro}. In this part of the study, the 3-dimensional structural models for these mutants were produced using computational techniques. The known structure of the \textit{P. falciparum} and \textit{P. cynomolgi} MSP-1_{19} were used as templates.

5.2 Results and discussion

For a comparative study of the molecular structure of \textit{P. falciparum} and \textit{P. cynomolgi} MSP-1_{19} protein, the ribbon, molecular surface and electrostatic potential images were created. Figure 5.3 shows the 3-dimensional molecular structure of \textit{P. falciparum} and \textit{P. cynomolgi} MSP-1_{19} that have previously been determined using NMR and X-ray crystallography techniques, respectively (Morgan \textit{et al.}, 1999; Chitarra \textit{et al.}, 1999). The 32 structures of MSP-1_{19} from the NMR study are aligned. Since most of the available anti-MSP-1_{19} antibodies bind to the first EGF domain of \textit{P. falciparum}, the mutagensis studies were focused on this domain. Thus, for the \textit{P. falciparum} MSP-1_{19} modelling study, the NMR model number 22 (in the 1cej.pdb data file) was used, as this model is shown to be the most representative for the first EGF domain of \textit{P. falciparum} in the NMR study (Dr. Bill Morgan, Division of Molecular Structure, NIMR, London; personal communication).

Alignments of the two structures from the two species (\textit{P. falciparum} in red, \textit{P. cynomolgi} in yellow) and the close up of the region in which residues would form the 2-4 disulphide bond in \textit{P. falciparum}, which are Val12 and Trp28 in \textit{P. cynomolgi}, are shown in Figures 5.4A and B, respectively. From the \textit{P. cynomolgi} crystal structure data, the distances between the $\alpha$ and $\beta$ carbon atom pairs in Val12 and
Trp28, residues that align with the 2-4 cysteines, are 6.6 Å and 5.1 Å, respectively, while the corresponding distances for the 2-4 disulphide bond in the other EGF domains vary from 5.4 to 6.1 Å between α carbon atoms and from 3.4 to 4.1 Å between β carbon atoms (Chitarra et al., 1999). Along with a number of conserved residues across the amino acid sequence, these results explain why the conformations of these two proteins studied by two different approaches show their structural similarities; particularly in the first domain where the *P. cynomolgi* protein has only 2 disulphide bonds, compared to 3 disulphide bonds in the *P. falciparum* protein.

Figure 5.5 shows the molecular surface images of *P. falciparum* and *P. cynomolgi* MSP-119 proteins in two views of the top and bottom of the molecules (A and B, respectively) and four views of 90° rotation around the y-axis (C to F) to provide the overall display of the surface of the molecules. The molecular surface is coloured by amino acid type; red = acidic, blue = basic, yellow = non-charged polar and grey = hydrophobic. The position of each domain in the images is indicated; D1 = domain 1, D2 = domain 2. The electrostatic potential of *P. falciparum* and *P. cynomolgi* MSP-119 proteins were calculated as shown in Figures 5.6 and 5.7, respectively. The electrostatic interactions, caused by charged side chains and bound ions, play a role in protein folding and stability, or specific protein-protein interaction such as antigen-antibody binding. As shown in Figure 5.6, the negative electrostatic potential of *P. falciparum* MSP-119 is clustered in the second EGF domain and on one side of the molecule (Figure 5.6D) with patches of positive charge around the molecule. In contrast, the first domain of *P. cynomolgi* MSP-119 showed clusters of negative electrostatic potential of higher magnitude than the second domain (Figure 5.7). The electrostatic potential information may assist in understanding how residues
Studies on molecular structure of MSP-1\textsubscript{19} mutant proteins

on different surfaces are involved in interactions between MSP-1\textsubscript{19} with the rest of the MSP-1 precursor, the protease(s) associated with the processing, and the binding of different antibodies.

The focus of the study in this chapter is the molecular structure studies of \textit{P. falciparum} MSP-1 mutant proteins. Studies in previous chapters have demonstrated that the binding of both inhibitory and blocking antibodies to recombinant MSP-1\textsubscript{19} proteins can be profoundly affected by the substitution of single and multiple amino acids in the protein. Although the positions of the amino acids that have been substituted could be identified on the known 3-dimensional structure of MSP-1\textsubscript{19} (as shown in chapter 3), it is also important to know whether or not and how the substitutions will affect the molecular structure or the surface of the protein.

Here the 3-dimensional molecular models of mutant proteins 12+28 and 27+31+43, the mutant proteins that were shown to induce protective antibodies, have been reproduced using the known structure of the \textit{P. falciparum} MSP-1\textsubscript{19}. The structures of mutant proteins 12+28 and 27+31+43 compared with the structure of the wild type protein, showing the positions and side chain of the amino acids of interest, are shown in Figures 5.8 and 5.9, respectively. Images are shown in four views that optimised the visibility of the amino acids of interest which are coloured as indicated in the figure legends. Cysteine residues are coloured in CPK mode, and the disulphide bonds formed between them are in yellow. The structures give an idea of how and where the side chain of substituted amino acids in the mutant proteins interact in the structure as compared to those of the wild type protein.
The molecular surface images of the mutant proteins 12+28 and 27+31+43 were created and compared with those of the wild type protein as shown in Figures 5.10 and 5.11, respectively. The molecular surface images are shown in four views that optimise clear visibility of the changes on the surface of the molecule. As shown by the molecular surface images, the substitutions of amino acids in these mutants are predicted to have resulted in changes on the surface and changes of electrostatic charge at the positions of interest without affecting the overall structure of the molecule. The changes are indicated (in green circles) on the molecular surface images of the mutant proteins. The symbol * on the wild type protein images indicates the C-terminal free oxygen atoms present in the pdb data file. These atoms do not exist in the models created by the modelling process. Thus the differences in colour and the surface at this position do not mean that these are changes that have occurred in this study.

As shown in Figure 5.10B, minor changes are seen on the surface of the mutant protein 12+28 molecule where Cys12 was replaced with hydrophobic residue Ile. An obvious change on the molecular surface is seen on another side of the molecule where Cys28 was replaced with the aromatic residue Trp (Figures 5.10C and D). The change has covered the normally accessible polar residue Ser16 from the surface. However, these changes are not as significant as, the disruption, by the substitutions, of the disulphide bond formed by the two cysteines at this position, although this can not be seen on the molecular surface. Cys28 in *P. falciparum* is replaced by Trp in all other species. The aromatic side chain of Trp may help maintain the EGF fold in the absence of the 2-4 disulphide bonds (Chitarra *et al.*, 1999). As discussed in chapter 4, the mutant protein 12+28 can induce antibodies that inhibit
both MSP-1 secondary processing and erythrocyte invasion *in vitro*. The reduction of this disulphide bond, as well as the changes on the protein surface, may disrupt the induction and/or the binding of blocking antibodies to the protein and expose epitopes for the inhibitory antibodies to the immune system. Furthermore, the mutant protein, that now has one less disulphide bond than the wild type protein, may be easier processed and presented to specific T cells as compared to the wild type protein.

The mutant protein 27+31+43 has significant changes on its surface as shown in Figure 5.11. Replacement of Glu27 with aromatic polar residue Tyr has filled the space between Glu26 and Gln36, and covered Cys28 as shown in Figures 5.11A and D. Replacement of Leu31 with Arg has introduced a positive charge and minor change on the surface to the molecule (Figures 5.11B, C and D), while replacement of Glu43 with Leu has abolished the negative charge on the top of molecule (Figures 5.11B and C). Altogether, there are significant changes on the molecular surface and to the polarity at the substituted positions. These changes may contribute to the disruption of the binding of blocking mAbs, as shown in chapter 3 that mAbs 2.2, 1E1 and 111.4 bind to epitopes including Glu27, Leu31 and Glu43, respectively. The changes may also be involved in the induction of antibodies that significantly inhibit erythrocyte invasion, but not MSP-1 secondary processing, *in vitro*.

The molecular modelling studies of the mutant proteins, as well as of the wild type protein, in this chapter provide the basis for the study of local structural changes introduced by amino acids substitutions, in which these local structural changes and antibody binding data were correlated. This study also provides a rationale for future
mutagenesis studies for improving of immunogenicity and antibody binding properties of these proteins.
Figure 5.1

Epidermal growth factor (EGF)-like domains of proteins

Representatives of different sub-types of EGF-like domains from Structural Classification of Proteins (SCOP) are shown. Cysteine residues are coloured in red. Beta-strands are in yellow. EGF: mouse EGF module, 1egf.pdb; Laminin: mouse Laminin γ1, 1klo.pdb; BasementMP: human Basement membrane protein (BM-40), 1nub.pdb; and *P. falciparum* MSP-119 domain 1 (1) and domain 2 (2), 1cej.pdb.
EGF 1egf.pdb
Laminin 1klo.pdb
BasementMP 1nub.pdb
P. falciparum MSP-119 1cej.pdb
Figure 5.2

Structural classification of EGFs

Structural classification of EGFs as shown in SCOP is shown. MSP-1 is classified into its own family, not in the EGF-type module family.
Structural Classification of EGFs

SCOP Database r1.5

Class: Small proteins
usually dominated by metal ligand, heme, and/or disulfide bridges

Fold: Knottins (small inhibitors, toxins, lectins)
disulphide-bound fold; contains beta-hairpin with two adjacent disulphides

Superfamily: EGF/Laminin

Families:
- EGF-type module (20)
- Laminin-type module (1)
- Domain of BM-40/SPARC/osteonectin (1)
- Merozoite surface protein 1 (MSP-1) (2)

Plasmodium falciparum
Plasmodium cynomolgi

- Factor IX (IXa)
- Coagulation factor VIIa
- E-selectin
- Factor X, N-terminal module
- Activated protein C
- Factor VII, N-terminal domain
- Prostaglandin H2 synthase-1
- P-selectin
- Epidermal growth factor
- Transforming growth factor alpha
- Heparin-binding EGF
- Plasminogen activator (urok-type)
- Heregulin-alpha
- Thrombomodulin
- Fibrillin-1 fragment
- Complement protease C1R
- Plasminogen activator (tissue-type)
Figure 5.3

Molecular structure of MSP-1_{19}

Molecular structure of both *P. falciparum* and *P. cynomolgi* MSP-1_{19} determined using NMR and X-ray crystallography techniques, respectively, are shown. The colours indicate structural groups of amino acids by their position on the model, from blue at the N-terminus through green, yellow and red at the C-terminus.
*P. falciparum* 1cej.pdb
NMR - 32 structures


*P. cynomolgi* 1b9w.pdb
X-ray - 1.8 Å

Figure 5.4

Structural alignment of *P. falciparum* and *P. cynomolgi* MSP-1$_{19}$

The molecular structure of *P. falciparum* (1cej, NMR model number 22, in red) and *P. cynomolgi* (1b9w, crystal model, in yellow) MSP-1$_{19}$ are aligned (A). The positions of cysteine residues in the structures are shown and labelled. The close up of the region in the first domain where Cys12 and Cys28 in *P. falciparum* are located to form the second disulphide bond, and which are Val12 and Trp28 in *P. cynomolgi*, is shown (B).
Figure 5.5

The molecular surface images of *P. falciparum* and *P. cynomolgi* MSP-1<sub>19</sub>

The molecular surface images of both molecules were created and shown in top (A), bottom (B) views, and in 4 views of 90° rotation around the y-axis. The molecular surface is colored by amino acid type; red = acidic, blue = basic, yellow = polar, grey = hydrophobic. The position of each domain in the images is indicated; D1 = domain 1, D2 = domain 2.
**P. cynomolgi**  
**P. falciparum**
P. cynomolgi  P. falciparum
Figure 5.6

The electrostatic potential images of *P. falciparum* MSP-1<sub>19</sub>

The electrostatic potential of *P. falciparum* MSP-1<sub>19</sub> was calculated and is shown, on both ribbon and molecular surface images, in 4 views of 90° rotation around the y-axis. Red and blue clouds show the areas with negative and positive potential, respectively. The molecular surface is colored by amino acid type; red = acidic, blue = basic, yellow = polar, grey = hydrophobic. The position of each domain in the images is indicated; D1 = domain 1, D2 = domain 2.
Figure 5.7

The electrostatic potential images of *P. cynomolgi* MSP-1\textsubscript{19}

The electrostatic potential of *P. cynomolgi* MSP-1\textsubscript{19} was calculated and is shown, on both ribbon and molecular surface images, in 4 views of 90° rotation around the y-axis. Red and blue clouds show the areas with negative and positive potential, respectively. The molecular surface is colored by amino acid type; red = acidic, blue = basic, yellow = polar, grey = hydrophobic. The position of each domain in the images is indicated; D1 = domain 1, D2 = domain 2.
Figure 5.8

Comparison of the structures of *P. falciparum* MSP-119 wild type and mutant protein 12+28

The structures of *P. falciparum* MSP-119 wild type and mutant protein 12+28 are compared in 4 views rotated around the y-axis. The cysteine residues are colored in CPK mode and the disulphide bonds formed by cysteine pairs are in yellow. The replacement amino acids in the mutant protein 12+28 are shown and coloured; Ile12 = blue, Trp28 = magenta.
12+28

Wild type
Figure 5.9

Comparison of the structures of *P. falciparum* MSP-1\textsubscript{19} wild type and mutant protein 27+31+43

The structures of *P. falciparum* MSP-1\textsubscript{19} wild type and mutant protein 27+31+43 are compared in 4 views rotated around the y-axis. The cysteine residues are colored in CPK mode and the disulphide bonds formed by cysteine pairs are in yellow. The amino acids at substitution positions in both wild type and mutant protein 27+31+43 are shown and colored; Wild type/mutant = color; Glu/Tyr\textsubscript{27} = pink, Leu/Arg\textsubscript{31} = red, Glu/Leu\textsubscript{43} = blue.
27+31+43

Wild type

A.

B.
Figure 5.10

Comparison of the molecular surface images of *P. falciparum* MSP-1₁⁹ wild type and mutant protein 12+28

The molecular surface images of *P. falciparum* MSP-1₁⁹ wild type and mutant protein 12+28 are compared in 4 views of 90° rotation around the y-axis. The effects of substitutions on the molecular surface are indicated by green circles. The molecular surface is coloured by amino acid type; red = acidic, blue = basic, yellow = polar, grey = hydrophobic. The symbol * on the wild type protein images indicates the C-terminal free oxygen atoms present in the pdb data file. These atoms do not exist in the models created by the modelling program. The position of each domain in the images is indicated; D1 = domain 1, D2 = domain 2.
Figure 5.11

Comparison of the molecular surface images of *P. falciparum* MSP-1\textsubscript{19} wild type and mutant protein 27+31+43.

The molecular surface images of *P. falciparum* MSP-1\textsubscript{19} wild type and mutant protein 27+31+43 are compared in 4 views of 90° rotation around the y-axis. The effects of substitutions on the molecular surface are indicated by green circles. The molecular surface is colored by amino acid type; red = acidic, blue = basic, yellow = polar, grey = hydrophobic. The symbol * on the wild type protein images indicates the C-terminal free oxygen atoms present in the pdb data file. These atoms do not exist in the models created by the modelling program. The position of each domain in the images is indicated; D1 = domain 1, D2 = domain 2.
27+31+43
Wild type
Chapter Six

General discussion and further work

The fine specificity of different antibodies to MSP-1, is important for understanding their function during malaria infection. It has been shown that MSP-1 can induce different kinds of specific antibodies; a) inhibitory antibodies that can inhibit the secondary processing of MSP-1 and also erythrocyte invasion, b) blocking antibodies that cannot inhibit both processes but instead block the inhibitory activities of the inhibitory antibodies, and c) neutral antibodies that do not have either inhibitory or blocking activities. In the first part of this study, by using the site-directed mutagenesis technique, amino acids that are important for the binding of different MSP-1-specific antibodies were identified. It was shown that, by combining the single substitutions that were found to prevent the binding of blocking mAbs, individual proteins with multiple amino acid substitutions continued to bind inhibitory mAbs, but not the targeted blocking mAbs. This study showed the possibility of the production of an additive effect in a single protein by combining individual point mutations. However a suitable combination of the mutations is required to maintain the correct molecular structure of the protein. The mutation that disrupted a disulphide bond leaving one free cysteine in the molecule, and the mutations of more than six amino acids in the protein abolished most of the antibody binding. Thus, this observation may be crucial for an effective vaccine design.
By mapping the binding data for each antibody on the MSP-1\textsubscript{19} molecular model, information on the sites that are important on the protein for antibody binding were obtained, which will lead to more detailed understanding of the interactions between the protein and its antibodies, and also between antibody and antibody. Until now, only 3 inhibitory antibodies and 4 blocking antibodies were identified. Thus the monoclonal antibodies used in this study represent only a subset of the antibodies induced by the protein during natural infection. The information on antibody binding and its specificity obtained from this study may still not be a complete picture. Therefore further study should be concentrated on the identification of more inhibitory and blocking antibodies. More studies into the properties and functions of neutral antibodies should also be carried up. Although neutral antibodies do not compete with inhibitory antibodies to block the inhibitory activities, they may interfere with the activities of blocking antibodies. Competitive binding between blocking and neutral antibodies is being investigated using ELISA or BIAcore techniques.

In the second part of this study, the effects of amino acid substitutions on the proteins' immunogenicity and their ability to induce protective antibodies were investigated. Selected mutant proteins were used to immunised laboratory animals to raise polyclonal antibodies. It was shown that purified IgG from mice and rabbit immunised with mutant protein 12+28 (Cys12\rightarrow Ile and Cys28\rightarrow Trp substitutions) significantly inhibited the secondary processing of MSP-1 and erythrocyte invasion by the parasite. Furthermore Rabbit IgG induced by the mutant protein 27+31+43 (Glu27\rightarrow Tyr, Leu31\rightarrow Arg and Glu43\rightarrow Leu substitutions) significantly inhibited erythrocyte invasion by the parasite, but not MSP-1 secondary processing. These
results suggest that the mechanism of inhibition of erythrocyte invasion by inhibitory antibodies is not mediated only by the inhibition of the secondary processing of MSP-1, and other mechanisms may be involved. Detailed characterisation of the protective antibodies induced by the mutant proteins is underway. The newly proposed MSP-1 secondary processing-independent mechanism may involve the binding of MSP-1\textsubscript{19} specific antibodies to MSP-1\textsubscript{19} protein, and this binding may also interfere with erythrocyte receptor-parasite ligand interactions; for example the binding of other surface proteins that are involved during erythrocyte invasion by the parasite. The cross binding of MSP-1\textsubscript{19} specific antibodies to other proteins on the merozoite surface can not be ruled out. Further investigation of this hypothesis will be carried out by conducting experiments on competitive or cross binding activities between antibodies on the merozoite surface using IFA. Since the protective antibodies induced in this study were polyclonal antibodies, they might not be suitable for the specific antibody binding assays, because they may produce high background signals. Specific monoclonal antibodies induced by these mutant proteins will be produced for future studies. Therefore, an important step for MSP-1\textsubscript{19} mutant protein-based malaria vaccine development will be further elucidation of this new inhibitory mechanism mediated by MSP-1\textsubscript{19} specific antibodies induced by the mutant proteins. It has also been shown in this study that it is possible to selectively induce protective antibodies by subsequent boosting immunisations with candidate proteins, from an antibody repertoire primarily induced by the wild type protein. These findings suggest the possibility to induce protective antibodies by immunisation with a malaria vaccine of individuals already exposed to malaria.
In the last part of the study, by using a computational biology approach, the molecular structures and molecular surfaces of the 12+28 and 27+31+43 mutant proteins, the proteins that induce protective antibodies, were studied and compared to those of the wild type protein. It was shown that although the overall structures of mutant proteins and the wild type protein were not different, the molecular surfaces, polarity and electric charges of the mutant proteins at the substituted sites were changed. These changes may contribute to the disruption of the binding or induction of blocking antibodies, and be involved in the induction of protective antibodies that significantly inhibit secondary processing and erythrocyte invasion \textit{in vitro}. The molecular modelling studies of the mutant proteins, as well as of the wild type protein provide a basis for the study of local structural changes introduced by amino acid substitutions which will be useful for mutant protein-based vaccine design and development. The advantages of this approach will then be further exploited. Future mutagenesis studies should be carried out only after they have been studied and predicted using this computational method. However, the computational studies' predictions will always have to be confirmed by experimentation, for example comparison of the mutant and wild type structures by NMR.

Malaria is still one of the most deadly infectious diseases that is responsible for the deaths of 2 million people every year. It is often cited as a pull-back factor to economic and social development in endemic regions. The growing risk and spread of drug-resistant parasites and insecticide-resistant mosquitoes, along with the absence of effective vaccines, have put enormous pressure on the scientific community to work towards the development of an effective malaria vaccine. Many efforts have been made in several laboratories and field research groups with the aim of obtaining
and testing an efficient malaria vaccine. However this goal has not yet been accomplished. The concept of a multistage/multivalent vaccine is another attractive approach, which is believed to be a solution to combat against the antigenic polymorphism and variation of the parasite. The data presented in this study have, again, supported the importance of inclusion of MSP-1 in the multivalent malaria vaccine. The study has increased our knowledge on the fine specificity of anti-MSP-119 antibodies, which is crucial for further understanding of their functions. Importantly, the data from the immunisation study has shown that the mutagenesis approach can be used to improve protective immune responses elicted by MSP-1. In conclusion, the data obtained in this study have provided a strong rationale for the design and development of an MSP-119-based vaccine against *P. falciparum* malaria.
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