ANTIBODY RESPONSES TO, AND THE STRUCTURE OF PLASMODIUM FALCIPARUM MEROZOITE SURFACE PROTEIN-1; A CANDIDATE MALARIA VACCINE ANTIGEN.

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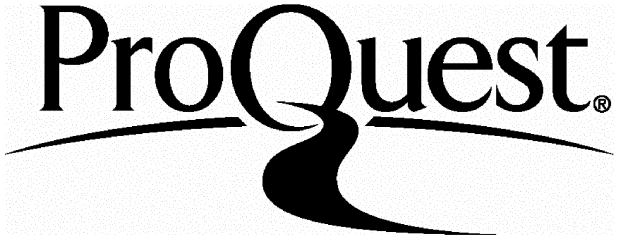

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Dedicated to Rebeca and my beautiful children Alejandra and Gabriel.

In memory of Ligia Ortega, La Tía Ana and Don Pedro Patiño.

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ABBREVIATIONS.

A_x	Absorbance measures at a wavelength of x nanometers.
BSA	Bovine serum albumin.
CBB	Coomassie brilliant blue.
CNBr	Cyanogen bromide.
cpm	Counts per minute.
Da	Dalton.
DTT	Dithiotheitol.
EDTA	Ethylenediaminetetracetic acid.
EGF	Epidermal growth factor.
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid.
ELISA	Enzyme-linked immunoadsorbent assay.
g	Acceleration due to gravity.
GPI	Glycosyl phosphatidylinositol.
HPLC	High performance liquid chromatography.
HRP	Horse-radish peroxidase.
IFA	Indirect immunofluorescence assay.
kDa	Kilodalton.
mAb	Monoclonal antibody.
Mbq	Mega becquerel.
MSP-1	Merozoite surface protein-1.

$MSP\text{-}1_{19}$	19 kDa processing fragment of merozoite surface protein-1_{42}.
$MSP\text{-}1_{33}$	33 kDa processing fragment of merozoite surface protein-1_{42}.
$MSP\text{-}1_{30}$	30 kDa processing fragment of merozoite surface protein-1.
$MSP\text{-}1_{42}$	42 kDa processing fragment of merozoite surface protein-1.
$MSP\text{-}1_{38}$	38 kDa processing fragment of merozoite surface protein-1.
$MSP\text{-}1_{83}$	83 kDa processing fragment of merozoite surface protein-1.
MSP1-pME	Specific expression constructs of MSP-1.
NCP	Nitrocellulose membrane.
NP-40	Nonidet P40.
OPD	o-phenylenediamine dihidrochloride.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
P	*Plasmodium.*
PMSF	Phenylmethylsulphonyl fluoride.
PVC	Polyvinyl chloride.
RBCs	Red blood cells.
SDS	Sodium dodecyl sulphate.
T	Tween-20.
TLCK	N-α-p-tosyl-L-lysine chloromethyl ketone.
U	Units.
v/v	Volume per unit volume.
w/v	Weight per unit volume.
w/w	Weight per unit weight.

INDEX OF CONTENTS

Page.

2 Acknowledgements.

3 Abbreviations.

4 Index to Figures.

15 Index to Tables and Panels.

16 Abstract.

CHAPTER ONE: INTRODUCTION.

21 1.1 Life cycle of the malaria parasite within the human host.

23 1.2 Antimalarial immunity.

25 1.3 Malaria vaccine design.

26 1.4 Anti-disease vaccine.

28 1.5 Transmission blocking vaccine candidates.

29 1.5.1 Pfs25.

30 1.6 Pre-erythrocytic vaccine candidates.

31 1.6.1 Circumsporozoite protein.

32 1.6.2 SSP2/TRAP.

33 1.7 Blood-stage vaccine candidates.

33 1.7.1 MSP-2

34 1.7.2 RESA

35 1.7.3 Synthetic peptide blood stage vaccines.

35 1.7.3.1 SPf66.

36 1.7.4 MSP-1.

41 1.8 Acquired immune response to MSP-1.

42 1.9 MSP-1-based malaria vaccine candidates.

42 1.9.1 GST-MSP-1_{19}.

43 1.9.2 P30P2 MSP-1_{19}.

43 1.9.3 BVp42.

44 1.10 Aims of this project.

CHAPTER TWO: MATERIALS AND METHODS.

50 2.1 Polyclonal and monoclonal antibodies.

51 2.2 SDS PAGE and visualisation of proteins.

52 2.3 *In vitro* culture of *P. falciparum.*

52 2.4 Synchronisation of *P. falciparum* cultures.

54 2.5 Purification of schizonts and naturally released merozoites.

55 2.6 Biosynthetic radiolabelling of *P. falciparum.*

56 2.7 Quantitation of antibody-mediated inhibition of MSP-1 secondary processing.

58 2.8 Pepsin digestion of mAb 12.10 to produce $F(ab')_2$

58 2.9 Reduction and alkylation of $F(ab')_2$ to produce Fab.

59 2.10 Preparation of recombinant antigens.

60 2.11 Coupling GST-MSP-1_{19} to the sensor chip CM5.

61 2.12 Binding of mAb 12.10, or its $F(ab')_2$ and Fab fragments to immobilised GST-MSP-1_{19}.

61 2.12 Binding of mAb 12.10, or its $F(ab')_2$ and Fab fragments to immobilised GST-MSP-1_{19}.

61 2.13 Determination and evaluation of kinetic binding constants of mAb 12.10 and its $F(ab')_2$ and Fab fragments to immobilised GST-MSP-1_{19} by BIAcore.

62 2.14 Radioiodination of antibodies.

63 2.15 Preparation of merozoite antigen sonicate for immunoassays.

64 2.16 ELISA.

64 2.17 Competitive radioimmune assay (RIA).

65 2.18 Affinity purification of human antibodies reactive with pME6.

66 2.19 Lectin-binding analysis of *P. falciparum* MSP-1_{19}.

67 2.20 Proteolytic digestion of recombinant MSP-1_{19}.

CHAPTER THREE: SECONDARY PROCESSING OF *P. FALCIPARUM* MSP-1 IS ESSENTIAL FOR SUCCESSFUL ERYTHROCYTE INVASION.

68 3.1 Introduction.

70 3.2 Results.

70 3.2.1 Visualisation of antibody-mediated inhibition of MSP-1 secondary processing.

70 3.2.2 Production of active F(ab′)$_2$ and Fab fragments from mAb 12.10.

71 3.2.3 Analysis of binding kinetics of mAb 12.10 and its F(ab′)$_2$ and Fab fragments to immobilised GST-MSP-1_{19} by BIAcore.

74 3.2.4 Analysis of the processing-inhibitory activity of mAb 12.10 and its F(ab′)$_2$ and Fab fragments.

75 3.2.5 Simultaneous analysis of the processing-inhibitory activity and erythrocyte invasion-inhibitory activity of mAb 12.10 and its $F(ab')_2$ and Fab fragments in the *in vitro* invasion parasite assay.

76 3.3 Discussion.

CHAPTER FOUR: ASSOCIATION BETWEEN *IN VIVO* PROTECTION FOLLOWING IMMUNISATION WITH MSP-1-BASED VACCINES AND INDUCTION OF PROCESSING-INHIBITORY ANTIBODIES.

85 4.1 Introduction.

87 4.2 Sera from primates vaccinated with experimental *P. falciparum* MSP-1 vaccines.

90 4.3 Results.

90 4.3.1 Analysis of the processing-inhibitory activity of sera from primates vaccinated with experimental *P. falciparum* MSP-1 vaccines and correlation with the *in vivo* protective response against blood-stage challenge.

90 4.3.2 Analysis of primate sera from published vaccine trials.

93 4.3.3 Analysis of primate sera from unpublished vaccine trials.

94 4.4 Discussion

CHAPTER FIVE: ANTIBODIES THAT INHIBIT MALARIA MSP-1 PROCESSING AND ERYTHROCYE INVASION ARE BLOCKED BY NATURALLY ACQUIRED HUMAN ANTIBODIES.

107 5.1 Introduction.

109 5.2 Results.

109 5.2.1 Development and validation of an assay to quantitate antibody-mediated inhibition of MSP-1 processing.

111 5.2.2 Blocking antibodies act by competitively preventing the binding of processing-inhibitory mAbs to merozoites.

112 5.2.3 Antibodies against the N-terminal region of MSP-1 can block the binding of processing-inhibitory mAbs directed against epitopes within MSP-1_{19}.

113 5.2.4 Naturally-acquired human antibodies specific for epitopes within the N-terminal domain of MSP-1 block the activity of processing-inhibitory anti-MSP-1_{19} antibodies.

117 5.3 Discussion.

CHAPTER SIX: STRUCTURAL STUDIES ON MSP-1_{19}.

135 6.1 Introduction.

138 6.2 Results.

138 6.2.1 Determination of possible oligosaccharides on $MSP1_{19}$.

140 6.2.2 Digestion of recombinant MSP-1_{19} by well characterised proteases.

144 6.3 Discussion.

CHAPTER SEVEN: GENERAL DISCUSSION AND FURTHER WORK.

CHAPTER EIGHT: REFERENCES.

INDEX TO FIGURES.

46 Figure 1. Life cycle of *Plasmodium* in mosquito and human.

47 Figure 2. Processing of MSP1.

48 Figure 3. Schematic representation of *P. falciparum* MSP1.

49 Figure 4. Proposed EGF-like structure of MSP-1_{19}.

79 Figure 5. Inhibition of MSP-1 secondary processing by anti-MSP-1_{19} mAbs.

80 Figure 6. SDS PAGE analysis of the mAb 12.10 and its F(ab′)$_2$ and Fab fragments.

81 Figure 7. Binding of mAb 12.10 and its F(ab′)$_2$ and Fab fragments to GST-MSP-1_{19} analysis by BIAcore.

82 Figure 8. BIAcore, two step competitive assay.

83 Figure 9. Analysis of the processing-inhibitory activity of mAb 12.10 and its F(ab′)$_2$ and Fab fragments.

84 Figure 10. Analysis of mAb 12.10, F(ab′)$_2$ and Fab fragments in the *in vitro* parasite invasion assay.

99 Figure 11. Analysis of processing-inhibitory activity of serum from primates vaccinated with BVp42 (Chang *et al.*, 1996).

100 Figure 12. Analysis of processing-inhibitory activity of serum from primates vaccinated with GST-MSP-1_{19} (Burghaus *et al.*, 1996).

101 Figure 13. Analysis of processing-inhibitory activity of serum from primates vaccinated with P30P2 MSP-1_{19} (Kaslow *et al.*, unpublished).

102 Figure 14. Analysis of processing-inhibitory activity of serum from primates vaccinated with BVp42 (Chang *et al.*, unpublished).

122 Figure 15. Conversion of MSP-1_{42} into MSP-1_{33} over 1 hour.

123 Figure 16. Quantitation of MSP-1 secondary processing.

124 Figure 17. Percentage antibody-mediated inhibition of MSP-1 secondary processing.

125 Figure 18. Monoclonal antibodies directed against the native MSP1 recognise a merozoite membrane extract in ELISA.

126 Figure 19. The binding of processing-inhibitory mAbs 12.8 and 12.10 to FCB-1 merozoite-derived MSP-1 is competitively prevented by certain other anti-MSP-1 mAbs.

127 Figure 20. Schematic of recombinant (pME) MSP-1 constructs relative to the MSP-1 gene and its products.

128 Figure 21. Rabbit polyclonal antibodies raised against defined domains of MSP-1 have blocking activity.

129 Figure 22. SDS PAGE analysis of the affinity purified human Ig anti MSP1-pME6.

130 Figure 23. Specificity of affinity-purified human anti-PME6 antibodies shown by western blot analysis.

131 Figure 24. Affinity-purified, naturally-acquired human anti-pME6 antibodies are potent blocking antibodies.

132 Figure 25. Processing-inhibitory anti-MSP-1_{19} mAbs can prevent MSP-1 and erythrocyte invasion *in vitro* culture, and can be rendered ineffective by the simultaneous presence of anti-pME6 blocking antibodies.

148 Figure 26. Lectin analysis for glycosylation of the *P. falciparum* MSP-1_{19}.

149 Figure 27. Reverse phase HPLC analysis of the recombinant MSP-1_{19}.

150 Figure 28. Digestion of the recombinant MSP-1_{19} by well characterised proteases.

151 Figure 29. Atomic mass of the recombinant MSP-1_{19}.

INDEX TO TABLES.

73 Table 1. Biacore analysis of kinetics of binding and dissociation of mAb 12.10 and its $F(ab')_2$ and Fab fragments to/from immobilised GST-MSP-1_{19}.

103 Table 2. Trial 1.

104 Table 3. Trial 2.

105 Table 4. Trial 3.

106 Table 5. Trial 4.

134 Table 6. Regions of the MSP1 gene included within the expression constructs.

ABSTRACT.

The malaria merozoite expresses a number of surface proteins, one or more of which are thought to mediate the initial interaction between parasite and host cell. Recent work in this laboratory has focused on the proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). Initially synthesised as a large (approximately 200 kDa) precursor during intracellular merozoite development, MSP-1 is present on the surface of the released merozoite in the form of a multicomponent protein complex derived *via* proteolytic processing. At some point between merozoite release and completion of erythrocyte invasion, the membrane-bound component ($MSP\text{-}1_{42}$) of this surface complex is further cleaved at a single site to form two fragments, $MSP\text{-}1_{33}$ and $MSP\text{-}1_{19}$. This results in the majority of the complex being shed from the parasite surface, leaving only $MSP\text{-}1_{19}$, representing the extreme end of the MSP-1 precursor and comprised of two epidermal growth factor (EGF)-like motifs, to be taken into the invaded cell on the parasite surface. This so-called secondary processing of MSP-1 is conserved across the genus and invariably goes to completion when a merozoite successfully invades a red blood cell, suggesting that it is a necessary step in the invasion pathway.

Certain antibodies directed against $MSP\text{-}1_{19}$ can prevent erythrocyte invasion by *P. falciparum* merozoites. In a previous study of a panel of anti-$MSP\text{-}1_{19}$ monoclonal antibodies (mAbs), it was found that those antibodies which most effectively prevent invasion can, upon binding to MSP-1 on the surface of merozoites, completely prevent secondary processing of the molecule. Of those mAbs which do not affect the processing,

some can interfere with the processing-inhibitory activity of the first group of antibodies. This second group of antibodies was referred to as blocking antibodies. In the first part of this project the mechanisms involved in, and the significance of antibody-mediated inhibition of MSP-1 processing, were investigated. It was found that both the processing-inhibitory activity and erythrocyte invasion-inhibitory activity of the anti-MSP-1_{19} mAb 12.10 was completely abrogated following removal of its Fc domain, clearly indicating that processing inhibitory antibodies directly interfere with erythrocyte invasion by inhibiting secondary processing of MSP-1.

In the second part of the project, an assay which measures antibody-mediated inhibition of MSP-1 processing was used to evaluate individually the ability of a panel of sera from primates vaccinated with recombinant MSP-1 constructs to inhibit the secondary processing of MSP-1. Significant processing inhibitory activity was detected in some primate sera; it was found that these sera corresponded to those animals that were protected against subsequent blood-stage parasite challenge. The data obtained indicate that vaccination with recombinant MSP-1 may induce protection *in vivo* by inducing processing-inhibitory antibodies, and that an assay which measures antibody-mediated inhibition of MSP-1 processing can be used to effectively predict protective immunity *in vivo* following immunisation with candidate MSP-1-based vaccines.

The third part of this project focused on the phenomenon of blocking antibodies. It was shown that blocking antibodies act by competing with processing-inhibitory mAbs for binding to the merozoite surface. Naturally-acquired human antibodies specific to the N-

terminal domain of MSP-1 were found to be potent blocking antibodies which can completely abolish the activity of invasion-inhibitory antibodies *in vitro*. The observations reveal a mechanism by which the parasite can avoid the action of a class of protective antibodies, and have important implications for the optimal design, evaluation and administration of MSP-1-based malaria vaccines.

In the fourth part of the study, the post-translational modification of MSP-1_{19} was investigated. Lectin binding studies did not detect any glycosylation of native MSP-1_{19}. Attempts were made to determine the molecular mass of merozoite-derived MSP-1_{19} and the identity and position of any post-translational modifications by a combination of peptide-mapping and liquid chromatography-electrospray mass spectrometric analysis.

CHAPTER 1.

INTRODUCTION.

There is an urgent need for a vaccine against malaria, since the disease represents a major and growing cause of mortality and morbidity in tropical and subtropical areas of the world. According to World Health Organisation estimates, there are some 300-500 million clinical cases of malaria per year, 90% of them in Africa, and 1.5-3 million deaths, one million of them in African children under five years of age. Of the 10% of clinical cases outside Africa, India alone accounts for 39% of these cases, and Brazil, Venezuela and Colombia account for a further 11% of these cases (2.2-5.6 million clinical cases per year, mainly in Amazonia) (W.H.O. Weekly Epidemiological Record, no. 34, pp 245-252, 1993; and no. 35, pp 253-258, 1993).

There are four species of malaria parasite, restricted to the family *Plasmodiae* within the order *Coccidiida,* sub-order *Haemosporidiidea,* which cause human malaria: *Plasmodium falciparum,* a major human pathogen, the causative agent of the most severe form of human malaria and responsible for practically all malaria-related deaths; *P. vivax,* also widespread and causing considerable morbidity; and the generally less prevalent species, *P. malariae,* and *P. ovale.*

The increase and spread of drug resistant malaria represents a major health problem. The use of chloroquine as a mass drug for therapy or prophylaxis has been

prevalent over the last 40 years, leading to an increase in the development and spread of drug resistant infections. *P. falciparum* infections resistant to 4-aminoquinolines (chloroquine and amodiaquine) were first observed in Thailand in 1957 and on the Colombia-Venezuela border in 1959, and resistance to choloroquine is also now well recognised in East Africa and is spreading in several countries of West Africa, including Sierra Leone, Senegal and Liberia (Lege-Oguntoye *et al.,* 1989; 1991; Brinkmann *et al.*, 1991; Brasseur, *et al.*, 1995). It seems unavoidable that chloroquine resistance in *P. falciparum* will eventually be a global problem. Alternative drugs such as pyrimethamine and sulphonamide combinations are in use, but parasites resistant to these treatments have also continued to emerge and are widespread in South-east Asia (Phillips *et al.,* 1984). While artemisinin and its derivatives continue to be developed, mefloquine and halofantrine provide the main alternatives to chloroquine for the treatment of multi-drug-resistant *P. falciparum* infections. However, mefloquine-resistence has been reported in West Africa, (Brasseur *et al.*, 1990; Ringwauld *et al.*, 1990), and in Thailand (Brodeau *et al.*, 1982).

Given the limited knowledge and lack of detailed understanding of the epidemiology and genetic basis of drug resistance, the biochemical basis of the action of some chemotherapeutic agents, and the biochemical processes involved during the complex life-cycle of the malaria parasite, it has been difficult to develop rational prophylactic strategies and effective anti-malarial therapies in response to the emergence of drug resistance. By understanding better the biology and pathogenesis of malaria, we

will be more able to develop strategies to prevent and treat the most severe forms of malaria.

1.1 Life cycle of the malaria parasite within human host.

To begin to understand malaria, it is necessary to understand the life cycle of the parasite. The life cycle of the malaria parasite begins when a female *Anopheles* mosquito, feeding on a person with malaria, ingests blood containing gametocytes. These cells undergo sexual development (sporogony) within the mosquito, culminating as infective sporozoites located in the insect's salivary glands. When the infected mosquito takes a blood meal the sporozoites are released into the blood stream of the host. Following the inoculation of sporozoites by the mosquito there is a brief period of approximately half an hour when sporozoites can be detected in blood. The sporozoites then disappear; many are destroyed but some successfully invade liver parenchymal cells, where the parasite multiplies asexually (the exoerythrocytic phase). After a maturation period ranging from days to months (an average of 2 to 4 weeks), merozoites are released and invade red blood cells (RBCs), initiating the erythrocytic (blood stage) phase of the life cycle. *P. vivax* and *P. ovale* exoerythrocytic forms may persist in the liver cells, periodically producing new merozoites to cause relapse. *P. falciparum* and *P. malariae* do not persist in the liver; however, in untreated or inadequately treated infections, erythrocytic parasites may persist from months (*P. falciparum*) to years (*P. malariae*) and produce recrudescent clinical disease. Parasites multiply asexually within RBCs in a process known as schizogony to produce a new generation of merozoites. When infected RBCs rupture,

these merozoites are released into the plasma to reinvade circulating RBCs. Male and female gametocytes, rather than merozoites, are formed in some infected RBCs. These gametocytes, which cannot self-replicate, die unless ingested by an *Anopheles* mosquito (Figure 1).

The life-cycle of the *P. falciparum* therefore contains a number of obligate intracellular stages, in which the parasite can replicate and differentiate and is protected from the immune response of the host. In addition, in order to perpetuate the life cycle, the parasite also has a number of highly specialised invasive stages (eg. sporozoites and merozoites) which have the specific function of allowing the parasite to invade host cells.

Replication of the parasite in the vertebrate host, and the production of sexual forms capable of perpetuating the life cycle of the organism in the insect vector, is crucially dependent on the ability of the parasite to invade and replicate within erythrocytes. Erythrocyte invasion takes place in the form of several steps, as follows. Initial attachment between merozoites and erythrocytes can occur between any point on the merozoite surface and the erythrocyte. Reorientation of the merozoite then takes place, so that its apical end, which contains a number of secretory organelles - paired rhoptries and several micronemes - comes into contact with the erythrocyte. Discharge of contents of the apical organelles then appears to take place, into the space between the apical complex and the erythrocyte membrane (Sinnis *et al.*, 1997). Although there is no direct evidence that rhoptry release is essential for erythrocyte invasion, it is thought that these events alter the properties of the host cell membrane to permit its rapid distension to

enclose the entering parasite in a parasitophorous vacuole (Mitchell and Bannister, 1988). The outer surface coat of the merozoite is shed in the process of parasite entry, and the merozoite is internalised within an erythrocyte within 20-30 seconds of initial contact and attachment.

It is important to stress the relevance of the blood-stage in the life cycle of the parasite. The parasite must go through successive cycles of replication in erythrocytes in order to complete its life cycle. Erythrocyte invasion is therefore critical to the survival of the parasite. The additional fact, that it is the blood-stage cycle which is responsible for the clinical manifestations of the disease, has resulted in the process of RBC invasion being of great interest to those investigating both malaria vaccine design and targets for novel drug intervention strategies. Though the biochemical mechanisms involved in invasion are poorly understood, it is clear that RBC invasion is initiated by receptor-ligand interactions between molecules on the surface of the parasite and the host cell. Merozoite surface molecules are therefore of particular interest.

1.2 Antimalarial immunity.

The most encouraging evidence that vaccination against malaria may be eventually realised is the well documented fact that residents of areas which are endemic for malaria can develop some form of protective immunity against the disease. In holoendemic areas high levels of morbidity and mortality are present in young children but in adults malaria is relatively mild. This is due to the acquisition of specific immunity. Children born to

immune mothers appear themselves to be relatively immune to malaria for a period of between 3 and 6 months following birth. This phenomenon is generally believed to be due to the transfer of immune maternal gamma-globulin (IgG) *via* the placenta (Edozien *et al.*, 1962). If malaria transmission is heavy the child may become parasitaemic during this early period in its life, and sometimes have fever, but rarely manifests any severe features of malaria.

Following this period of relative protection, children become increasingly susceptible to the more severe clinical manifestation of malaria, severe anaemia and cerebral malaria. The age of maximum susceptibility for these two clinical syndromes is different, with anaemia typically most prevalent in children under 18 months and cerebral malaria having a peak incidence in the third year of life. From around the fourth year of life the severity of clinical attacks begins to decline, although parasite rates and densities remain high. In holoendemic areas fully developed but non-sterile immunity is generally present by ten years of age (McGregor, 1986). Death from malaria is rare after the fifth year and clinical attacks becomes less frequent and less severe until adulthood, when significant disease is rare. A few experiments have shown that passive administration of IgG purified from clinically immune adults can confer protection in children and adults suffering from severe clinical malaria (Cohen *et al.*, 1961 and McGregor *et al.*, 1963; Druihle *et al.*, 1991).

These findings have encouraged the scientific community to make a considerable effort towards identifying either immunological targets or essential biochemical processes

of the parasite. The main aim of any putative malaria vaccine would be to induce an adequate immune response capable of effectively interfering with the life cycle of the parasite. The cultivation of human malaria parasites in the laboratory, first described by Trager and Jensen (1976), represented a significant advance in the development of drug targeting and testing, and vaccine design. Nevertheless, it is generally accepted that the large scale production of parasite material from *in vivo* cultures is neither safe nor practical for use as a vaccine. Recombinant technology has provided a potential route to solving this problem by allowing the generation of recombinant proteins which can be used as subunit vaccines.

1.3 Malaria vaccine design.

Significant progress has been made in the development of the malaria vaccine during the last 20 years. The life-cycle of the parasite involves multiple stages but it is widely assumed that an effective vaccine must prevent the establishment or amplification of the blood stage infection, since it this which is responsible for the disease. Furthermore, due to the intracellular location of the parasite for the major part of the asexual cycle, the extracellular merozoite - and in particular the surface components of this stage - are thought to be particularly accessible to the host's humoral immune system. However, approaches to vaccine-mediated disruption of other stages of the parasite life-cycle, distinct from the asexual blood-stage, are also under investigation.

Essentially, two approaches to malaria vaccine design have been taken. The first of these is the classical approach to vaccination, currently used for the majority of current malaria vaccine candidates, in which the main aim is to raise host immunity against the parasite so as to reduce parasite densities or to sterilise an infection. Within this group are included transmission blocking vaccines, based on the concept of inducing antibodies which block sporogony within the mosquito. Pre-erythrocytic vaccines target the sporozoite or liver stages of the parasite in order to disrupt the continuity of the parasite life cycle effectively. In the second approach to malaria vaccine design, so-called anti-disease vaccines are under development, the aim of which are to alleviate morbidity by suppressing immunopathology in the host. This second approach has been successfully used in other pathogens which mediate pathogenesis primarily through the production of toxic factors; diphtheria and tetanus are classical examples. Anti-disease vaccines are based on neutralising parasite components that induce host pathology, leaving the parasite itself unaffected. Some of these approaches are discussed further in the next section.

1.4 Anti-disease vaccines.

One important observation in malaria-endemic areas has been that, following repeated attacks of malaria, children develop a progressive reduction in the severity of the clinical manifestation of a malaria infection, independently of the parasitaemia presented (McGregor *et al.*, 1956). A more complete understanding of this phenomenon could lead to the development of new approaches - such as an anti-disease vaccine - to moderate the

vaccine - to moderate the intensity of the clinical presentation and potentially reducing the risk of such life threatening conditions as cerebral malaria.

During the blood-stage cycle, schizont rupture and merozoite release occurs simultaneously with release of lipids, glycoproteins, glycolipids and other soluble parasite components into the blood stream of the host. These factors have been analysed for their ability to mediate the clinical symptoms of malaria. Studies by Playfair *et al.* (1990) and Taverne *et al.* (1990) have demonstrated that secretion of tumour necrosis factor-alpha (TNFα) can be induced by substances found in *P. falciparum* culture supernatant. Interestingly, mice vaccinated with such malaria "toxins" following parasite challenge, have been found to survive in spite of high parasitaemia, a presentation that resembles the initial observation in African children. Schofield *et al.* (1993) more recently made the observation that purified glycosyl phosphatidyl inositols (GPI) from several merozoite surface proteins which are anchored by a GPI moiety have the ability to induce the secretion of TNFα and IL-1 by macrophages *in vitro.* The suggestion that TNFα may be an endogenous mediator of the pathology associated with malaria has been supported by the finding that TNFα levels are increased in children that have developed cerebral malaria, compared with TNFα levels in children with mild malaria and similar parasitaemia (Kwiatkowski *et al.*, 1990). Additionally studies by Udomsangpetch *et al.* (1997) suggest an association between the histopathology found in the brain of cases of fatal cerebral human malaria and focal accumulation of cytokines (TNFα, IFNγ and IL-1β).

Cerebral malaria is a life threatening multifactorial consequence of a malaria infection that is only infrequently observed in humans suffering from malaria. However, due to the lethality of this complication, the pathogenesis of cerebral malaria has been the focus of multiple studies. McGuire *et al.* (1994) have shown in Gambian children a genetic element that appears to establish an association between TNF and cerebral malaria. Two allelic forms of the TNF gene exist, referred to as TNF1 and TNF2. TNF2 is associated with higher levels of TNFα . This study found an association between children suffering from cerebral malaria and the presence of the TNF2 allele. However, experimental data (Kwiatkowski *et al.*, 1993) showed that administration of a neutralising anti-TNF monoclonal antibody can reduce fever, but does not reduce the risk of developing cerebral malaria. Combined use of an anti-disease vaccine coupled with (an) anti-parasite vaccine(s) would target growth of the parasite by disrupting its life cycle and in addition could alleviate the clinical outcome and potentially serious complications of a malaria infection.

1.5 Transmission blocking vaccine candidates.

Transmission blocking vaccines are designed to interrupt fusion and development of gametes in the mosquito itself. They do not protect individuals directly, but are aimed at reducing transmission, which is why they are sometimes called 'altruistic' vaccines (Greenwood, 1996). Transmission blocking candidates have been identified on the surface of gametocytes, ookinetes and zygotes and are thought to elicit a protective humoral immune response. Specifically, antibodies raised against these proteins would be ingested

by the mosquito in the course of a blood meal, which would then act on the specific parasite stages in the mosquito, interfering with the parasite maturation. Transmission blocking vaccines have the primary goal of reducing morbidity and mortality as a consequence of reducing parasite transmission (Kaslow, 1993; 1996). Several *P. falciparum* gametocyte surface proteins have been identified as a targets of transmission-blocking monoclonal antibodies (Carter *et al.*, 1985; Graves *et al.*, 1985; Quayi *et al.*, 1987). Carter and Chen (1976) showed that immunisation of the host with extracellular gametes of the parasite can induce suppression of further development of the parasite within the mosquito, suggesting that the inhibitory agents were ingested during the blood meal. The proposed mechanism by which these antibodies act was either through complement mediated lysis of gametes or by directly inhibiting the fertilisation process leading to sterilisation of the parasite in the vector.

1.5.1 Pfs25.

Pfs25 is a 25 kDa cysteine rich and lipid anchored ookinete surface protein (Kaslow *et al.*, 1988; 1989; Fries *et al.*, 1990). It is a leading candidate antigen for a transmission blocking vaccine, and has been suggested to act as a ligand for a mosquito midgut cell receptor. Kaslow *et al.* (1991) developed a recombinant vaccinia virus, expressing Pfs25, and used this to immunise mice, inducing antibodies that blocked transmission. Barr *et al.* (1991) have shown that recombinant Pfs25 expressed in yeast, can induce the production of transmission-blocking antibodies when used to immunise monkeys or mice. Complete reduction of the cysteines within the protein abrogated the

binding of these antibodies (Fries *et al.*, 1992) indicating that epitopes recognised by these antibodies are conformation dependent. The nucleotide sequence, encoding the regions within Pfs25 associated with the epitopes for blocking antibodies, has been found to be highly conserved. However, some structural diversity has been suggested based on the fact that transmission blocking antibodies react with several parasite isolates but not with others (Foo *et al.*, 1991). To date, no human trial with recombinant Pfs25 has been carried out. However mice and monkey trials strongly suggest that a protective immune response can be achieved. Currently, studies are attempting to evaluate the immunogenicity and length of the immune response induced by new vaccine-adjuvant formulations (Bathurst *et al.*, 1992).

1.6 Pre-erythrocytic vaccine candidates.

Pre-erythrocytic vaccines are designed to induce a protective immune response against the sporozoite stage or liver stages of the parasite life cycle through several mechanisms. Following the bite of the mosquito, sporozoites circulate in the bloodstream for a brief period, before reaching the liver and entering hepatocytes, a period of time in which they are potential targets for the host immune response. Antibodies directed to components on the surface of the sporozoite can exert protective effects by opsonization, complement-mediated lysis or by blocking the invasion of hepatocytes and thus preventing the infection. Infected hepatocytes in turn can be targeted by cell-mediated immunity. MHC molecules that can present processed parasite antigens are on the surface of the hepatocyte, leading to recognition and killing of the infected cell by either direct lysis or

by the action of multiple cytokines. Early studies by Clyde *et al.* (1975) demonstrated that immunisation of animals and humans with irradiation-attenuated sporozoites can elicit a protective immune response against infection with infected mosquitoes. Other studies (eg. Ramsey *et al.*, 1982) in which volunteers were immunised by repeated bites from irradiated *P. falciparum*-infected mosquitoes, demonstrated complete, short-lived stage and species-specific protection following challenge with the same strain of parasite. Egan *et al.* 1987 also demonstrated that antibodies against a protein present on the surface of mature sporozoites, termed circumsporozoite protein (CSP) can passively protect *in vivo*.

1.6.1 Circumsporozoite protein (CSP).

Circumsporozoite protein or CSP is a surface protein present on the surface of sporozoite and has been found in all species of *Plasmodium*. The open reading frame of the *P. falciparum* CSP gene encodes a sequence of 412 amino acids, the central region of which consists of 41 tandem repeats of a tetrapeptide, Asn-Aln-Asn-Pro (NANP) and four additional Asn-Val-Asn-Pro (NVNP) repeated units. Studies by Nussenzweig *et al.* (1989) have shown that, in animal models, antibodies to these repeats mediate protection against sporozoite-induced infections. Human trials (Ballou *et al.*, 1987; Fries *et al.*, 1992; Herrington *et al.*, 1992) showed such antibody responses to be poorly effective in inhibiting sporozoite invasion into hepatocytes. Failure of these early CSP-based vaccines has been attributed to the poor immunogenicity of the preparations. However, further efforts have been made in order to improve the efficacy of this vaccine by reformulating the adjuvants used for these preparations. Recently, Stoute *et al.* (1997) published the

results of a trial in which six out of seven human volunteers vaccinated with a formulation called RTS,S consisting of a hybrid of *P. falciparum* CSP (central tandem repeats and C-terminal epitopes) and hepatitis B (hepatitis B surface antigen) emulsified in a water oil preparation containing monophosphoryl lipid A (immune stimulating agent) and QS21 (synthetic adjuvant) were protected following challenge with infected mosquitoes; this represents an astonishing 85% efficacy. RTS,S has been shown to induce a good cellular and humoral immune response against the CSP central tandem repeats and C-terminal epitopes; however the mechanism by which this vaccine offers an effective protective-immune response remains unclear.

1.6.2 SSP2/TRAP.

Charoenvit *et al.* (1987) identified a 140 kDa protein on the surface of sporozoites subsequently named Sporozoite Surface Protein 2 (SSP2) or thrombospondin-related anonymous protein (TRAP). The protein was later shown to also reside in sporozoite micronemes and to be present on the hepatocyte surface following invasion (Rogers *et al.*, 1992). Mice immunised with both CSP and SSP2 were completely protected against malaria infection whereas each individual protein induced only partial protection. Complete protection in mice was observed in mice that receive a passive transfer of $CD8^+$ CTL specific for *P. yoelii* SSP2, before parasite challenge (Khusmith *et al.*, 1991). Rogers *et al.* (1992) demonstrated that murine antibodies against recombinant PfSSP2 could inhibit sporozoite invasion and development in hepatocytes *in vitro.* However, further

studies will provide more data on the development of new approaches for immuno-prophylaxis of malaria infection.

1.7 Blood-stage vaccine candidates.

After a period of maturation within the erythrocyte, schizont rupture occurs and merozoites are released into the blood stream to infect new red blood cells. During this brief period merozoites are accessible to the host immune response. Antibody-mediated immune protective response could be directed against surface proteins of the merozoite, in which case they could act by blocking essential steps in the invasion process or by rendering the merozoite susceptible to secondary effects such as phagocytosis or complement-mediated damage. Several merozoite surface proteins have been identified and their apparent function studied; however only a few of these are currently studied for their capacity to induce an adequate protective immune that may disrupt the asexual blood-stage replication of the parasite. Among these proteins are several promising candidates found either on the surface or within secretory organelles of the merozoite. Some of these are briefly discussed in the course of the next section.

1.7.1 MSP-2.

Merozoite surface protein-2 (MSP-2) in *P. falciparum* has been identified as a parasite glycoprotein varying in size between 35 and 53 kDa. The protein contains a central repeat region flanked on both sides by conserved domains (Smythe *et al.*, 1991).

The protein is probably held on the surface of the merozoite *via* a GPI moiety (Ramasamy, 1987); Smith *et al.* 1988; Gerold *et al.*, 1994; 1996). Clark *et al.* (1989) showed that polyclonal sera raised against purified MSP-2 (Miettinen *et al.*, 1988), inhibited merozoite invasion *in vivo* although the inhibition was significantly lower than that observed using a similar concentration of human IgG obtained from residents of a malaria endemic area. Similar levels of strain specific invasion inhibition *in vitro* have been observed using MSP-2 specific monoclonal antibodies (Epping *et al.*, 1988; Clark *et al.*, 1989; Ramasamy *et al.*, 1990). Currently, MSP-2 has been incorporated into a single vaccine preparation in combination with another malarial blood-stage protein, Ring-infected Erythrocyte surface antigen (RESA), and a C-terminal fragment of merozoite surface protein-1 (see below); this combination is currently being tested for immunogenicity and safety in humans (Genton *et al.*, 1997).

1.7.2 RESA.

Ring-infected Erythrocyte Surface Antigen (RESA) or Pf155 is a 155 kDa molecular mass protein, localised within dense granules in the merozoite (Aikawa *et al.*, 1990). The protein contains two immunogenic oligopeptides repeat regions near its carboxy terminus. RESA is released from dense granules immediately following invasion of erythrocytes (Culvenor *et al.*, 1991). A panel of RESA-specific, affinity-purified immunoglobulins from human IgG were found to effectively inhibit parasite growth *in vitro* (Wahlin *et al.*, 1984). Monoclonal and polyclonal antibodies directed against epitopes within RESA can inhibit invasion by merozoites *in vitro* (Ruangjirachuporn *et*

al., 1988). However, *Aotus* monkeys immunised with recombinant RESA or synthetic peptides corresponding to repeat regions of RESA (Collins *et al.*, 1986; 1991) have been only partially or not at all protected against a subsequent blood-stage challenge infection. Nevertheless, numerous epidemiological studies on residents of endemic areas have correlated the presence of serum antibodies against the immunodominant repeat regions of RESA with the acquisition of immunity against malaria (Chumpitazi *et al.*, 1991; Deloron and Chougnet, 1992). The mechanism by which antibodies are able to interact with a protein such as RESA has been the subject of much controversy and has not yet been explained. However, RESA has been proposed to play a role in stabilising the erythrocyte membrane by interacting with erythrocyte cytoskeleton-spectrin following invasion, which may aid in the survival of the parasited erythrocyte (Da Silva *et al.*, 1994).

1.7.3 Synthetic Peptide blood stage vaccines.

1.7.3.1 SPf66

SPf66 malaria vaccine is a synthetic protein based on a number of partial amino acid sequences derived from pre-erythrocytic and asexual blood-stage proteins of *P. falciparum*. The construct includes two NANP sequences of the CS protein, 9 amino acids from the conserved N-terminal region of *P. falciparum* MSP-1 and two peptides of unknown origin. Patarroyo *et al.* (1987) showed that *Aotus* monkeys vaccinated with SPf66 were significantly protected against blood-stage challenge. Initial small scale trials in humans using SPf66 (Patarroyo *et al.*, 1988) showed a markedly delayed course of

infection following parasite challenge. In 1992 Patarroyo and co-workers demonstrated the safety and immunogenicity of SPf66 in children 1 to 14 years of age (Patarroyo *et al.*, 1992). Valero *et al.* (1993), in further large-scale vaccination trials in an endemic area, showed encouraging results in which SPf66 had an estimated efficacy against the first clinical episode of *P. falciparum* malaria of 38.8%. Alonso *et al.* (1994) in accordance with previous studies, reported an efficacy of 31% in Tanzanian children between 1 to 5 years of age. More recent studies by Valero *et al.* (1996) carried out in Rio Rosario, Colombia showed an efficacy of 36%. However, recent studies in 547 Gambian children failed to elicit protection (D'Alesandro *et al.*, 1995); an estimated efficacy of 3% was reported. Similarly, Nosten *et al.* (1996), found in a study carried out in 610 Karen children an overall efficacy of -9%. These results are in clear disagreement with the Colombian (La Tola, Rio Rosario) and Tanzanian trials. The mechanisms involved in SPf66-mediated protection have not been clarified, nor have the reasons for the marked differences between the efficacy reported in the mentioned trials. However, a second generation of SPf66 vaccines is currently under development, and Patarroyo and co-workers continue to study the immunogenicity and safety of new polypeptides and adjuvants.

1.7.4 MSP-1.

The merozoite is briefly accessible to antibody between the time of schizont rupture and erythrocyte invasion, and therefore the surface may be the target of antibody

that interferes with the recognition and invasion of the red blood cell. Merozoite surface protein 1 (MSP-1) is one of the best characterised malarial proteins and the most promising current candidate for a vaccine against the blood stage of the *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 weight, antigenically polymorphic glycoprotein synthesised by the intracellular schizont as a 185-205 kDa precursor protein, (Holder and Freeman, 1982), and detectable in all *Plasmodium* species (Holder *et al.*, 1992). MSP-1 on the merozoite surface is present in the form of a complex of 4 major polypeptides of 83 kDa ($MSP\text{-}1_{83}$), 30 kDa ($MSP\text{-}1_{30}$), 38 kDa ($MSP\text{-}1_{38}$) and 42 kDa ($MSP\text{-}1_{42}$) resulting from proteolytic processing of the precursor (Figure 2; Lyon *et al.*, 1986; Holder *et al.*, 1987; McBride *et al.*, 1987). This primary processing occurs at or just prior to merozoite release (Holder, 1988). Secondary proteolytic cleavage then takes place, mediated by a calcium-dependent serine protease (Blackman and Holder, 1992); the membrane bound C-terminal 42 kDa component of the merozoite surface MSP-1-derived complex is cleaved into two polypeptide fragments of approximately 19 kDa and 33 kDa. The NH_2-terminal, 33 kDa cleavage product ($MSP\text{-}1_{33}$) is shed in a soluble form with other components of the merozoite surface complex, which also contains at least three parasite polypeptides - denoted p19, p22 and p36 - which are not MSP-1-derived (Stafford *et al.*, 1996). The membrane bound C-terminal, 19 kDa fragment ($MSP\text{-}1_{19}$) is carried into the invaded erythrocyte on the merozoite surface (Blackman *et al.*, 1990; 1991). Secondary processing goes to completion when a merozoite successfully invades an erythrocyte. Processing also takes place in free merozoites isolated from culture, independent of erythrocytes available for invasion.

The deduced MSP-1 precursor protein sequence begins with a predicted hydrophobic signal peptide to direct the gene product to the merozoite surface, as experimentally demonstrated by Edman sequencing of the N-terminal region of MSP-1_{83} (Heidrich *et al.*, 1983; Stafford *et al.*, 1994). The C-terminal region of the deduced primary sequence is hydrophobic, suggesting a membrane-anchor domain; however, it is proposed that this region is cleaved for GPI anchor attachment, which functions to anchor MSP-1 to the merozoite surface (Haldar *et al.*, 1985; Gerold *et al.*, 1994; 1996). Studies on the complete DNA sequence of different *P. falciparum* strains carried out by Tanabe *et al.* (1987) and Miller *et al.* (1993) suggest the presence of two allelic types referred to here as Wellcome-type and MAD20-type (which refers to two parasite isolates, Wellcome and MAD20, expressing MSP-1 alleles typical of the two major alleles). The nucleotide sequence has been divided into 17 blocks (Figure 3) according to the degree of homology between the alleles at the amino acid level. These blocks of sequence correspond to regions of high homology (87-97% homology; blocks 1, 3, 5, 12 and 17), intermediate homology (65-77% homology; blocks 7, 9, 11, 13 and 15) and low homology (10-38% homology; blocks 2, 4, 6, 8, 10, 14 and 16) between the alleles. The relatively highly conserved and hydrophobic C-terminal region of MSP-1 (block 17) is cysteine-rich, containing 12 of the 19 cysteine residues of the MSP-1 sequence, and the binding of mAbs and polyclonal antibodies reactive with this domain is abolished when native MSP-1 is pre-treated with a reducing agent as such dithiothreitol (Chappel and Holder, 1992; Burghaus and Holder, 1994; Egan *et al.*, 1995). Reduction and alkylation of MSP-1 also abolished the production of growth-inhibitory antibodies (Locher and Tam, 1993). This suggests that the C-terminus of MSP-1 is a highly folded structure maintained by

disulphide bonds. MSP-1_{19}, which essentially corresponds to block 17 of the nucleotide sequence, is considered to adopt a structure similar to two contiguous epidermal growth factor (EGF)-like domains (Blackman *et al.*, 1991), defined by the characteristic disulphides which link cysteine residues C1 to C3, C2 to C4 and C5 to C6, and which consists of several loops connected together by β-sheet structures (Figure 4). Preliminary nuclear magnetic resonance (NMR) studies in the Institute on recombinant MSP-1_{19} support this prediction (Shabih Syed, personal communication).

An immune response directed against the complete MSP-1 can protect against blood-stage challenge (Holder and Freeman, 1987; Holder, 1988; Hui *et al.*, 1993; Siddiqui *et al.*, 1987) and a humoral immune response to MSP-1_{19} alone can provide partial or complete protection against blood-stage challenge (Chang *et al.*, 1992; Ling *et al.*, 1994; 1995; Kumar *et al.*, 1995; Chang *et al.*, 1996). However, the immune mechanisms involved in this protection are unclear. Using an assay which measures secondary processing, it has been shown that certain monoclonal antibodies, upon binding the C-terminal domain of MSP-1 on the merozoite surface, effectively inhibit the secondary processing of the MSP-1 and in addition these same antibodies inhibit erythrocyte invasion by *P. falciparum* merozoites *in vitro* (Blackman *et al.*, 1994a), whereas other antibodies, recognising adjacent epitopes, do not prevent MSP-1 processing and have no effect on erythrocyte invasion. This secondary processing of MSP-1 has been postulated to be a prerequisite for invasion to occur and inhibition of processing may therefore be a mechanism by which antibodies to MSP-1 can interrupt

erythrocyte invasion, and thereby protect against malaria (Blackman *et al.*, 1993a; Holder *et al.*, 1994).

A monoclonal antibody recognising an epitope close to the C-terminus of the *P. yoelii* MSP-1 is able to passively protect mice against blood-stage malaria infection (Burns *et al.*, 1989; Daley and Long, 1993). In addition, the C-terminal EGF-like domains of the *P. yoelii* MSP-1 expressed in *Escherichia coli* and *Saccharomyces cerevisiae* can immunise rodents against massive challenge infections (Ling *et al.*, 1994; Hirunpetcharat *et al.*, 1997). Similarly, rabbits immunised with a recombinant protein expressed in baculovirus produced antibodies that inhibit parasite growth *in vitro* (Chang *et al.*, 1992). *Aotus* monkey trials (Kumar *et al.*, 1995; Chang *et al.*, 1996) have also demonstrated that induction of *in vivo* protection against blood-stage challenge can be achieved in primate models followed administration of vaccines based on the C-terminal domain of *P. falciparum* MSP-1. These results indicate that the use of recombinant MSP-1_{19} as a vaccine can provide an efficient and protective immune response against the malaria parasite, which probably (though not conclusively) blocks the life cycle of the parasite by preventing erythrocyte invasion.

Some antibodies against MSP-1_{19} that are known to prevent erythrocyte invasion and/or intracellular development can also prevent the secondary processing of MSP-1 (Blackman *et al.*, 1990); other antibodies (blocking antibodies), which bind to MSP-1_{19} and other regions of the MSP-1 can interfere with this inhibitory activity. An earlier, particularly interesting observation was that antibodies to MSP-1_{83} could block the

binding of MSP-1_{19} specific antibodies to the precursor protein (Wilson *et al.*, 1987); it was postulated that such antibodies may contribute to immune evasion by preventing the binding of antibodies to conserved structures with a biological function. Inhibition of MSP-1 processing on merozoites may be a mechanism by which protective anti-MSP-1 antibodies can interrupt invasion and the life-cycle of the malaria parasite.

1.8 Acquired immune response to MSP-1.

In general, both humoral and cellular responses to the *P. falciparum* MSP-1 (MSP-1) appear to be acquired in an age-dependent manner with peak prevalence, or maximum intensity of the response, in late childhood or early adolescence (Riley *et al.*, 1992). This suggests that repeated exposure to infections is required to induce responses in the majority of the population, and may reflect a cumulative response to different polymorphic forms of MSP-1. High titres of antibody to the C-terminal MSP-1_{42} domain are significantly associated with resistance to both clinical malaria and high parasitaemia, indicating that T- and B- cell epitopes within the C-terminal region of MSP-1 are associated with the induction of protective cellular and humoral responses (Riley *et al.*, 1992, 1993; Egan *et al.*, 1997). Studies in human populations in malaria endemic areas have revealed an association between the presence of antibodies against epitopes within the MSP-1_{19} and the lack of clinical disease (Riley *et al.*, 1991; Egan *et al.*, 1995; al-Yaman *et al.*, 1996).

1.9 MSP-1-based malaria vaccine candidates.

The *P. falciparum* MSP-1 is the major candidate for a blood-stage malaria vaccine. Data from epidemiological studies, discussed above, suggest that MSP-1_{19} is an important target of antimalarial immunity. Additionally, epidemiological studies on naturally acquired immune responses indicate a significant association between anti-MSP-1_{19} antibody titer and reduction in malaria morbidity (Egan *et al.*, 1996). Siddiqui *et al.* (1987) and Etingler *et al.* (1991) have shown that vaccination with MSP-1 isolated from cultured parasites can protect monkeys against *P. falciparum* infection. Vaccination experiments with the C-terminal domain of MSP-1 from *P. yoelii* expressed in *E. coli* have shown that mice immunised with this antigen are protected (Ling *et al.*, 1994; Daley and Long, 1993). Currently, efforts are being made in the development of a recombinant MSP-1-based vaccine that can induce a protective level of immunity. Three of the major candidates will be briefly discussed.

1.9.1 GST-MSP-1_{19} (Burghaus and Holder, 1993; Burghaus *et al.*, 1996).

GST-MSP-1_{19} is a recombinant fusion protein expressed in *E. coli,* based on the sequence of the C-terminal domain of MSP-1 (Wellcome allelic type). The protein appears to have similar folding and antigenicity to the native MSP-1_{19} (Burghaus and Holder, 1993). However, immunisation-challenge experiments in which *Aotus nancymai* were vaccinated with GST-MSP-1_{19} in liposomes and alum adjuvant prior to blood-stage

challenge showed that immunisation did not induce protection in this animal model (Burghaus *et al.*, 1996).

1.9.2 P30P2 MSP-1_{19} (Kumar *et al.*, 1995).

P30P2 MSP-1_{19} is a fusion protein secreted in *S. cerevisiae.* The protein contains two universal T-helper-cell epitopes from tetanus toxoid (P30 and P2) fused to the amino terminus of the Wellcome-type *P. falciparum* MSP-1_{19} (Kumar *et al.*, 1995). Kumar *et al.* have shown in immunisation-challenge experiments, in which *Aotus nancymai* were vaccinated with P30P2 MSP-1_{19} in complete Freund's adjuvant prior to challenge with *P. falciparum* FVO strain, that the animals self-resolved an otherwise lethal infection.

1.9.3 BVp42 (Chang *et al.*, 1992; 1996).

BVp42 is a baculovirus-derived recombinant polypeptide corresponding to the 42 kDa C-terminal fragment of MSP-1 (MAD20 allelic type). The protein appears to conserve the disulphide-dependent conformation of the C-terminal region of MSP-1 critical for its immunogenicity. The efficacy and immunogenicity of BVp42 has been evaluated. Rabbit sera raised against BVp42 inhibited *P. falciparum* growth *in vitro* (Chang *et al.*, 1992). Recently, BVp42 was tested in immunisation-challenge experiments (Chang *et al.*, 1996). It was shown that all the animals immunised with BVp42 in complete Freund's adjuvant, produced antibodies that inhibited parasite growth *in vitro* and the animals were protected against blood-stage challenge with *P. falciparum*.

1.10 Aims of this project.

The project has three main aims; to study the role of inhibition of MSP-1 processing in antibody-mediated inhibition of erythrocyte invasion; to investigate the location, within the merozoite surface MSP-1 complex, of epitopes recognised by processing inhibitory and "blocking" antibodies; to establish whether there is any correlation between the immune status of primates vaccinated with MSP-1 based vaccine, and serum levels of processing inhibitory anti-MSP-1 antibodies; and to investigate further the structure of the C-terminal domain of MSP-1. In the first part of the project, the mechanism by which processing inhibitory antibodies interfere with erythrocyte invasion by the merozoite was meticulously studied. For this purpose an assay capable of measuring quantitatively the processing-inhibitory activity of antibodies was developed. Also, the prevalence of processing-inhibitory antibodies in sera from primates vaccinated with MSP-1 based vaccines have been measured, and the association between the level of such antibodies and clinical status of the immunised animals has been investigated. In the second part of the project, antibodies to various regions of MSP-1 were tested for their ability to either inhibit processing of MSP-1, or to act as blocking antibodies preventing the binding of inhibitory antibodies. This part of the project also allowed the identification of domains of MSP-1 which are targets for inhibitory or blocking antibodies. In the third part of the project, structural studies were carried out on the native and recombinant $MSP\text{-}1_{19}$.

This project is very relevant to ongoing studies on the potential of the MSP-1 as a vaccine against malaria, especially if a simple assay which measures the processing inhibitory activity of an antibody response can provide a quantifiable prediction of antibody-mediated resistance to blood-stage infection in human beings.

Figure 1.

Life cycle of *Plasmodium* in mosquito and human.

There are two distinct phases: sexual stages and asexual stages. An infected mosquito feeds and injects sporozoites into the bloodstream. Sporozoites invade hepatocytes and undergo growth and asexual division to form exoerythrocytic schizonts. Infected liver cells burst and release 10 to 40,000 merozoites. Merozoites invade red blood cells, develop through ring and schizonts to form the multinucleate mature erythrocytic schizonts. Erythrocytic schizonts burst and merozoites are released to invade new red blood cells. Some merozoites undergo sexual differentiation to form gametocytes and these are taken up by feeding mosquitoes and produce female and male gametes in the mosquito gut. After fertilisation, the motile zygote (ookinete) penetrates the stomach wall and an oocyst develops. Inside the oocyst repeated divisions (sporogony) produce many sporozoites. Sporozoites migrate to and mature in the insect salivary glands from where they can be transmitted to a host during feeding.

Figure 1.

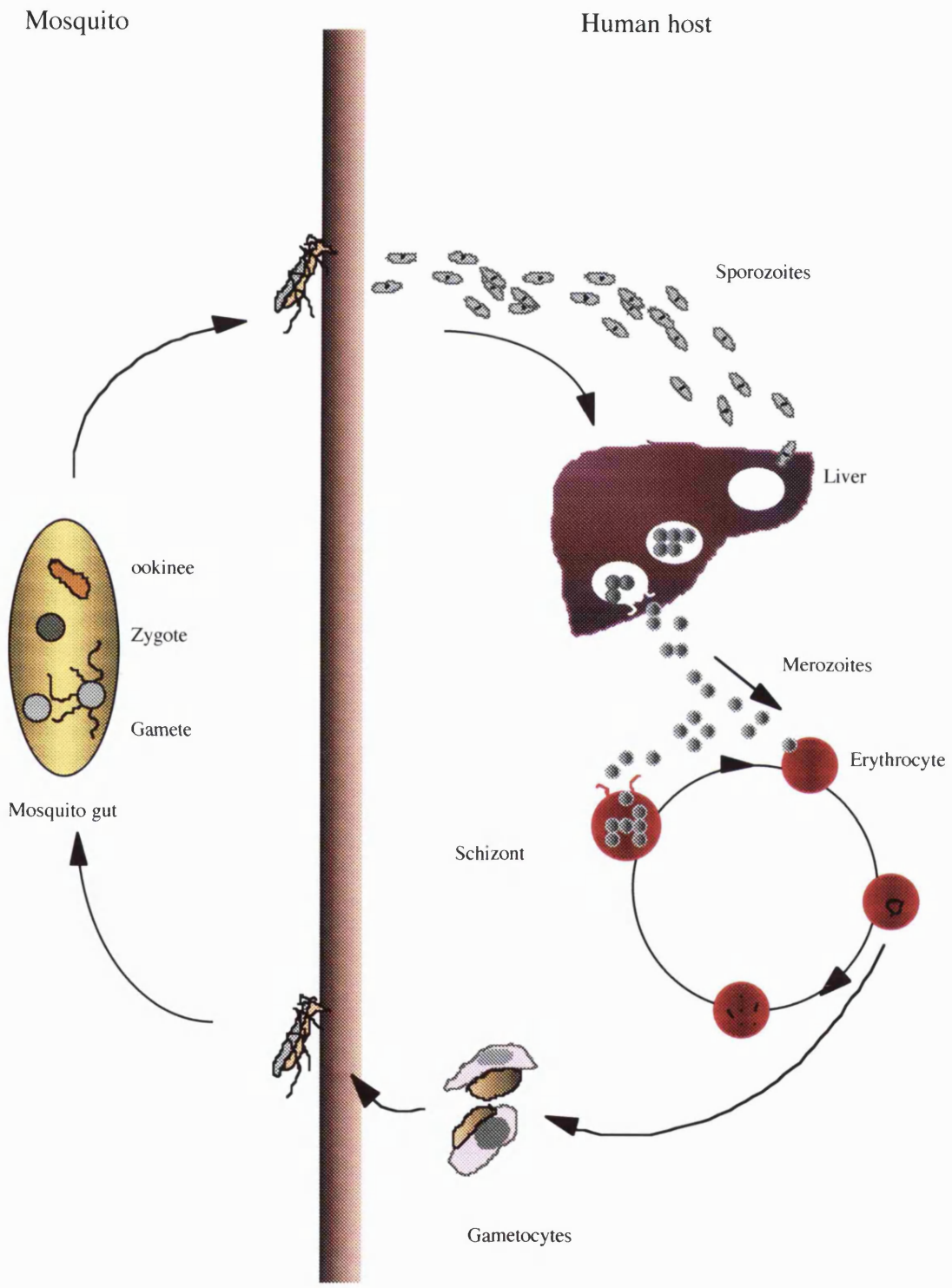

Mosquito
Human host
Sporozoites
Liver
Merozoites
Erythrocyte
Schizont
Gametocytes
ookinee
Zygote
Gamete
Mosquito gut

Figure 2.

Processing of MSP1.

The membrane-bound intact MSP1 (A) is processed into small fragments which are found on the surface of the free merozoite (B). At or just before erythrocyte invasion a secondary processing step cleaves $MSP1_{42}$ to produce $MSP1_{33}$ and $MSP1_{19}$ (C). $MSP1_{19}$ is carried into the newly invaded erythrocyte. $MSP1_{33}$ is shed in a soluble form from the merozoite surface as part of a complex with the other MSP1-derived polypeptides.

Figure 2.

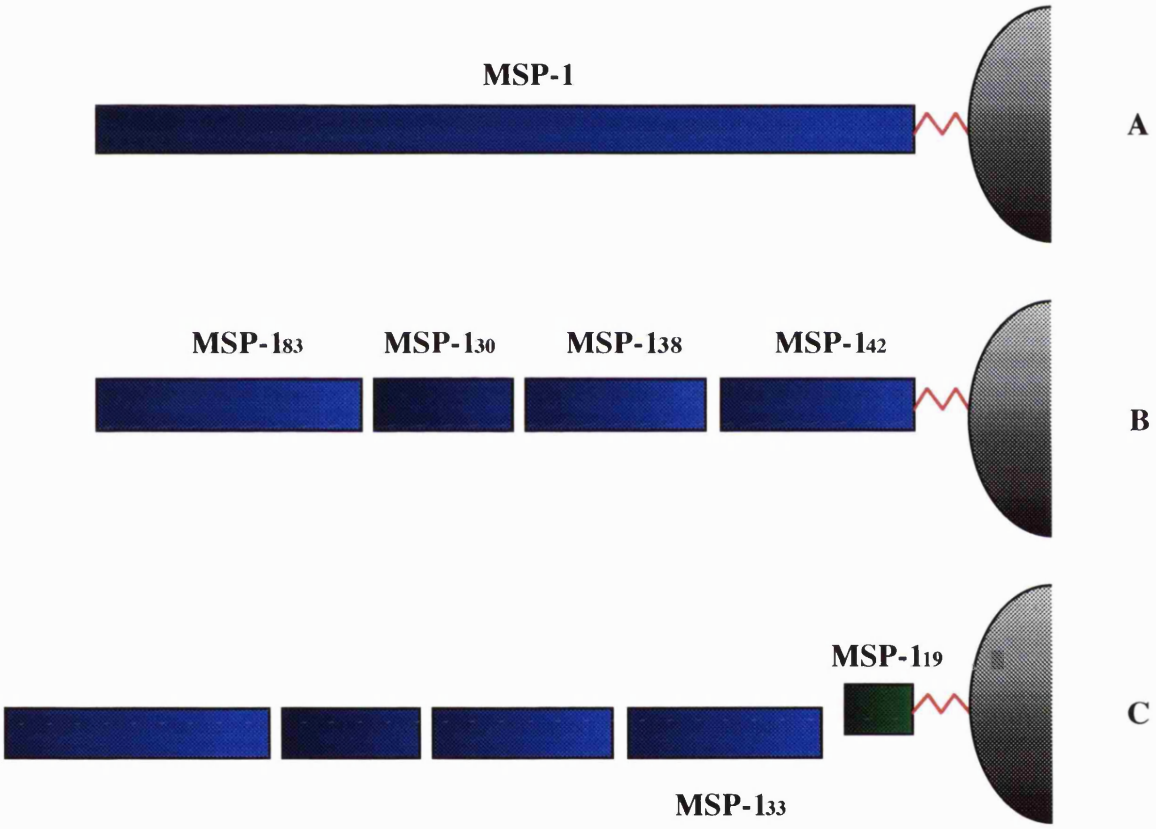


Figure 3.

Schematic representation of *P. falciparum* MSP1.

This figure shows the 17 sequence blocks defined by Tanabe *et al.*, (1987) and the position within the primary sequence of the major proteolytic processing sites. The sequence blocks have been defined as high homology, medium homology (semiconserved) and low homology (variable) in comparisons of genes from different dimorphic types. The amino acid sequence, where known, at the N-terminus of each processing fragment is indicated by each of the cleavage points; the apparent molecular masses of the fragments are indicated.

Figure 3.

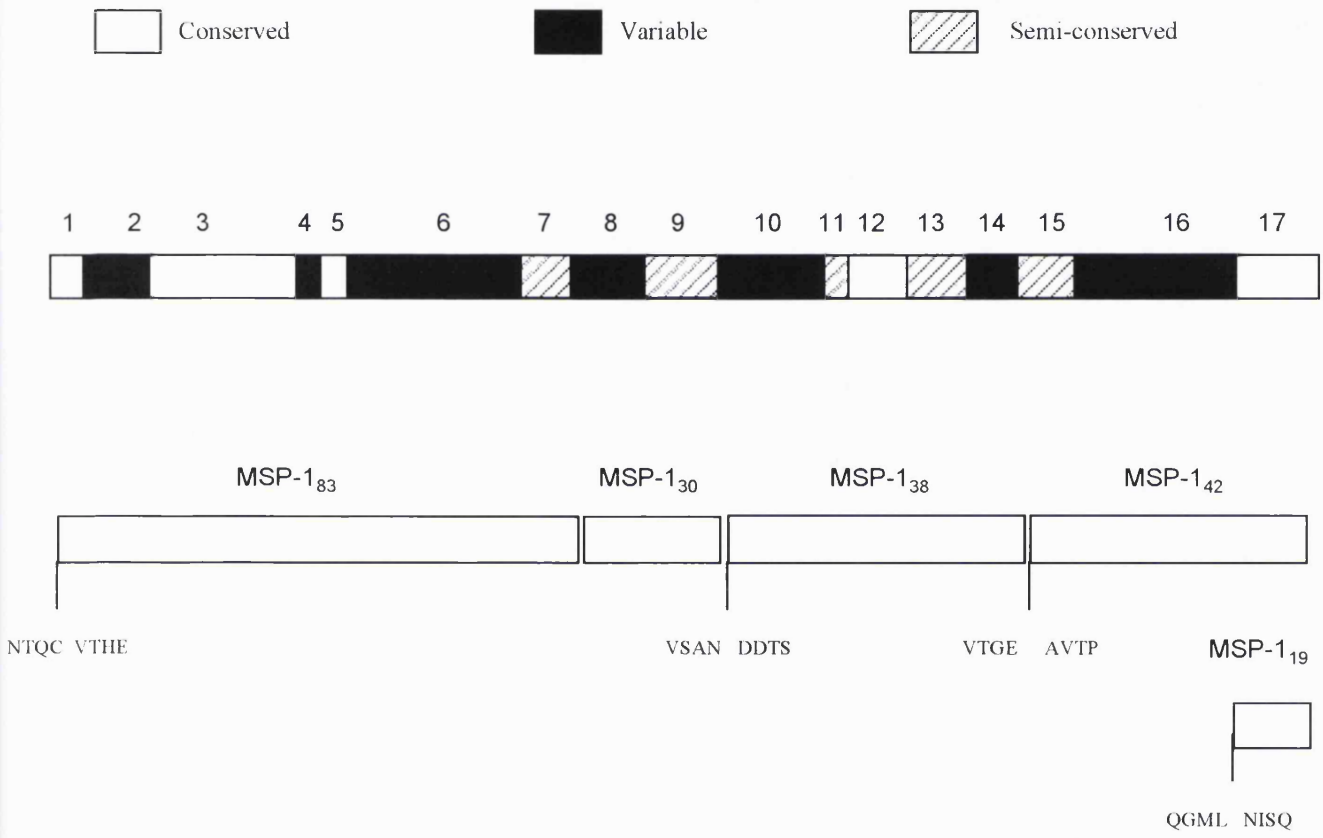
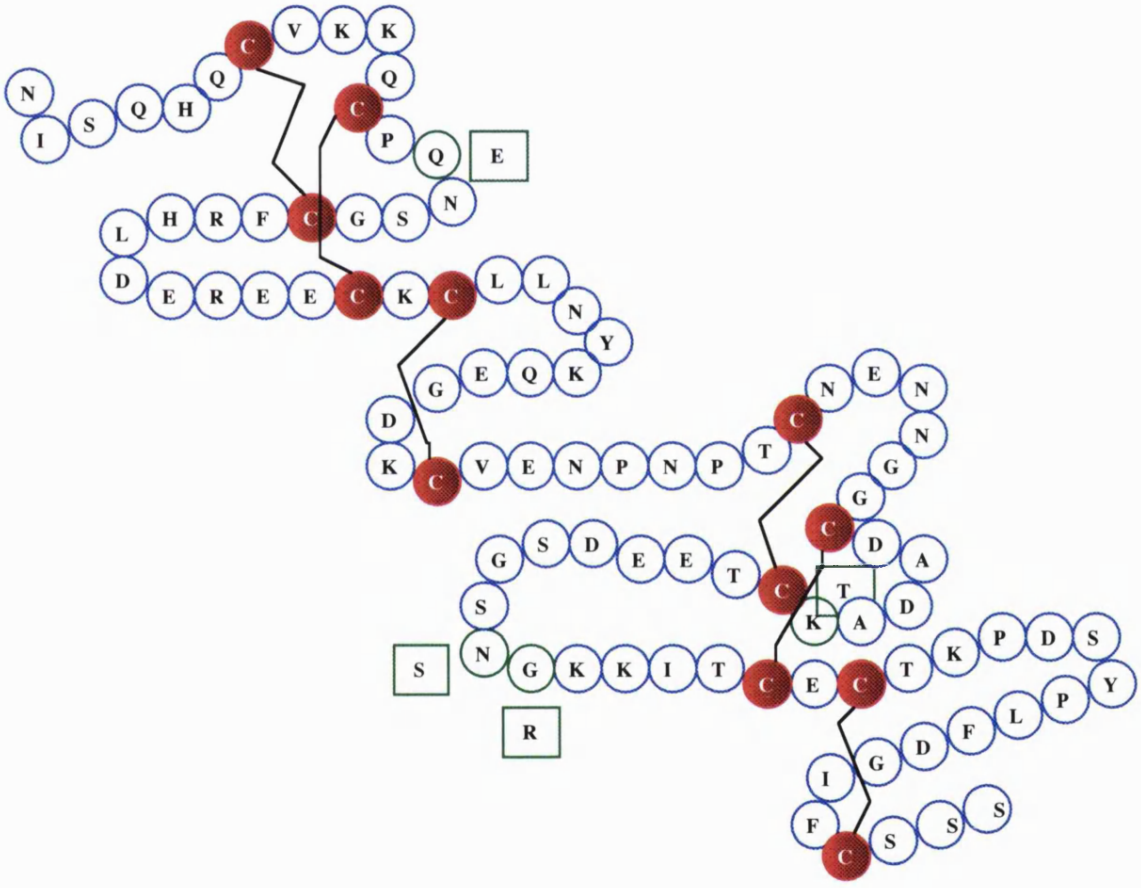

Conserved
Variable
Semi-conserved
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
MSP-1 83
MSP-1 30
MSP-1 38
MSP-1 42
NTQC VTHE
VSAN DDTS
VTGE AVTP
MSP-1 19
QGML NISQ

Figure 4.

Proposed EGF-like structure of MSP-1_{19}.

$MSP1_{19}$ is a highly folded structure maintained by disulphide bonds and containing 12 of the 19 cysteine residues of the all MSP1 sequence and is considered structurally as two contiguous epidermal growth factor-like domains (Blackman *et al.*, 1991). Cysteines are paired in disulphides (shown as solid lines); C1 to C3, C2 to C4 and C5 to C6. In the first EGF-like the motif of *P. falciparum* MSP-1, only one amino acid varies between strains; Q_{1641} with E. In the second EGF-like domain three amino acids vary; K_{1688} with T, N_{1497} with S, and G_{1498} with R.

Figure 4.


C V K K
N Q Q
I S Q H C
P Q E
L H R F C G S N
D E R E E C K C L L N Y
G E Q K
D
K C V E N P N P T C
N E N
N
G
G
C D A
G S D E E T C T
S K A D
S N G K K I T C E C T K P D S
S
R
L P Y
D F
G
I
F C S S S

CHAPTER 2.

MATERIAL AND METHODS.

2.1 Polyclonal and monoclonal antibodies.

Murine anti-MSP-1_{19} mAbs 2.2, 7.5, 12.8, 12.10, 111.4, 117.2, 1E1, 2F10, 7E5, 8A12, and 12D11; the anti-MSP-1_{83} mAb 89.1 and the mAb 25.1, which is specific for *P. yoelii* MSP-1; and the human anti-MSP-1_{33} mAb X509, have all been described previously (McBride and Heidrich, 1987; Blackman *et al.*, 1990; 1991; 1994a; Burghaus and Holder, 1994; Holder and Freeman, 1981). All mAbs were purified by affinity chromatography on Protein A or Protein G Sepharose (Pharmacia, U. K.) before use (Harlow and Lane, 1988). A panel of polyclonal anti-MSP-1 antisera was a kind gift of Dr. A. Holder; the sera were raised in rabbits against defined regions of MSP-1 expressed as fusion proteins in *E. coli* (Holder *et al.*, 1987). IgG was purified from these sera by ion exchange chromatography on DEAE Sephadex (Pharmacia, U.K.) using standard methods (Harlow and Lane, 1988). A polyclonal rabbit antiserum reactive with the MSP-1_{33} fragment of the Wellcome MSP-1 (Rb anti-MSP-1_{33}) was raised against a recombinant protein expressing a 93 amino acid region from within the N-terminal half of MSP-1_{42}; the antibodies therefore recognise both MSP-1_{42} and MSP-1_{33}, and show absolutely no reactivity with MSP-1_{19} (Blackman *et al.*, 1993a). Pooled human serum obtained from adult Gambian donors clinically immune to *falciparum* malaria was a kind gift of Dr. H. Whittle (Medical Research Council Laboratories, Fajara, The Gambia, West Africa).

Human serum from European donors never exposed to malaria (non-immune sera) was obtained from the U.K. Blood Transfusion Service and pooled. Sera from vaccinated primates were obtained from Dr. D. Kaslow (Washington University), Dr. S. Chang (Hawaii University), and Dr. A. Holder (NIMR).

2. 2 SDS PAGE and visualisation of proteins.

Samples were solubilised by boiling in SDS sample buffer; this is 0.125 M Tris-HCl pH 6.8, glycerol 20% (v/v), sodium dodecyl sulphate (SDS) 4.6% (w/v), bromophenol blue 0.01% (w/v), with or without 0.1 M DTT as reducing agent. Proteins were fractionated on polyacrylamide gels using two different methods; either homogeneous 15% and 12.5% polyacrylamide gels were run by the method of Laemmli (1970); alternatively, for optimal resolution, polypeptides were electrophoresed using the Tricine-SDS PAGE method of Shagger and von Jagow, (1987). Depending on the requirements of the experiment, molecular weight markers used were obtained from Pharmacia (low molecular weight; 14,400-94,000 Daltons) or Gibco BRL (high molecular weight prestained; 14,300-200,000 Daltons).

Following electrophoresis, proteins were visualised by either staining with Coomassie blue (Coomassie Brilliant Blue R-250; Sigma), or by western blotting (immunoblotting).

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

P. falciparum was maintained *in vitro* in human A+ erythrocytes at a 1-2% haematocrit in RPMI 1640 medium supplemented with 25 mM HEPES, 25 mM $NaHCO_3$, 0.2% (w/v) glucose, 25 μg ml^{-1} gentamicin, 20 μg ml^{-1} hyponxanthine (complete medium) and 0.5% (w/v) Albumax™ (Gibco BRL). Cultures were gassed with a mixture containing 7% CO_2, 5% O_2, 88% N_2 and incubated at 37 °C. The medium was replaced daily. Cultures were resuspended, transferred to 250 ml conical-bottomed polypropylene centrifuge tubes (Corning 25350-250), and pelleted in a Beckman J-6B centrifuge at 2,200 rpm for 5 minutes. The supernatant was aspirated and the pelleted cells were resuspended and returned to culture using 175 cm^2 (800 ml) Nunclon flasks (Nunc), using 100 ml of culture per flask at a parasitaemia of 10-15%. Parasitaemias were monitored by taking smears of cultures each day. These were air dried, fixed in 100% methanol and stained for 10 minutes with 10% (v/v) Giemsa in phosphate buffer pH 7.2.

2.4 Synchronisation of *P. falciparum* cultures. (Blackman *et al.*, 1990)

The following methodology is a combination of flotation on gelatin (Pasvol *et al.*, 1978) and disruption with sorbitol (Lambros and Vanderberg, 1979). Parasites cultures were routinely synchronised once weekly in order to maintain synchrony. The parasites were synchronised at 48 hour intervals (i.e. once per cycle) until four days before merozoite isolation.

Cultures containing mature, segmented schizonts were pelleted in a Beckman J-6B centrifuge at 2,200 rpm for 5 minutes at room temperature, and the supernatant discarded. Up to 5 ml of packed cells were resuspended in 30-40 ml of warm complete medium plus 10% decomplemented human A+ serum. Cells were transferred to a 50 ml polypropylene screw-cap tube and pelleted again at 560 g for 5 minutes on a bench centrifuge. The volume of the pellet was estimated, then multiplied by 2.4 and warm medium added to the tube to bring the total volume up to the calculated figure. A volume of warm Plasmagel equal to the total volume of cells and medium, was then added to the tube. The contents of the tube were carefully but completely resuspended and transferred to a fresh tube, sealed without gassing, and placed in a 37 °C incubator for 30 minutes without disturbing.

At this stage two phases were visible; the top, brownish layer containing mature forms of the parasite, whilst the lower contained uninfected red cells and immature forms of the parasite. 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 450 g for 5 minutes on a bench centrifuge (Labofuge M, Hearaus). The pellet was carefully resuspended in fresh medium and added to a culture flask containing fresh medium and fresh, washed red cells added such that the parasitaemia was 2-20% and the final haematocrit was 20-25%. The flask was gassed and incubated without disturbing at 37°C for 3-4 hours. During this period schizont rupture and erythrocyte reinvasion occurred.

Following the incubation step, the culture was treated with sorbitol to remove residual schizonts. The cells were pelleted at 560 g for 5 minutes, the supernatant was aspirated, and the pellet resuspended into at least 10 volumes of 5% (w/v) sorbitol in water. The suspension was left for 10 minutes at room temperature, then pelleted at 560 g for 5 minutes, washed once in serum free medium and recultured. Cultures treated in this way contained only ring forms of the parasite.

2.5 Purification of schizonts and naturally released merozoites.

Mature schizonts from synchronous cultures were purified on Plasmagel as described above (Blackman *et al.*, 1990, 1994b). The schizonts were 40-44 hours old, containing about 8 nuclei. The parasitaemia of such enriched preparations is at least 80%. The schizonts were recultured in warm complete medium at a 0.25% haematocrit, changing the medium at 4 hours intervals until merozoites release began; this was assessed by making Giemsa-stained smears of the culture at 1-2 hours intervals.

Cultures were transferred to 50 ml tubes and centrifuged at 440 g for 6 minutes at room temperature to pellet the schizonts. The supernatant from the pelleted culture, which contained free merozoites, was passed under negative pressure through first a 3 μm pore size and then a 1.2 μm pore size acrylic supported membrane filter, held in filter units, (Gelman Sciences). Filter units used were Sterifil System filter units (47 mm), obtained from Millipore. The schizont pellet was immediately resuspended in warm complete medium and recultured for further merozoite production. The filtered supernatant was

transferred to 50 ml flanged polycarbonate tubes (Beckman) and centrifuged in a Beckman HB-4 swing out rotor at 3000 g for 10 minutes at 4 °C. The supernatant was aspirated from the tubes, and the merozoite pellet resuspended in ice-cold phosphate-buffered saline pH 7.2, Ca^{2+} and Mg^{2+} free, supplemented with 2 mM EGTA, and protease inhibitors (leupeptin, 10 μg ml^{-1}; antipain, 10 μg ml^{-1}; aprotinin, 10 μg ml^{-1}; and tosyl-L-lysyl chloromethyl ketone [TLCK] at 74 μg ml^{-1}). For use in MSP-1 processing assays, the merozoites were stored in aliquots at -70 °C. Examination by light microscope of Giemsa-stained smears showed that the purified merozoite preparations were morphologically intact in the complete absence of contaminating schizonts or erythrocytes, although some contamination with hemozoin was often evident.

2.6 Biosynthetic radiolabelling of *P. falciparum.*

When required, schizont-enriched cultures were metabolically radiolabeled with [^{35}S]methionine and cysteine (Pro-mix™, Amersham U.K.), placed back into culture in medium containing 0.5% (w/v) Albumax™ (GIBCO BRL, U.K.), and allowed to undergo merozoite release in the presence of fresh erythrocytes as described (Blackman *et al.*, 1991). Labeled MSP-1_{33} was immunoprecipitated from harvested culture medium using mAb X509 coupled to Sepharose, and analysed by SDS PAGE and fluorography as described by Blackman *et al.* (1991; 1992). When appropriate, ring-stage parasitemia in cultures following reinvasion was assessed by microscopic examination of Giemsa-stained thin blood films.

2.7 Quantitation of antibody-mediated inhibition of MSP-1 secondary processing.

Analysis and quantitation of secondary processing of MSP-1 in merozoite preparations was by a modification of an assay described previously (Blackman *et al.*, 1993, 1994a). Washed merozoites were resuspended in ice-cold 50 mM Tris-HCl pH 7.5 containing 10 mM $CaCl_2$ and 2 mM $MgCl_2$, supplemented with protease inhibitors antipain, leupeptin, aprotinin and TLCK (reaction buffer). Aliquots of about 1 x 10^9 merozoites were dispensed into 1.4 ml Eppendorf tubes on ice, and the parasites pelleted in a microfuge at 12,000 g for 2 minutes at 4 °C. The buffer was aspirated, and individual merozoite pellets were then resuspended on ice in 20 µl of reaction buffer, further supplemented with protease inhibitors or antibodies as appropriate. Merozoites were maintained on ice for 15 minutes to allow antibody binding, then transferred to a 37 °C water bath for one hour to allow processing to proceed. Assays always included the following controls; a "positive processing" control sample of merozoites, resuspended in reaction buffer only; a negative "no processing" sample of merozoites, resuspended in reaction buffer plus 1 mM PMSF; and a zero time (0h) control, in which processing was immediately stopped before the 37 °C incubation step by the addition of an equal volume of 2% (v/v) Nonidet P40 (NP40; BDH, U.K.) Samples were vortexed and extracted on ice for 1 hour, then centrifuged for 15 minutes at 12,000 g. The supernatant was removed to a new tube containing an equal volume of 2 x SDS PAGE sample buffer, and 5-20 µl of each sample was subjected to electrophoresis under non-reducing conditions on 12.5% or 15% polyacrylamide minigels (Hoefer, U.K.), before being transferred electrophoretically

to nitrocellulose (NCP) (Schleicher and Schuell, U.K., 0.2 μm pore size). Blots were blocked in PBS containing 7% (w/v) BSA and probed with a 1/100 dilution of Rb anti-MSP-1_{33}. After washing three times in PBS containing 0.05% (v/v) Tween-20 (PBS/T), bound antibody was detected by further incubation with radioiodinated anti-rabbit IgG. Blots were washed for 1 hour with several changes of PBS/T, then dried. Bands on the blot corresponding to MSP-1_{33} and MSP-1_{42} were visualised by autoradiography; direct quantitation of the radioactivity associated with these bands was then performed by excising the appropriate regions from the blots and measuring the associated radioactivity (in cpm) in a gamma counter. Merozoite samples were routinely assayed in triplicate, and results were expressed as mean percentage MSP-1_{42} processing, using the formula [{X-B}/{A-B} x 100], where A was the mean amount of MSP-1_{33} (in cpm) in control samples incubated in reaction buffer alone, B was the mean amount of MSP-1_{33} in the 0h control (i.e. background levels of MSP-1_{33} present at the start of the assay), and X was the mean amount of MSP-1_{33} produced in the presence of the antibody under test or protease inhibitor. Alternatively, for standard visualisation of MSP-1_{42} and MSP-1_{33} following incubation with either rabbit anti serum anti MSP-1_{33} (MSP-1 Wellcome-like allele) or mAb X509 specific for an epitope within MSP-1_{33} (MSP-1 MAD20-like allele), bound antibody was detected using horseradish peroxidase-conjugated (HRP)-rabbit anti-mouse IgG or HRP-mouse anti-rabbit IgG, or HRP-rabbit anti-human IgG (Sigma, U.K.) as appropriate. Blots were developed in the presence of 4-chloro napthol substrate for 15 minutes.

2.8 Pepsin digestion of mAb 12.10 to produce $F(ab')_2$.

Monoclonal antibody 12.10 was digested with pepsin according to the method of Nisonoff *et al.* (1975). An appropriate amount of a 1 M citrate buffer, pH 3.5 was added to 5 mg of mAb 12.10 to give a final concentration of 0.1 M. Lyophilised pepsin was dissolved at 1 mg ml^{-1} in 0.1 M citrate, pH 3.5 and added to the antibody solution to a final pepsin concentration of 25 μg ml^{-1}. Following incubation for 6 hours at 37 °C, the reaction was halted by raising the pH to 8.0 by the addition of 3 M Tris-HCl, pH 8.6. The incubated preparation was then extensively dialysed against PBS. Digestion was monitored by SDS PAGE under non-reducing conditions.

2.9 Reduction and alkylation of $F(ab')_2$ to produce Fab.

Mild conditions of reduction with cysteine were used to break the inter-heavy chain disulphide bonds and leave intact the disulphide between the antibody heavy and light chain. For the reduction and alkylation of $F(ab')_2$ to Fab fragments, a 0.1 M solution of cysteine base (Sigma) in 0.1 M Tris-HCl, pH 7.5 and a 0.1 M solution of iodoacetamide (Calbiochem) in Tris-HCl, pH 7.5 were made just before use. Cysteine was added to $F(ab')_2$ to a final concentration of 10 mM, mixed and incubated for 2 hours at 37 °C. Then iodoacetamide was added to a final concentration of 150 mM and incubated for 30 minutes at room temperature. The mixture was then desalted by gel filtration on a PD-

10 column (Pharmacia), pre-equilibrated with PBS. The sample was then concentrated by ultrafiltration to a volume of 5 ml using a Centricon 10, and the antibody fragments were separated by size exclusion chromatography on a 2.6 x 77 cm column of Sephacryl S-200 HR equilibrated in PBS containing 0.02% NaN_3. The column was run at room temperature at a flow rate of 30 ml h^{-1} and 10 ml fractions collected.

2.10 Preparation of recombinant antigens.

Production of a recombinant pGEX-3X plasmid (Smith and Johnson, 1988) to express the MSP-1_{19} domain of the *P. falciparum* (Wellcome strain) MSP-1 fused to *Schistosoma japonicum* glutathione S-transferase has been described previously (Burghaus and Holder, 1994). Fusion protein (GST-MSP-1_{19}) was adsorbed to glutathione agarose (Sigma), and the malarial portion (rMSP-1_{19}) cleaved *in situ* from the carrier protein (Abath and Simpson, 1990) by overnight incubation with Factor Xa (Boehringer Mannheim) at 4 °C. Eluted protein was further purified by gel filtration in PBS on Sephadex G50 Superfine (Pharmacia), and concentrated by ultrafiltration using a YM1 membrane (Amicon, U.K.).

Recombinant expression plasmid pME6 encodes Leu_{208} to Asp_{416} of the *P. falciparum* Wellcome strain MSP-1 gene (numbering according to Miller *et al.*, 1993), as an N-terminal fusion with β-galactosidase (Holder *et al.*, 1987). Fusion protein (also referred to as pME6; Holder *et al.*, 1987) was purified by affinity chromatography on *p*-

aminophenyl-β-D-thiogalactopyranoside-agarose (Steers *et al.*, 1971) and stored as a precipitate in 50% (w/v) ammonium sulphate.

2.11 Coupling GST-MSP-1_{19} to the sensor chip CM5.

GST-MSP-1_{19} was used to coat a carboxymethyl dextran hydrogen sensor chip (Pharmacia Biacore) by the followed methodology. The binding of the GST-MSP-1_{19} to the carboxymethyl dextran hydrogen layer is via its amino groups, the binding being aided by electrostatic attraction between the negatively charged carboxyl groups on the dextran and the positively charged protein, using EDC/NHS chemistry (Buckle *et al.*, 1993). Immobilisation was done with the amine coupling kit (Pharmacia Biacore). The CM5 dextran surface was activated with 35 µl of 200 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 5 mM N-hydroxysuccinimide (NHS) (Pharmacia Biacore) for 7 minutes. GST-MSP-1_{19} was then coupled to the BIAcore sensor surface, using 20 µl of a solution at 100 µg ml^{-1} in coating buffer (0.01 M sodium acetate buffer, pH 3.5) for 4 minutes. Unreacted carboxyl groups were blocked by adding 35 µl of 1 M ethanolamine, pH 8.5 for 7 minutes. The cells were washed with two pulses of 20 µl of 10 mM glycine-HCl buffer pH 2.8 for 8 minutes in total to remove any non-covalently bound protein. The immobilisation procedure was carried out at a flow rate of 5 µl min^{-1}. Measurements were performed on the Biacore 2000 instrument (Pharmacia Biacore) with a CM5 sensor chip (certified). 8300 resonance units response was seen, following coupling of the GST-MSP-1_{19}. According to the manufacturer's guidelines (Pharmacia), this corresponds to immobilisation of approximately 8.3 ng protein mm^{-2}.

2.12 Binding of mAb 12.10, or its F(ab′)$_2$ and Fab fragments to immobilised GST-MSP-1$_{19}$.

The binding assays were performed with a constant flow rate of 5 ml min^{-1} at 25 °C. The mAb 12.10 and its F(ab′)$_2$ and Fab fragments were used at equimolar concentrations with respect to the valency of the fragment. For binding, mAb 12.10 at 160 μg ml^{-1} final concentration and F(ab′)$_2$ and Fab at 100 μg ml^{-1} final concentrations in HBS were allowed to interact with the immobilised GST-MSP-1$_{19}$ for 2 minutes, then dissociation was followed for a further 15 minutes. In order to study the ability of F(ab′)$_2$ and Fab fragments to prevent the binding of mAb 12.10 to the immobilised GST-MSP-1$_{19}$, F(ab′)$_2$ or Fab fragments were bound to the immobilised GST-MSP-1$_{19}$, then mAb 12.10 was added and its binding measured. The regeneration steps were performed with 20 μl of 10 mM glycine-HCl buffer pH 2.8, for 4 minutes.

2.13 Determination and evaluation of kinetic binding constants of mAb 12.10 and its F(ab′)$_2$ and Fab fragments to immobilised GST-MSP-1$_{19}$ by Biacore.

Association and dissociation binding kinetics for mAb 12.10 and its F(ab′)$_2$ and Fab fragments were analysed using the Bia-analysis software (Biacore, Pharmacia). Areas of the graph (analysis regions) covering the base line, association and dissociation phase of the reaction were selected. The association analysis region was selected such that the first point was five seconds after the addition of the antibody sample. The end of the association analysis region was selected at the end of the 15 minutes of binding. The

dissociation analysis region was started approximately five seconds after the end of the addition of antibody sample and ended immediately before the regeneration of the flow cells.

In a pseudo first order model, the dissociation phase is described by $dR/dt=-K_{diss}R$.

An association phase, assuming an homogeneous 1:1 interaction on the sensor chip surface may be described by the equation:

$$A + B \underset{k_{off}}{\overset{k_{ass}}{\rightleftharpoons}} AB$$

were A is the analyte (mAb or fragments) and B is the surface-bound ligand (recombinant MSP-1_{19}). Assuming pseudo-first order interaction kinetics, the rate of complex formation during sample injection in given by $d[AB]/dt=K_{ass}[A][B]-[AB]$, which may be expressed in terms of the surface plasmon resonance signal (SPR) as $dR/dt=K_{ass}CR_{max}-(K_{as}C+K_{diss})R$, where dR/dt is the rate of change of the SPR signal, C is the concentration of analyte, R_{max} is the maximum analyte binding capacity in resonance units (RU) and R is the SPR signal in RU at time t.

2.14 Radioiodination of antibodies.

Protein G-purified mAbs 12.8, 12.10, and X509, and purified rabbit anti-IgG antibodies (Sera-Lab, U.K.) were labelled at 4 °C with ^{125}I by the Iodogen (Pierce)

method (Fraker, and Speck, 1978). Labelled antibody was separated from free isotope by gel filtration on a PD-10 column (Pharmacia) pre-equilibrated with PBS containing 1% BSA and 0.02% (w/v) sodium azide. The specific activity of the labelled antibody was approximately 3.1×10^6 cpm μg^{-1}. Labelled antibody was stored at 4 °C.

2.15 Preparation of merozoite antigen sonicate for immunoassays.

This study investigated recognition by antibodies of MSP-1 in the form in which it exists on the surface of the free merozoite. Since the MSP-1 precursor undergoes proteolytic modification at or before merozoite release, possibly resulting in conformational differences between the precursor molecule and the merozoite surface complex, it was decided to avoid the use of detergent-solubilised precursor protein for experiments exploring the mechanisms involved in blocking antibody activity, and to use merozoite-derived, non-detergent solubilised antigen instead. Purified merozoites were suspended on ice in 0.1 M carbonate/bicarbonate buffer, pH 9.6, 0.02% (w/v) sodium azide (coating buffer), containing the protease inhibitors leupeptin, antipain, TLCK, and 1 mM PMSF. The suspension was sonicated in a Kerry KS 1000 sonicating waterbath for 1 minute, centrifuged at 12,000 g for 15 minutes, and then the resulting supernatant was diluted further (usually 100-fold) in coating buffer before being used to coat ELISA or radioimmune assay plates.

2.16 ELISA.

An ELISA was used to titrate the binding of antibodies to native or recombinant MSP-1 and to determine saturating antibody concentrations under these conditions. Serially diluted mAbs, rabbit antibodies or human antibodies were added to ELISA plates (Immulon 4, Dynatech, U.K.) coated with an optimal concentration of purified rMSP-1_{19} or merozoite antigen sonicate. Bound antibody was detected with HRP-rabbit anti-mouse IgG or HRP-mouse anti-rabbit IgG, or HRP-rabbit anti-human IgG (Sigma, U.K.) as appropriate. Assays were otherwise performed and developed as described (Blackman and Holder, 1993). In preliminary experiments, titration curves obtained using anti-MSP-1_{19} mAbs in the two ELISA systems (rMSP-1_{19} and merozoite sonicate) were indistinguishable.

2.17 Competitive radioimmune assay (RIA).

A competitive solid-phase RIA was used to determine whether or not anti-MSP-1 mAbs or rabbit antibodies could competitively block the binding of processing-inhibitory mAbs 12.8 and 12.10 to their epitopes. Wells of PVC microtiter plates (Falcon, Becton Dickinson) were coated overnight at 4 °C with 100 µl of merozoite antigen sonicate, or rMSP-1_{19} at a final concentration of 10 µg ml^{-1} in coating buffer. Plates were then washed 3 times in PBS/T and treated overnight at 4 °C with PBS/T containing 1% (w/v) bovine serum albumin (PBS/T/BSA). The plates were then washed and 50 µl PBS/T/BSA containing serum or purified antibody at a saturating concentration (predetermined by

ELISA; see above) was added to wells in triplicate. Plates were incubated for 2 hours at room temperature, then washed again, and 50 µl of optimally diluted radioiodinated mAb 12.8 or 12.10 was added in PBS/T/BSA. Optimal concentrations of radiolabeled mAbs were determined in preliminary radioimmune titration assays; the concentration of radiolabeled mAbs finally used in the competitive RIAs corresponded to those in the linear part of the dose response curve, so that changes in 12.8 and 12.10 binding in the presence of blocking antibodies would be readily apparent. Plates were incubated for a further 2 hours at room temperature, then washed as before. Individual wells were excised and counted for 1 minute in a gamma counter. Samples were routinely assayed in triplicate, and the binding of radiolabeled mAbs was expressed as a percentage of that obtained in the absence of pre-treatment of wells.

2.18 Affinity purification of human antibodies reactive with pME6.

Purified pME6 protein was bound to cyanogen bromide-activated Sepharose 4B (Pharmacia) at 5 mg ml^{-1} swollen gel according to the manufacturer's instructions. Thirty millilitres of pooled serum derived from adult Gambian donors was diluted 1:4 in 50 mM Tris-HCl pH 8.0 containing 0.02% (w/v) sodium azide, clarified by passage through a 0.45 µm filter, then passed over a 5 ml affinity column at a flow rate of 10 ml h^{-1}. The column was washed extensively in 50 mM Tris-HCl pH 8.0, and bound Ig eluted in the same buffer containing 8 M urea. Samples of eluate fractions were subjected to SDS PAGE under reducing conditions, and assessed for the presence and purity of IgG by examination of Coomassie blue stained gels. Peak fractions were pooled, dialyzed

exhaustively against PBS, concentrated in an ultrafiltration cell using an XM10 membrane (Amicon), and stored at 4 °C. Yield of IgG was quantified by spectrometry assuming an A_{280} for human IgG of 1.4 at 1.0 mg ml^{-1} (1 cm path length).

2.19 Lectin-binding analysis of the *P. falciparum* MSP-1_{19}

Washed merozoites were resuspended in 20 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$, 2 mM $MgCl_2$, in the presence of protease inhibitors as described before, then incubated for 1 hour at 37 °C. Immediately after incubation samples were resuspended in an equal volume of NP-40, incubated for 1 hour on ice, and then solubilised in SDS sample buffer and boiled for 5 minutes. 15 µl of the extract was loaded onto tracks of 15% SDS minigels and subjected to SDS PAGE under non-reducing conditions. Proteins were transferred onto NCP and the membrane was blocked with 0.1% Tween 20 in PBS overnight at 4 °C. Non-stained high molecular markers were used as glycosylation controls. Membranes were incubated in the presence of biotinylated lectins jacalin, lycopersicon, *Solanum tuberosum*, *Vicia villosa*, *Bandereira*, and *Datura stramonium* (Vector). Lectin binding was detected following addition HRP-streptavidin (Pierce) at a 1:2000 dilution in 0.1% Tween 20 in PBS. Blots were developed in the presence of 4-chloro napthol substrate for 15 minutes.

2.20 Proteolytic digestion of recombinant MSP-1_{19}.

Five μg samples of recombinant MSP-1_{19} dissolved in 1M Tris-HCl pH 8.0 were incubated at room temperature for 30 minutes, in the presence of an empirically determined amount of the proteases pepsin, trypsin, and chymotrypsin (Boehringer) in a total volume of 12 μl. Reactions were halted by the addition of 1 mM PMSF and non-reducing SDS sample buffer. Pronase in the range of 0.002-0.01U, and chymotrypsin or trypsin in the range 0.2-1U were used (U; activity units as described by the manufacturer). Equivalent samples of digestion products were subjected to SDS PAGE under reducing conditions on SDS-Tricine gels. The gels were fixed in a solution containing 50% methanol and 10% acetic acid for 30 minutes, before they were stained with 0.025% Serva blue G (Serva) in 10% acetic acid for 1 hour. Destaining was achieved by shaking the gels in 10% acetic acid for 1 hour, then gels were dried at 60 °C.

CHAPTER 3.

SECONDARY PROCESSING OF *P. FALCIPARUM* MSP-1 IS ESSENTIAL FOR SUCCESSFUL ERYTHROCYTE INVASION.

3.1 Introduction.

In previous work by Blackman *et al.* (1994a) designed to study the ability of a panel of antibodies to inhibit the secondary processing of *P. falciparum* MSP-1 and erythrocyte invasion, it has been shown that within a panel, mAb 12.8 and mAb 12.10, specific for epitopes within MSP-1_{19}, have the ability of inhibiting secondary processing of MSP-1. Both mAbs are also capable of interfering with erythrocyte invasion by the merozoite. On the basis of these findings, it was postulated that MSP-1 secondary processing is a pre-requisite for erythrocyte invasion to occur and that both mAbs block erythrocyte invasion by inhibiting MSP-1 secondary processing. The mechanisms involved in this invasion inhibitory activity were not completely identified, however. It is possible that processing-inhibitory antibodies may elicit this, "protective activity" by effectively interfering with the proteolytic activity of the protease responsible for the secondary processing of MSP-1, by means of steric hindrance. Alternatively, it is conceivable that the ability of the mAbs to interfere with erythrocyte invasion could be due to a disrupted RBC receptor-ligand interaction which may be required for invasion; binding of the mAbs to (an) epitope(s) involved in recognition of a putative RBC receptor might prevent

successful erythrocyte invasion. The aim of this section of the present study was to elucidate the mechanism by which antibodies against epitopes within *P. falciparum* MSP-1_{19} block erythrocyte invasion by the merozoite.

For this purpose mAb 12.10 was enzymatically and chemically modified in an attempt to reduce its size without affecting its binding properties. The Fc domain of mAb 12.10 was removed by pepsin digestion according to the method of Nisonoff *et al.* (1975), yielding a $F(ab')_2$ molecule, which was then reduced and alkylated under mild conditions to obtain Fab fragments. The intact mAb 12.10, its corresponding $F(ab')_2$, and the Fab fragments were purified by size exclusion chromatography, then evaluated by SDS PAGE for purity and correct size and by surface plasmon resonance analysis to determine binding properties. The preparations were then tested for their ability to inhibit MSP-1 secondary processing and erythrocyte invasion. The $F(ab')_2$ and the Fab fragments of mAb 12.10 were shown to have the expected molecular mass and retained their binding properties, but both failed to significantly inhibit either MSP-1 secondary processing or erythrocyte invasion.

3.2 Results.

3.2.1 Visualisation of antibody-mediated inhibition of MSP-1 secondary processing.

A panel of anti-MSP-1_{19} mAbs were assayed for processing-inhibitory activity. Purified merozoites were washed, incubated on ice with individual mAbs, then transferred to 37 °C to allow processing to occur. The merozoite suspension was then solubilised in SDS and subjected to SDS PAGE under non reducing conditions. Following transfer to NCP, visualisation of MSP-1_{42} and MSP-1_{33} was carried out using a rabbit polyclonal anti- MSP-1_{33} serum, followed by a HRP-labelled anti-rabbit antibody. Figure 5 shows, in accordance with Blackman *et al.* (1994a) that of the ten mAbs assayed only mAb 12.8 and 12.10 inhibited MSP-1 secondary processing. Mab 1E1 appeared to induce abnormal processing rather than preventing the processing.

3.2.2 Production of active F(ab′)$_2$ and Fab fragments from mAb 12.10.

Pepsin digestion of mAb 12.10 to form F(ab')$_2$ fragments was followed by SDS PAGE, as shown in Figure 6A. Figure 6B shows an analysis of the products by SDS PAGE; under non-reducing conditions (in the absence of DTT), mAb 12.10 and the F(ab′)$_2$ were observed as single bands of apparent m.w. ~ 150,000 and ~ 120,000 Daltons respectively (tracks 1 and 2). Under reducing conditions (in the presence of DTT) the heavy chain of mAb 12.10 migrates with an apparent m.w. of 50,000-60,000 Daltons and a light chain band of approximately 25,000 Daltons (track 4). Reduction of F(ab′)$_2$

produced a light chain band of an identical size to that observed in the reduced intact IgG preparation, and a band corresponding to the truncated heavy chain fragment at a slightly slower mobility than the light chain band (track 5). Mild reduction and alkylation of $F(ab')_2$ produced Fab fragments that migrated under non-reducing conditions as a single band of 50,000 Daltons (track 3), and when reduced produced a pattern identical to reduced $F(ab')_2$ (track 6).

3.2.3 Analysis of binding kinetics of mAb 12.10 and its $F(ab')_2$ and Fab fragments to immobilised GST-MSP-1_{19} by BIAcore.

SDS PAGE analysis of mAb 12.10 and its corresponding fragments following pepsin digestion, reduction and alkylation, demonstrated the purity and correct size of the preparations. It was next decided to meticulously examine the binding properties of mAb 12.10 and its $F(ab')_2$ and Fab fragments by surface plasmon resonance analysis. A recombinant antigen corresponding to the C-terminal part of MSP-1 fused to GST (GST-MSP-1_{19}) was immobilised on the CM5 sensor surface, then mAb 12.10 or its $F(ab')_2$ or Fab fragments were allowed to interact with the immobilised protein individually, under similar conditions and at equimolar concentration with respect to valency, and the degree of binding measured. Kinetic data was obtained and compared using the BIA-analysis software (BIAcore, Pharmacia). Figure 7A, B and C show the actual sensorgram obtained during the analysis. Significant differences were observed between the association phase (given by K_{ass}) of mAb 12.10 and its $F(ab')_2$ and Fab fragments at equimolar concentration with respect to valency. The K_{ass} for $F(ab')_2$ ($7.1 \times 10^5 M^{-1}$ sec^{-1}) was higher that the K_{ass}

for mAb 12.10 (5.5×10^5 M^{-1} sec^{-1}) or Fab (6.7×10^5 M^{-1} sec^{-1}). However, as expected, during the dissociation phase the univalent Fab fragment was found to dissociate from immobilised GST-MSP-1_{19} ($K_{diss} \sim 8.9 \times 10^{-5}$ sec^{-1}) faster than the bivalent mAb 12.10 ($K_{diss} \sim 5.5 \times 10^{-6}$ sec^{-1}) or the bivalent F(ab′)$_2$ ($K_{diss} \sim 4.9 \times 10^{-6}$ sec^{-1}) (Table 1). In a two step competition assay, in which F(ab′)$_2$ or Fab fragments were allowed to bind to immobilised GST-MSP-1_{19} before the addition of mAb 12.10 it was found that either fragment can effectively prevent the binding of mAb 12.10 to the antigen (Figure 8). Indirect immunofluorescence (IFA) analysis additionally demonstrated that mAb 12.10 and its F(ab′)$_2$ and Fab fragments were able to recognise the native MSP-1 present on acetone-fixed strain T9/96 parasites from asynchronous cultures (not shown). These results show that despite the pepsin digestion, reduction and alkylation, the F(ab′)$_2$ and Fab fragments of mAb 12.10 retained their binding properties.

Table 1.

Biacore analysis of kinetics of binding and dissociation of mAb 12.10 and its $F(ab')_2$ and Fab fragments to/from immobilised GST-MSP-1_{19}.

	K_{ass}	K_{diss}	K_d	$t_{1/2}$
	(M^{-1} sec^{-1})	(sec^{-1})	(M)	(sec)
mAb 12.10	5.37×10^{5}	5.5×10^{-6}	1×10^{-11}	126026.7
$F(ab')_2$	7.1×10^{5}	4.9×10^{-6}	6.9×10^{-12}	141458.6
Fab	6.7×10^{5}	8.9×10^{-5}	1.3×10^{-10}	7788.1

K_{ass} is expressed in M^{-1} sec^{-1}, K_{diss} in sec^{-1}, K_d in M and $t_{1/2}$ in sec.

($K_d = K_{diss} / K_{ass}$)

($t_{1/2} = \ln 2 / K_{diss}$)

3.2.4 Analysis of the processing-inhibitory activity of mAb 12.10 and its F(ab′)$_2$ and Fab fragments.

In the previous section the binding properties of mAb 12.10 and its F(ab′)$_2$ and Fab fragments were determined. All the preparations were then tested in the MSP-1 processing assay for their ability to inhibit MSP-1 secondary processing. Figure 9 shows that in the presence of 400 μg ml^{-1} of mAb 12.10 processing was completed inhibited. Interestingly, no processing-inhibitory effect was detected when merozoites were incubated in the presence of equimolar concentrations (250 μg ml^{-1}) of F(ab′)$_2$ or F(ab) fragments. A two step competition assay was carried out in order to demonstrate that F(ab′)$_2$ and Fab fragments actively bind to MSP-1 and can effectively compete with mAb 12.10 for binding. Merozoites were pre-incubated with of F(ab′)$_2$ or Fab (at 250 μg ml^{-1}), and then with mAb 12.10 at 400 μg ml^{-1} (final concentration). It was found that when merozoites were pre-incubated with F(ab′)$_2$ or Fab, the processing-inhibitory activity of mAb 12.10 was significantly reduced, indicating that the F(ab′)$_2$ and Fab can effectively prevent mAb 12.10 from binding to MSP-1$_{19}$, thereby interfering with its processing-inhibitory activity.

3.2.5 Simultaneous analysis of the processing-inhibitory activity and erythrocyte invasion-inhibitory activity of mAb 12.10 and its $F(ab')_2$ and Fab fragments in the *in vitro* invasion parasite assay.

The above results clearly showed that the processing-inhibitory activity mediated by mAb 12.10 can be completely abrogated by enzymatically removing the Fc domain of the antibody. The next set of experiments was designed to investigate the ability of mAb 12.10 and its $F(ab')_2$ and Fab fragments to inhibit MSP-1 processing and to interfere with erythrocyte invasion under conditions in which merozoites are actively invading erythrocytes. For this purpose an *in vitro* invasion parasite assay was used in which conversion of MSP-1_{42} into MSP-1_{33} was quantitated in parallel with erythrocyte invasion.

Biosynthetically radiolabeled T9/96 schizonts were washed and placed in culture with fresh red cells. Merozoite release and red cell invasion was then allowed to proceed in the presence or absence of mAbs 12.10, or its $F(ab')_2$ or Fab fragments. MSP-1 processing in individual samples was then assessed by direct immunoprecipitation of MSP-1_{33} from the culture supernatants using mAb X509 coupled to Sepharose. The efficiency of erythrocyte invasion was also assessed by counting the number of new ring stage parasites formed over the course of the experiment.

Figure 10A and B shows that significant inhibition of both MSP-1 processing and erythrocyte invasion was observed when either EGTA or mAb 12.10 were added to individual microcultures. Surprisingly, neither the $F(ab')_2$ nor Fab preparations were

capable of interfering with MSP-1 processing or erythrocyte invasion. In a competition experiment, in which $F(ab')_2$ and Fab were added at the same time as an equimolar concentration of intact mAb 12.10, it was observed that both fragments can effectively interfere with the processing-inhibitory activity of mAb 12.10 and can also reverse the ability of mAb 12.10 to prevent erythrocyte invasion. These results indicate that modification of an antibody in a manner that abolishes its processing-inhibitory activity but does not affect its ability to bind its epitope, also abolishes its ability to prevent erythrocyte invasion.

3.3 Discussion.

This study provides strong evidence for a causal association between antibody-mediated inhibition of MSP-1 secondary processing, and inhibition of erythrocyte invasion. A processing-inhibitory antibody mAb 12.10 (which recognises a single, conformational epitope within MSP-1_{19}) was converted to its $F(ab')_2$ and Fab fragments. Both preparations, as well as the intact mAb, were then tested for their ability to inhibit secondary processing of MSP-1 and erythrocyte invasion. It was found that removal of the Fc domain of mAb 12.10 simultaneously abrogated both the processing-inhibitory activity and the erythrocyte invasion activity of the antibody.

How can these results be explained?. First, it is possible that following pepsin digestion, reduction and alkylation of mAb 12.10, the $F(ab')_2$ and Fab fragments had lost their ability to recognise their epitope, and thereby their ability to block erythrocyte

invasion and MSP-1 secondary processing. However, both surface plasmon resonance analysis and IFA (not shown) demonstrated that the $F(ab')_2$ and Fab retained their ability to bind to MSP-1_{19}, ruling out this possibility. Secondly, differences between the valence of mAb 12.10 and the Fab might could explain the absence of inhibitory activity in the latter, but would not explain the difference between the bivalent mAb 12.10 and $F(ab')_2$; both molecules have two binding regions. Third, differences in the affinity of the intact mAb 12.10 and the $F(ab')_2$ and Fab fragments could have significantly affected their inhibitory activity. Interestingly, in a study by Thomas *et al.* (1984) it was shown that Fab fragments of inhibitory monoclonal antibodies specific for a *P. knowlesi* merozoite surface antigen inhibited invasion of erythrocytes more effectively than the corresponding intact IgG. In this study it was suggested that this increased activity could be attributable to the size difference between the Fab fragments and IgG,; the smaller size and more rapid diffusion rate of the Fab fragment might allow it to have freer access to the epitope. It was also suggested that the reduced invasion-inhibitory activity intact of IgG compared to Fab and $F(ab')_2$ fragments may be due to a greater electrostatic repulsion between the negatively charged merozoite (Heidrich *et al.*, 1982) and the negatively charged Fc component of the intact antibody. Interestingly, in the present study, similarly to Thomas *et al.* (1984), both the Fab and $F(ab')_2$ fragments were found to exhibit a slightly increased rate of association to GST-MSP-1_{19}. However, in the present study it was found that although the Fab and $F(ab')_2$ fragments showed to have higher association rate (K_{ass}) than mAb 12.10 to the antigen, failed to elicit any inhibitory activity.

The data obtained strongly indicate that those anti-MSP-1_{19} antibodies which interfere with erythrocyte invasion do so by effectively interfering with the proteolytic activity of the protease responsible for the secondary processing of MSP-1, in agreement with the model proposed by Blackman *et al.*, (1990); (1992); (1994a). It is conceivable that by interfering with MSP-1 secondary processing, processing-inhibitory antibodies disrupt an essential MSP-1_{19} (ligand) erythrocyte (receptor) interaction, that is required for erythrocyte invasion. These results clearly demonstrate that secondary processing of MSP-1 is essential for successful erythrocyte invasion.

Figure 5.

Inhibition of MSP-1 secondary processing by anti-MSP-1_{19} mAbs.

Washed FCB-1 merozoites were either immediately detergent solubilised (0h) or incubated for 1 hour at 37 °C in the presence of no antibodies (No Ab), 1 mM PMSF as inhibitor control, or purified mAbs 12.8, 12.10, 1E1, 2.2, 7.5, 111.4, 8A12, 12D11, 7E5 or 117.2, all at a final concentration of 400 μg ml^{-1}. MSP-1 secondary processing in the samples was visualised as described in Materials and Methods.

Figure 5.

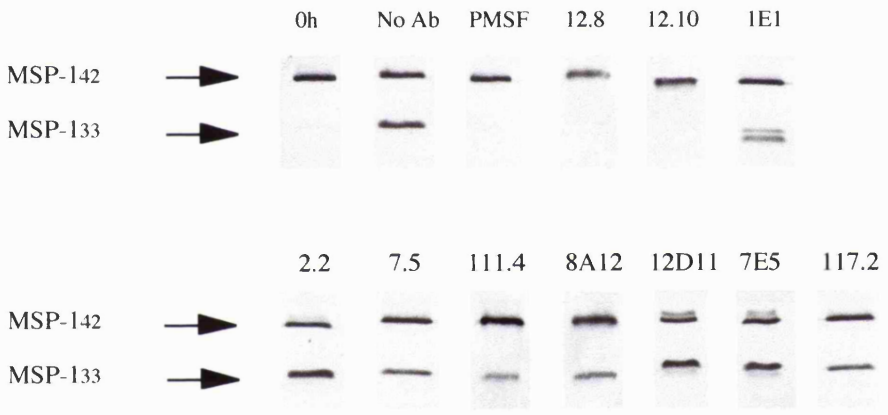

0h
No Ab
PMSF
12.8
12.10
1E1
MSP-142
MSP-133
2.2
7.5
111.4
8A12
12D11
7E5
117.2
MSP-142
MSP-133

Figure 6.

SDS PAGE analysis of the mAb 12.10 and its $F(ab')_2$ and Fab fragments.

Panel A. Pepsin digestion of mAb 12.10 was analysed for 6 hours. Aliquots corresponding to 1 hour intervals were run on a 12.5% SDS PAGE gel under non reducing conditions. **Panel B**. Mab 12.10 and its $F(ab')_2$ and Fab fragments were solubilised in SDS in the presence (tracks 4, 5 and 6) or absence (tracks 1, 2 and 3) of 50 mM DTT and analysed by SDS-PAGE. Molecular weight markers are shown in kDa.

Figure 6.

A

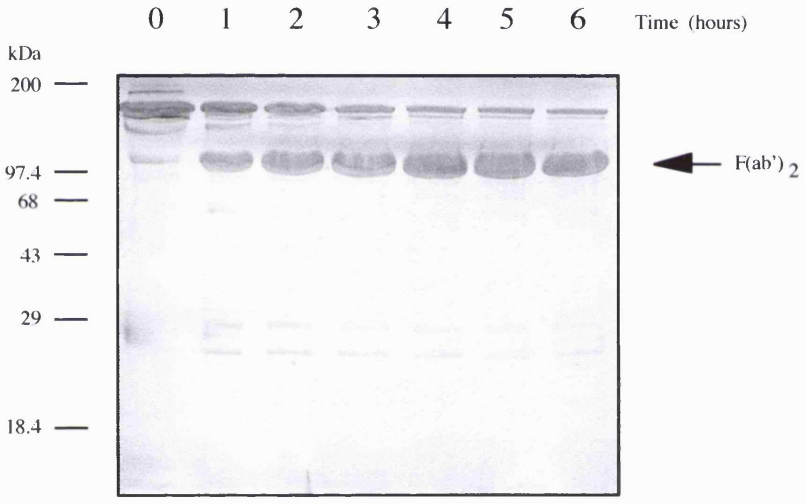


Figure 6.

B

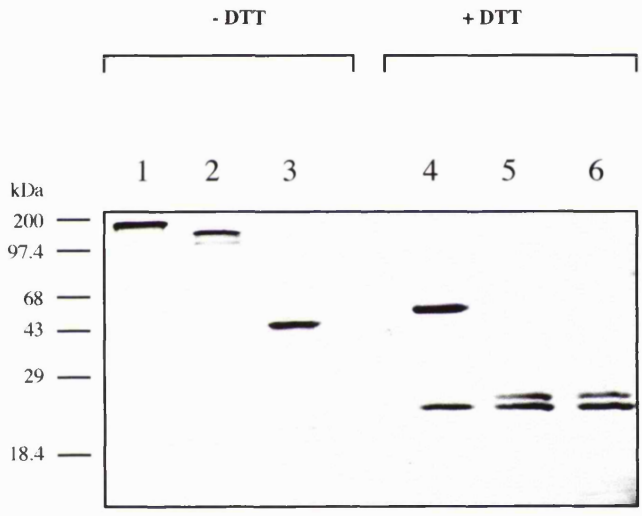


Figure 7.

Binding of mAb 12.10 and its F(ab′)$_2$ and Fab fragments to GST-MSP-1$_{19}$ analysis by BIAcore.

400 μg ml^{-1} mAb 12.10 (curve a) and 250 μg ml^{-1} F(ab′)$_2$ (curve b) and 250 μg ml^{-1} Fab (curve c) fragments were added individually to the cuvette containing immobilised GST-MSP-1$_{19}$. The Y axis represents the response in resonance units (RU), which is directly proportional to the mass bound to the sensor surface; according to the manufacturer's instructions, 1000 RU corresponds to 1 ng protein mm^{-2} of sensor surface. The X axis represents time in seconds. The association phase (ass), shown between red filled circles, was followed until saturation was reached. The dissociation phase, indicated as a blue line (diss) was studied for a further 2 minutes. At the end of this phase the cuvette was regenerated by washing in glycine buffer, pH 2.8.

Figure 7.

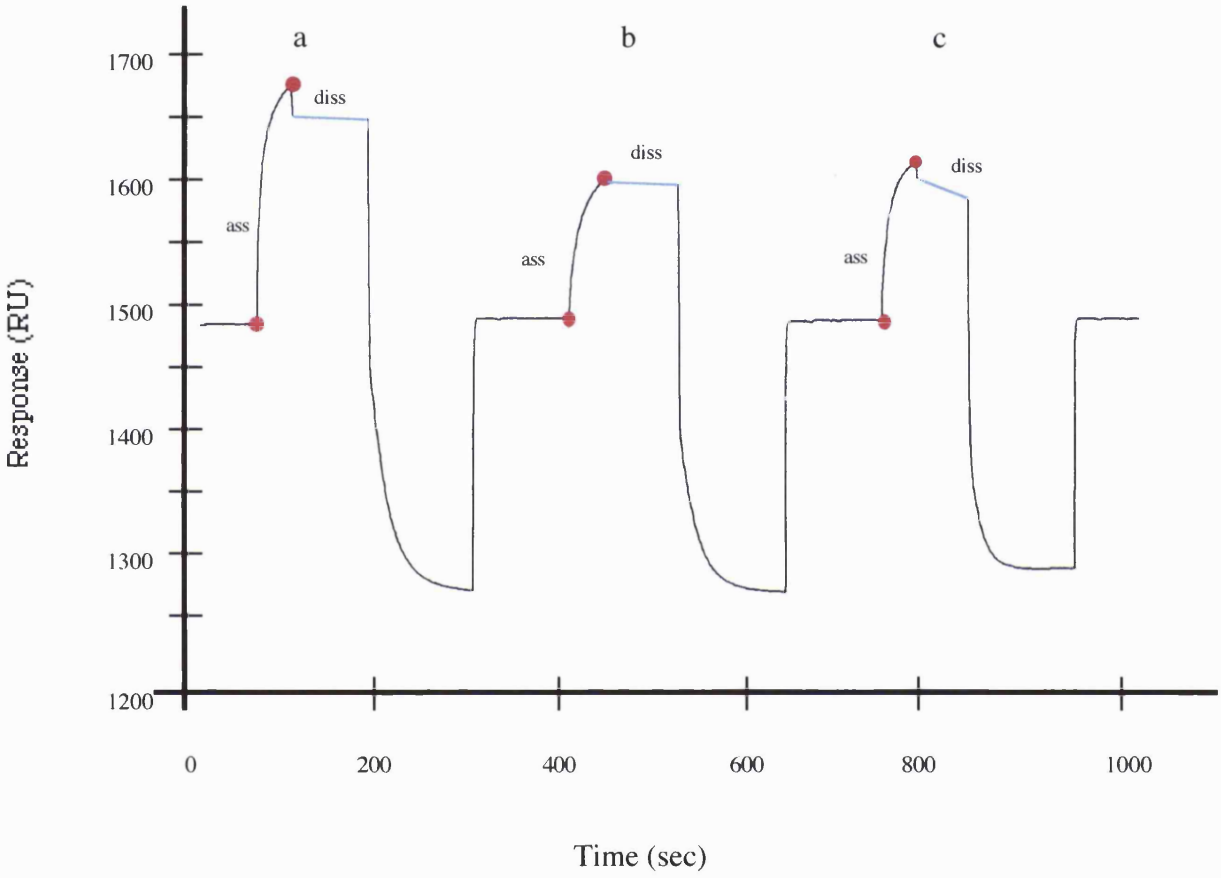

a
b
c
diss
diss
diss
ass
ass
ass
1700
1600
1500
1400
1300
1200
0
200
400
600
800
1000
Response (RU)
Time (sec)

Figure 8.

BIAcore, two step competitive assay.

$F(ab')_2$ (curve a) and Fab (curve b) fragments were added (shown between blue filled circles) to the cuvette to allow binding with GST-MSP-1_{19} until saturation was reached, followed by the addition of mAb 12.10 (shown between red filled circles). Intact mAb 12.10 binding was calculated as the difference between $F(ab')_2$ and Fab (filled green circle) maximum binding and mAb 12.10 maximum binding (filled black circle). The Y axis indicates response in resonance units (RU), and the X axis time in seconds.

Figure 8.

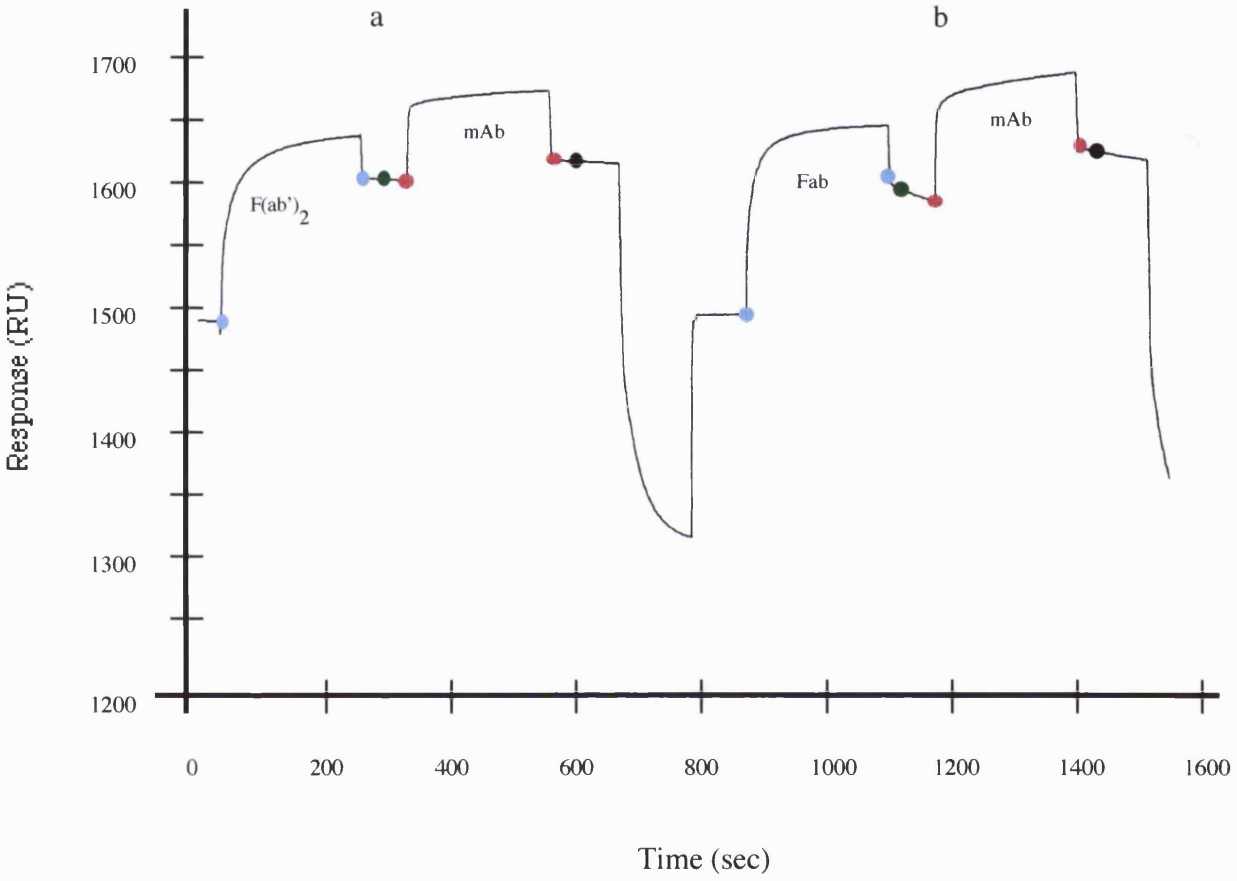

a
b
mAb
mAb
F(ab')2
Fab
Response (RU)
1700
1600
1500
1400
1300
1200
0
200
400
600
800
1000
1200
1400
1600
Time (sec)

Figure 9.

Analysis of the processing-inhibitory activity of mAb 12.10 and its $F(ab')_2$ and Fab fragments.

Isolated T996 merozoites were washed and either solubilised into SDS (track 1), or incubated for 1 hour at 37 °C in the presence of no antibody (track 2); PMSF (track 3); mAb 12.10 (track 4); $F(ab')_2$ (track 5) and Fab (track 6). In a competitive assay, merozoites were pre-incubated with $F(ab')_2$ followed by mAb 12.10 (track 7); or pre-incubated with Fab followed by mAb 12.10 (track 8). All samples were analysed by western blot using a monoclonal antibody specific for an epitope within $MSP\text{-}1_{33}$ (X509) followed by the addition of HRP anti-human polyclonal antibody.

Figure 9.

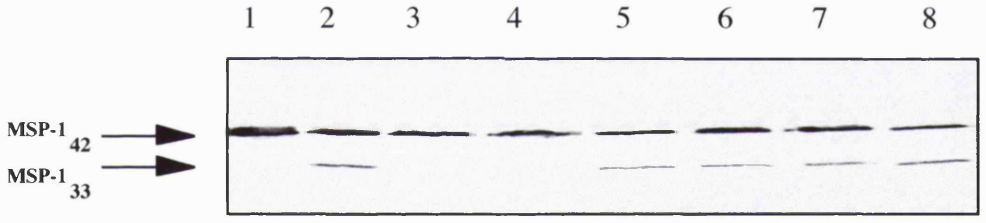


Figure 10.

Analysis of mAb 12.10, F(ab′)$_2$ and Fab fragments in the *in vitro* parasite invasion assay.

Mab 12.10 can effectively inhibit MSP-1 secondary processing and erythrocyte invasion, but its F(ab′)$_2$ and Fab fragments failed to elicit such an inhibitory activity. **Panel A**. Metabolically radiolabeled T996 schizonts were supplemented with fresh erythrocytes and medium to obtain a parasitaemia of approximately 2% and a haematocrit of 1%. The culture was then divided into equal aliquots and incubated at 37 °C in the presence of 5 mM EGTA; no antibody; 400 μg ml^{-1} mAb 12.10; 400 μg ml^{-1} F(ab′)$_2$; 250 μg ml^{-1}); 125 μg ml^{-1}; 62.2 μg ml^{-1}; 31 μg ml^{-1}, followed by 400 μg ml^{-1} mAb 12.10. Fab was added at 400 μg ml^{-1}; 250 μg ml^{-1}; 125 μg ml^{-1}; 62.2 μg ml-1; 31 μg ml^{-1}, followed by 400 μg ml^{-1} mAb 12.10. Culture supernatant was analysed by immunoprecipitation with mAb X509. **Panel B**. Erythrocyte invasion in individual microcultures was assessed by counting the number of ring-stage parasite in 5,000 red cells. Invasion is expressed as a percentage of the ring stage parasitaemia (10%) obtained in a control culture with non immune human sera (NI Hs). Maximum inhibition was obtained in a control culture with 5 mM EGTA. Mab 12.10, F(ab′)$_2$ and Fab inhibited erythrocyte invasion by 96.5%, 1% and 2% respectively. In a competitive assay, inhibition of erythrocyte invasion mediated by mAb 12.10 was abrogated by the presence of F(ab′)$_2$ or Fab. All samples were assayed in triplicate, and SE bars are indicated.

Figure 10.

A

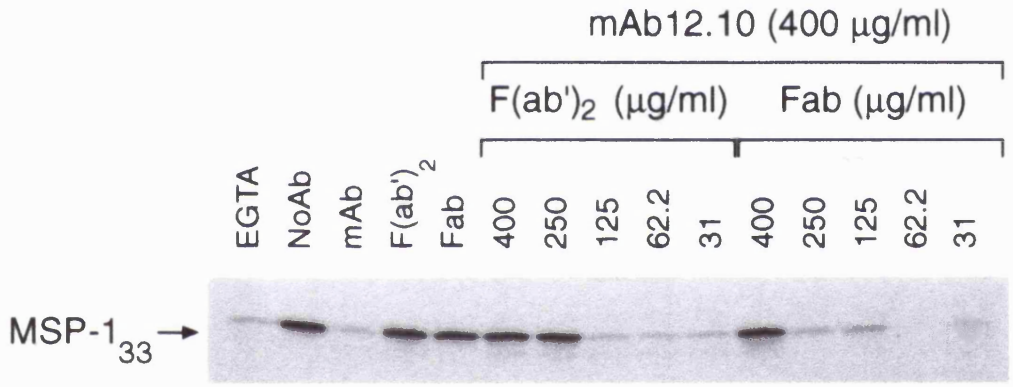
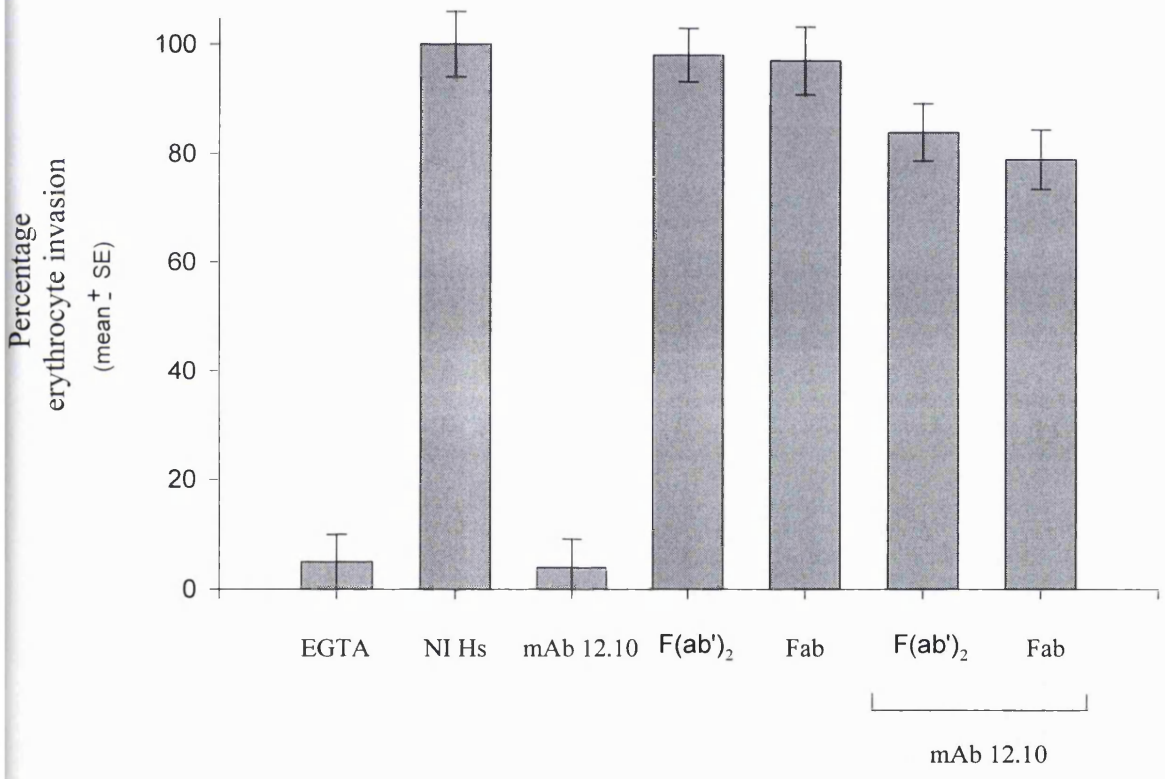

mAb12.10 (400 µg/ml)
F(ab')2 (µg/ml)
Fab (µg/ml)
EGTA
NoAb
mAb
F(ab')2
Fab
400
250
125
62.2
31
400
250
125
62.2
31
MSP-133

Figure 10.

B


Percentage erythrocyte invasion (mean + SE)
100
80
60
40
20
0
EGTA
NI Hs
mAb 12.10
F(ab')2
Fab
F(ab')2
Fab
mAb 12.10
Samples under test

CHAPTER 4.

ASSOCIATION BETWEEN *IN VIVO* PROTECTION FOLLOWING IMMUNISATION WITH MSP-1-BASED VACCINES AND INDUCTION OF PROCESSING-INHIBITORY ANTIBODIES.

4.1 Introduction.

It cannot be understated that one of the major difficulties in malaria vaccine development has been the lack of any laboratory assay capable of predicting protective immunity against malaria infection (Riley, 1995; Moreno and Patarroyo, 1995; Tanner and Facer, 1997; Miller *et al.*, 1997). Currently, the protective potential of a vaccine candidate, decisions on when to progress from experimental to field trials, and predictions of the likely outcome of those trials, can only be evaluated through the use of animal malaria models and *in vitro* assays based on the inhibition of parasite replication in the presence of antibodies (Hoffman, 1996; Miller *et al.*, 1997). The fact that primates are not the natural host of *P. falciparum,* (and therefore protective mechanisms identified in primate models may not necessarily reflect those which operate in the human host), the increasing cost of primates for experimentation, and controversy over the significance of *in vitro* parasite growth assays, have caused severe difficulties in the development of a malaria vaccine.

The results described in the previous chapter clearly showed a cause-effect association between antibody-mediated inhibition of MSP-1 secondary processing, and inhibition of erythrocyte invasion by the malaria merozoite, reinforcing the postulate that secondary processing of the *P. falciparum* MSP-1 is a prerequisite for erythrocyte invasion (Blackman *et al.*, 1992; 1994a). It seems therefore reasonable to propose that antibodies which possess processing-inhibitory activity may provide protection *in vivo* against replication of the blood-stage parasite. To address this question, it was decided to investigate the association between the presence of serum processing-inhibitory antibodies, and protection against blood-stage parasitaemia. To do this, a series of collaborative studies were initiated in which prechallenge sera from primates vaccinated with experimental *P. falciparum* MSP-1-based vaccines were obtained from a number of groups involved in development of MSP-1 vaccines and were tested for the presence of antibodies which inhibit MSP-1 secondary processing. It was found that some sera contained antibodies capable of inhibiting processing, and that these sera corresponded to those animals which showed protection against subsequent parasite challenge. These findings suggest that the MSP-1 processing assay may be used as a surrogate marker for a protective anti-MSP-1 antibody response.

4.2 Sera from primates vaccinated with experimental *P. falciparum* MSP-1 vaccines.

Several immunisation-challenge experiments have been carried out in *P. falciparum* malaria models with candidate malaria vaccine antigens based on the C-terminal domain of MSP-1. In some of these studies a protective immune response against blood-stage challenge was observed following immunisation of *Aotus* monkeys with recombinant *P. falciparum* MSP-1_{42} or MSP-1_{19}. Samples of prechallenge sera from these trials were made available to assess their ability to inhibit secondary processing of MSP-1. Sera were obtained from a number of immunisation/challenge trials, results of only two of which have been published. Details of these trials are as follows:

Ten serum samples were kindly provided by Dr. S. Chang from a published immunisation/challenge experiment (Chang *et al.*, 1996). This study is referred to here as trial 1. In this study three out of three *Aotus lemurinus* monkeys immunised with a recombinant baculovirus-derived polypeptide (BVp42) corresponding to MSP-1_{42} of the MAD20-type MSP-1 allele (isolate FVO), were protected against subsequent blood-stage challenge with homologous *P. falciparum* parasites. Purified IgG from immune sera was also observed to inhibit *in vitro* parasite growth (Chang *et al.*, 1996). The sera provided were assayed for processing-inhibitory activity, on four separate occasions.

Fourteen serum samples were provided by Dr. A. Holder from the study of Burghaus *et al.* (1996), referred to here as trial 2. In this study a recombinant GST-fusion protein corresponding to MSP-1_{19} of the Wellcome-type allele of the *P. falciparum* MSP-

1 expressed in *E. coli* (GST-MSP-1_{19}) was evaluated as a vaccine candidate. Of 5 *Aotus* monkeys vaccinated with GST-MSP-1_{19} none demonstrated a protective immune response against blood-stage parasite challenge. These sera were tested for processing-inhibitory activity only once.

A total of 31 primate sera from ongoing (unpublished) studies were also made available by Drs. D. Kaslow (NIH Bethesda, U.S.A.) and S. Chang (Hawaii University, U.S.A.) for analysis of processing-inhibitory activity. Details of these trials are as follows.

Sixteen serum samples were provided by Dr. S. Chang from an unpublished trial referred to here as trial 3. In this study the induction of protection against blood-stage *P. falciparum* parasite challenge in *Aotus* monkeys vaccinated with BVp42 was analysed. Prior to parasite challenge the immune sera were assayed for processing-inhibitory activity on three independent occasions.

Fifteen serum samples were provided by Dr. D. Kaslow from an unpublished immunisation/challenge experiment referred here as trial 4. This study aimed to establish the efficacy of a recombinant protein corresponding to the MSP-1_{19} of the MAD-20 allele of MSP-1, secreted in *S. cerevisiae* (P30P2), as a malaria candidate vaccine. The provided sera were assayed for processing-inhibitory activity, on two separate occasions.

In both trials 3 and 4, *Aotus* monkeys were vaccinated with the respective antigens (BVp42 and P30P2), and *prior* to challenge with *P. falciparum* parasites, primate sera were taken and evaluated for processing-inhibitory activity.

All analyses were carried out in a blind manner, with no prior knowledge of the identity of the samples tested. Briefly, T9/96 merozoites were washed, supplemented with protease inhibitors and 30 µl aliquots were then incubated in the presence of 3 µl of each primate serum for 20 minutes at 4 °C. Samples were then centrifuged at 14,000 g for 5 min at 4 °C, and 20 µl of supernatant removed, before incubation at 37 °C for 1 hour to allow processing to take place. MSP-1_{42} and MSP-1_{33} were detected using the standard MSP-1 processing assay as described in Material and Methods. It was found that only some sera were capable of effectively inhibiting MSP-1 secondary processing.

4.3 Results.

4.3.1 Analysis of the processing-inhibitory activity of sera from primates vaccinated with experimental *P. falciparum* MSP-1 vaccines and correlation with the *in vivo* protective response against blood-stage challenge.

4.3.2 Analysis of primate sera from published vaccine trials.

Trial 1.

Figure 11 shows that samples 2 and 5 from trial 1 were found to detectably inhibit MSP-1 secondary processing. After breaking the codes, it was found that samples 2 and 5 corresponded to those animals showing a significant level of *in vivo* protection against blood-stage challenge (table 2); furthermore in those cases where purified IgG from serum had been tested for their ability to inhibit parasite growth *in vitro* it was found that specifically, sample 2 inhibited erythrocyte invasion *in vitro* by 98% (and the corresponding animal also showed a markedly delayed course of infection after challenge), and sample 5 inhibited erythrocyte invasion *in vitro* by 99% (and the animal showed complete protection *in vivo* after challenge). Sample 9 was found not to inhibit processing, and inhibited erythrocyte invasion *in vitro* by 72%, but interestingly the animal showed complete protection *in vivo*.

In trial 1 the animals were vaccinated with a recombinant antigen (BVp42) based on the sequence of the MAD20 allele of the *P. falciparum* MSP-1. As described in the Introduction, the MSP-1 gene exists in two major allelic forms; the Wellcome-type and the MAD20-type family (Tanabe *et al.*, 1987; Miller *et al.*, 1993). The gene sequence has been divided into 17 blocks according to the grade of conservation. Block 16 corresponds to a region classified as dimorphic, with extensive variability between the two allelic families; there are, however, short regions within this block which are completely conserved. The MSP-1_{33} fragment is predominantly encoded by sequence lying within block 16 (Stafford *et al.*, 1996), and would therefore be expected to be essentially antigenically dimorphic. In contrast, the C-terminal domain of MSP-1 (MSP-1_{19}) is encoded by sequence within the highly conserved block 17; the deduced MSP-1_{19} sequences of the two allelic families, differ from each other at a maximum of only four amino acid positions (Tolle *et al.*, 1995; Kang and Long, 1995). Previous studies (Egan *et al.*, 1995; Dr. A. Egan, personal communication) investigating the human immune response to MSP-1_{42}, MSP-1_{33} and MSP-1_{19} in a malaria-endemic area (The Gambia, West Africa), have shown that in adult immune sera containing MSP-1_{42}-reactive antibodies, these antibodies are essentially directed to the MAD20-MSP-1_{33} sequence and show no reactivity with the alternative form of MSP-1_{33}; the sera also contain, at lower levels, antibodies against MSP-1_{19} that recognise both the MAD20-like and the Wellcome-like forms of MSP-1_{19}. Thus, as might be predicted, given the essentially polymorphic nature of MSP-1_{33}, the naturally-acquired humoral immune response against the MAD20-like MSP-1_{33} appears not to cross-react with the alternative, Wellcome-type MSP-1_{33} molecule. Consistent with this, in a study by Blackman *et al.* (1992) it was

shown that a rabbit serum raised against a recombinant protein covering almost all of the Wellcome-type MSP-1_{33} reacted only with the homologous parasite-derived MSP-1_{33} and not at all with the alternative MAD20-type MSP-1_{33} expressed by the T9/96 *P. falciparum* clone. In the present study, processing-inhibitory antibodies present in sera from primates vaccinated with BVp42 (an antigen corresponding to the MAD20 form of MSP-1_{42}) were detected when assayed against merozoites expressing the homologous allelic form of MSP-1. However it was not clear what the specificity of these processing-inhibitory antibodies was; i.e. whether the processing-inhibitory specificities were predominantly specific for MSP-1_{19} or MSP-1_{33}. It was reasoned that if the sera, raised against the MAD20-like MSP-1_{42}, could also inhibit secondary processing in merozoites expressing the Wellcome allele of MSP-1 (such as the FCB-1 *P. falciparum* isolate), this would strongly suggest that the observed processing-inhibitory activity is predominantly mediated by antibodies against MSP-1_{19}. In order to address this issue, sera from trial 1 were tested for their ability to inhibit secondary processing of MSP-1 using merozoites of the *P. falciparum* FCB-1 strain (Wellcome allelic type). Interestingly, it was found that serum samples 2 and 5 (and none of the others) retained their ability to inhibit secondary processing of MSP-1 (not shown).

Trial 2. (Burghaus *et al.*, 1996)

In the published report of trial 2, *in vivo* protection in *Aotus* monkeys was not achieved following vaccination with GST-MSP-1_{19}. In the present study it was found that

none of the sera from these animals was able to inhibit MSP-1 secondary processing (Figure 12), (Table 3).

4.3.3 Analysis of primate sera from unpublished vaccine trials.

Trial 3. Kaslow *et al.* (unpublished).

Serum samples, 1, 2, 3, and 8 were capable of effectively inhibiting secondary processing of MSP-1 (Figure 13), and corresponded to the animals in which *in vivo* protection was observed. Sample number 7 also showed significant processing-inhibitory activity; this animal showed a very low parasitaemia upon challenge, but required drug treatment due to anaemia. Serum samples 4, 5, 6 and 9-15 did not inhibit secondary processing of MSP-1, and the corresponding animals showed no *in vivo* protection upon challenge (Table 4).

Trial 4. Chang *et al.* (unpublished).

In this study some sera were capable of inhibiting secondary processing of MSP-1; specifically, sera 4, 5, 6, 7, 10 11, 12, 14 and 16 significantly inhibited MSP-1 secondary processing (Figure 14). However to date, no data regarding *in vivo* protection has been revealed (Table 5).

4.4 Discussion

This study has determined that, in primates immunised with recombinant proteins representing the C-terminal domain of MSP-1, there is a remarkable correlation between the induction of serum antibodies that inhibit secondary processing of MSP-1, and the induction of protective immunity *in vivo*. It is clearly not possible in a study such as this to determine whether processing-inhibitory antibodies are directly responsible for mediating the observed protection. However, a number of factors suggest that this may be the case.

Firstly, despite the small sample size (n=55) the correlation between the presence of serum antibodies that inhibit MSP-1 processing, and the induction of protective immunity *in vivo* appears very good. Only certain sera from immunised primates effectively inhibited processing and these sera corresponded to those animals protected against parasite challenge. Furthermore, within all the 55 samples tested in the MSP-1 processing assay, none of the primate sera associated with a lack of protection *in vivo*, - including pre-immune control sera - showed processing-inhibitory properties; in other words, no false positives were detected in the assay.

Secondly, the available evidence suggests that both protection and processing inhibition are predominantly mediated by antibodies against MSP-1_{19}. In trial 1 (Chang *et al.*, 1996), primates were vaccinated with an antigen corresponding to the MAD20-type MSP-1_{42} (BVp42) and elicited a humoral protective immune response against *P.*

falciparum. Chang *et al.* found that antibodies from protected *Aotus* predominantly recognise epitopes localised within MSP-1_{19}, suggesting the importance of anti-MSP-1_{19} antibodies in the control of parasite invasion. Furthermore, in a study by Kumar *et al.* (1995) a protective immune response against parasite challenge was observed in monkeys vaccinated with yeast recombinant MSP-1_{19}. In the present study, a dissection of the anti-BVp42 immune response indicates that anti-MSP-1_{19} antibodies appear to be responsible for the processing-inhibitory activity in the sera tested. Experiments in which sera from animals vaccinated with BVp42 (based on the MAD20 MSP-1 allele) were tested in the MSP-1 processing assay, showed that the same sera (samples 2 and 5; trial 1), capable of inhibiting secondary processing of MSP-1 in preparations of T9/96 strain merozoites (MAD20 allele), were also capable of inhibiting MSP-1 secondary processing when FCB-1 strain merozoites (Wellcome allele) were used, suggesting that anti-MSP-1_{19} antibodies and not anti-MSP-1_{33} antibodies may be responsible for the observed activity.

Thirdly, the results presented in the previous chapter provide compelling evidence that anti-MSP-1_{19} antibodies which inhibit invasion do so as a result of their ability to inhibit processing. Of a total of eleven anti-MSP-1_{19} mAbs screened to date, only mAbs 12.8 and 12.10 prevent either MSP-1 processing or erythrocyte invasion. No anti-MSP-1_{19} antibody has been identified which prevents invasion *in vitro* and does not prevent MSP-1 processing. Furthermore, the results described in the previous chapter have clearly shown that there is a causal correlation between antibody-mediated inhibition of processing, and inhibition of invasion.

Taken together, the data suggest that antibodies which prevent MSP-1 processing can be protective *in vivo*. However other immune effector mechanisms could be induced by vaccination with MSP-1 constructs. In trial 1, for example, serum sample 9 was found not to inhibit processing and to only partially affect parasite growth *in vitro*; however, the corresponding animal was completed protected *in vivo*. This *in vivo* protection could be attributed to an interaction between antibodies bound to MSP-1_{19} on the merozoite surface and other components of the immune system that can disrupt erythrocyte invasion by directly affecting the integrity of merozoites, such as macrophages, complement and/or non-antibody dependent protection, eg. CTL or cytokine-mediated killing (Lopez *et al.*, 1996). Nonetheless, the observations presented here suggest that measuring antibody-mediated inhibition of MSP-1 secondary processing may be used as a simple assay capable of predicting a protective anti-MSP-1 immune response.

The current western blot based MSP-1 processing assay is far from perfect, and modifications designed to improve the sensitivity, reproducibility and ease of use of the assay are currently under way. It is intended that these modifications will lead to an assay that can be used in any laboratory involved in the evaluation of MSP-1-based vaccines. Two alternative methodologies are proposed. First, a 96-well microtiter capture ELISA format would offer the possibility of semi-automation of the assay, and could provide quantitative data. Briefly, wells coated with MSP-1_{33}-specific antibody will be used to capture soluble fragments present in supernatant of incubated merozoites. The bound MSP-1_{33} will be detected with a second specific antibody (e.g. a rabbit anti-MSP-1 polyclonal serum, or mAbs specific for MSP-1_{83} or p22). Second, replacement of the

western blot assay with a 96-well microtiter plate format coupled to detection and quantitation of the released soluble MSP-1 derived complex by using surface plasmon resonance analysis (BIAcore), will provide an extremely sensitive method of measuring MSP-1 processing. The chip will be coated with capture antibody, and captured MSP-1_{33} will be detected directly for each sample, or else a second antibody will be used to amplify the signal by increasing the mass of the captured complex.

Preliminary attempts to develop these proposed modifications have been carried out using a rabbit anti-MSP-1 serum as a second antibody for detection of captured MSP-1 complex. In both ELISA capture format and by BIAcore, MSP-1_{33} was detectable (not shown). However, a very high background was observed, mediated by cross-reactivity of the rabbit anti-MSP-1 with the ELISA-plate surface and the BIAcore sensor chip surface. However, despite the background, secondary processing of MSP-1 could be monitored with the modified assay. Further efforts should be focused on the reduction of this background by, for example, using more specific reagents such as mAbs against MSP-1_{83} or p22.

Additional validation of the assay with serum from MSP-1 vaccine trials should be carried out in order to test the validity of the concept, outlined above, that *in vivo* protection is associated with the induction of processing-inhibitory antibodies. There are now a significant number of recombinant proteins, all based on the C-terminal domain of the *P. falciparum* MSP-1, under development as candidate vaccine antigens in a number of laboratories. This study has lead to a series of collaborative links with all the major

laboratories involved in this work. As and when available, serum samples from primate or human vaccine studies will be studied for their ability to inhibit processing, together with an assessment of their antibody titre against the parasite, results of *in vivo* growth inhibition or *in vivo* protection studies.

Figure 11.

Analysis of processing-inhibitory activity of serum from primates vaccinated with BVp42 (Chang *et al.*, 1996).

Washed T9/96 merozoites were dispensed into 30 µl aliquots and incubated for 20 minutes at 4 °C in the presence of 3 µl of primate serum (Chang *et al.*, 1996). Prior to incubation at 37 °C for 1 hour, each preparation was centrifuged at 14,000 g for 10 minutes at 4 °C and 20 µl of supernatant removed. Three aliquots of merozoites were either immediately solubilised in SDS sample buffer (0h), no serum (1h) or 1 mM PMSF as inhibitor control. Samples 2, 4, 5 and 9 correspond to animals vaccinated with BVp42 in complete Freund's adjuvant. Sample 1, 3, 6, 7 and 10 correspond to pre-immune sera. Sample 8 corresponds to a primate immunised with Complete Freund's adjuvant alone. Visualisation of MSP-1_{42} and MSP-1_{33} was carried out using the standard method as described in Material and Methods. All samples were tested in four independent experiments.

Figure 11.

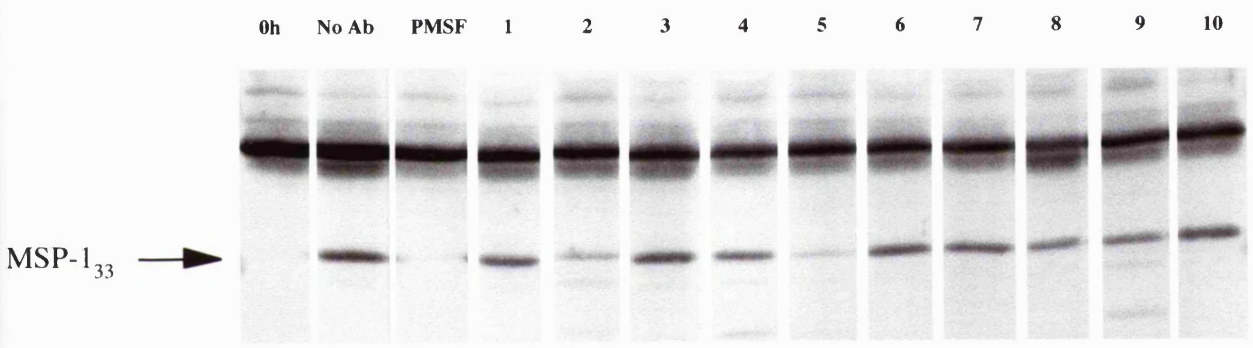


Figure 12.

Analysis of processing-inhibitory activity of serum from primates vaccinated with GST-MSP-1_{19} (Burghaus *et al.*, 1996).

Washed T9/96 merozoites were dispensed into 30 µl aliquots and incubated for 20 minutes at 4 °C in the presence of 3 µl of primate serum (Burghaus *et al.*, 1996). Prior to incubation at 37 °C for 1 hour, each mixture was centrifuged at 14,000 g for 10 minutes at 4 °C, and 20 µl of supernatant removed. Two aliquots of merozoites were either immediately solubilised in SDS sample buffer (0h) and no serum (1h). Samples 1, 2 and 3 correspond to primates vaccinated with GST alone. Sample 4-9 corresponded to primates vaccinated with GST-MSP-1_{19}. Samples 10-12 correspond to primates infected with *P. falciparum* CAMP parasites. Samples 13 and 14 correspond to animal immunised with liposomes alone. Visualisation of MSP-1_{33} was carried out using the standard method as described in Material and Methods.

Figure 12.

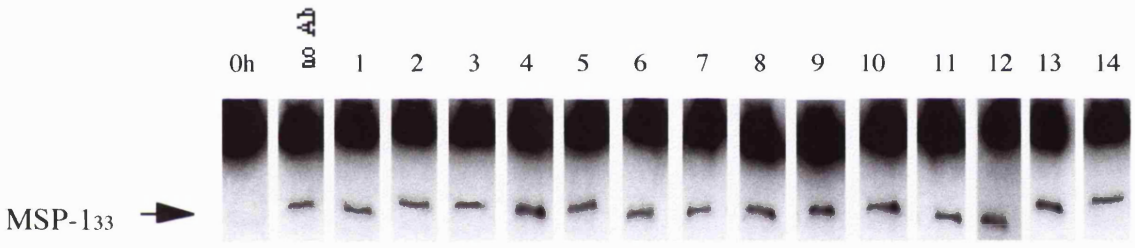


Figure 13 .

Analysis of processing-inhibitory activity of serum from primates vaccinated with P30P2 MSP-1_{19} (Kaslow *et al.*, unpublished).

Washed T9/96 merozoites were dispensed into 30 µl aliquots and incubated for 20 minutes at 4 °C in the presence of 3 µl of primate serum (Kaslow *et al.*, unpublished). Prior to incubation at 37 °C for 1 hour, each mixture was centrifuged at 14,000 g for 10 minutes at 4 °C, and 20 µl of supernatant removed. Two aliquots of merozoites were immediately solubilised in SDS sample buffer (0h) and no serum (1h). Samples 1-8 correspond to primates vaccinated with P30P2 MSP-1_{19}. Samples 9, 10, 13 correspond to primates vaccinated with TBV (Pfs25). Samples 11, 12, 14 and 15 correspond to non immunised primates. Visualisation of MSP-1_{33} was carried out using the standard method as described in Material and Methods. All samples were tested in two independent experiments.

Figure 13.

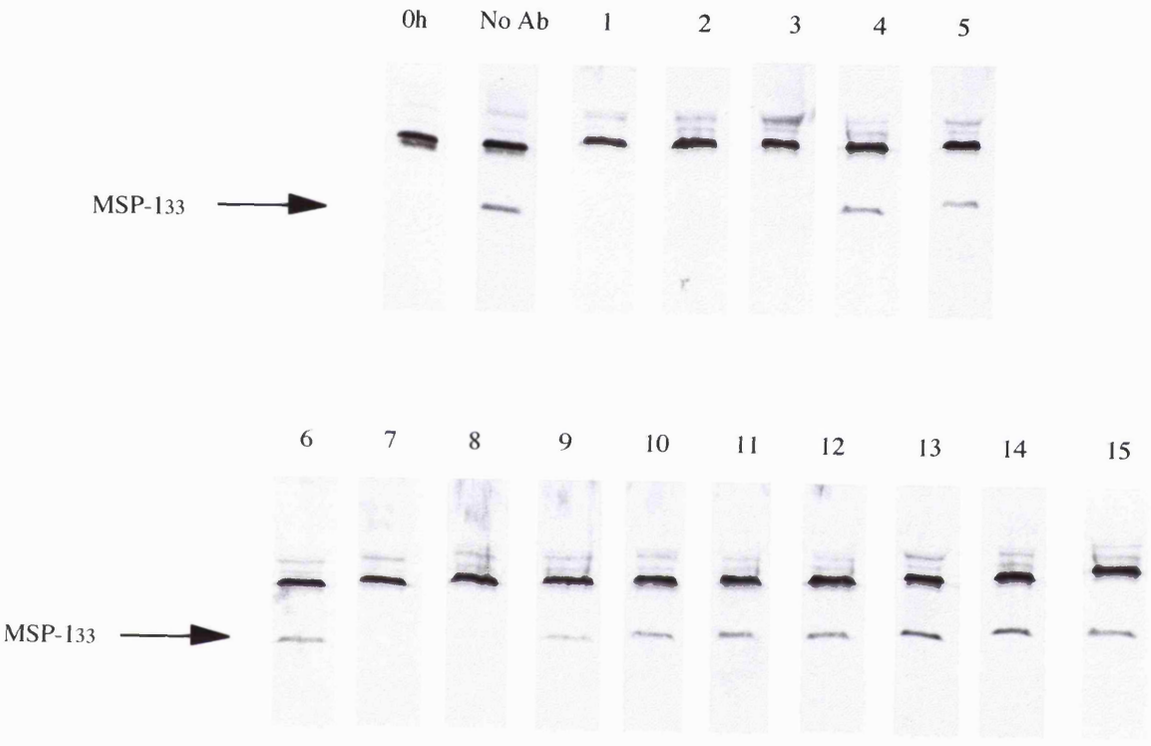


Figure 14.

Analysis of processing-inhibitory activity of serum from primates vaccinated with BVp42 (Chang *et al.*, unpublished).

Washed T9/96 merozoites were dispensed into 30 µl aliquots and incubated for 20 minutes at 4 °C in the presence of 3 µl of primate serum (Chang *et al.*, unpublished). Prior to incubation at 37 °C for 1 hour, each mixture was centrifuged at 14,000 g for 10 minutes at 4 °C, and 20 µl of supernatant removed. Two aliquots of merozoites were immediately solubilised in SDS sample buffer (0h) and no serum (1h). Samples 1, 4-7 and 10-16 correspond to primates vaccinated with BVp42. Samples 2, 3, 8 and 9 correspond to pre-immune primate sera. Visualisation of MSP-1_{33} was carried out using the standard method as described in Material and Methods. All samples were tested in three independent experiments.

Figure 14.

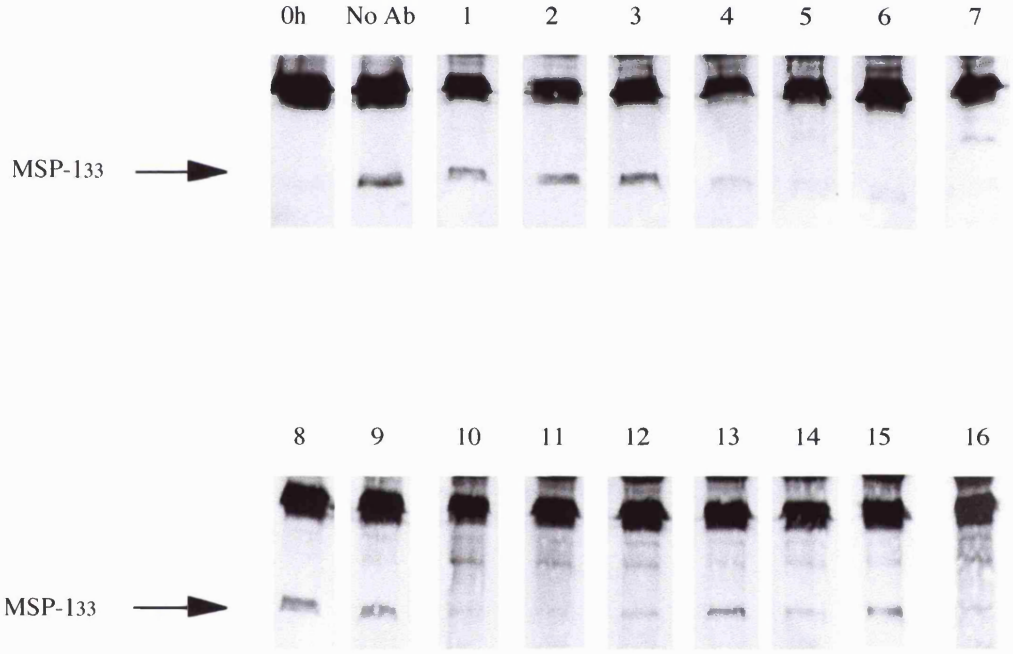


Table 2.

Trial 1

Source: Chang *et al.*, (1996) Infect Immun. 64 253-261

Aotus sera

No. times assayed: 4

Sample No.	**Treatment**	**Processing Inhibition**	**Growth Inhibition**	**Protection *in vivo*: Parasitaemia**
1	Normal (PI)	-	-	-
2	BVp42/CFA	+++	99%	markedly delayed course
3	Normal (PI)	-	-	-
4	Bvp42/SA	+	95%	moderately delayed course
5	BVp42/CFA	+++	98%	complete protection
6	Normal (PI)	-	-	-
7	Normal (PI)	-	-	-
8	CFA Control	-/+	6%	normal course
9	BVp42/CFA	-	72%	complete protection
10	Normal (PI)	-	-	-

Normal (PI): Aotus preimmune serum

CFA: Complete Freund's adjuvant

SA: Synthetic adjuvant A

Sera were also tested using FCB-1 parasites.

Results were consistent with T9/96.

Therefore, processing inhibitory activity is anti-MSP-1_{19} mediated.

Table 3.

Trial 2

Source: Burghaus *et al.*, (1996) Infect Immun. 64 3614-3619

Aotus sera

No. times assayed: 1

Sample No.	**Treatment**	**Processing inhibition**	**Growth inhibition**	**Protection *in vivo* Parasitaemia**
1	Liposome+GST	-	ND*	non-protected
2	Liposome+GST-MSP1$_{19}$	-	ND*	non-protected
3	Liposome+GST	-	ND*	non-protected
4	CAMP	-	ND*	non-protected
5	Liposome+GST	-	ND*	non-protected
6	Liposome+GST-MSP1$_{19}$	-	ND*	non-protected
7	CAMP	-	ND*	non-protected
8	CAMP	-	ND*	non-protected
9	Liposome+GST-MSP1$_{19}$	-	ND*	non-protected
10	Liposome+GST-MSP1$_{19}$	-	ND*	non-protected
11	Liposome	-	ND*	non-protected
12	Liposome+GST-MSP1$_{19}$	-	ND*	non-protected
13	Liposome	-	ND*	non-protected
14	Liposome+GST-MSP1$_{19}$	-	ND*	non-protected

Not done with purified Ig.

Table 4.

Trial 3

Source: David Kaslow *et al.*, (unpublished)

Aotus sera

No. times assayed: 2

Sample No.	Treatment	Processing inhibition	Growth inhibition	Protection *in vivo* Parasitaemia
1	P30P2 $MSP\text{-}1_{19}$	+++	ND ?	protected
2	P30P2 $MSP\text{-}1_{19}$	+++	ND ?	protected
3	P30P2 $MSP\text{-}1_{19}$	+++	ND ?	protected
4	P30P2 $MSP\text{-}1_{19}$	-	ND ?	non-protected
5	P30P2 $MSP\text{-}1_{19}$	-	ND ?	cured for anaemia, low parasitaemia
6	P30P2 $MSP\text{-}1_{19}$	-	ND ?	non-protected
7	P30P2 $MSP\text{-}1_{19}$	+++	ND ?	cured for anaemia, very low parasitaemia
8	P30P2 $MSP\text{-}1_{19}$	+++	ND ?	protected
9	TBV (Pfs25)	-	ND ?	cured for anaemia, low parasitaemia
10	TBV (Pfs25)	-	ND ?	non-protected
11	no vaccine	-	ND ?	non-protected
12	no vaccine	-	ND ?	non-protected
13	TBV (Pfs25)	-	ND ?	non protected
14	no vaccine	-	ND ?	non-protected
15	no vaccine	-	ND ?	non-protected

Table 5.

Trial 4

Source: Sandra Chang *et al.*, (unpublished)

Aotus sera

No. times assayed: 3

Sample No.	Treatment	Processing inhibition	Growth inhibition	Protection *in vivo* Parasitaemia
1	Bvp42	-	?	?
2	normal (PI)	-	?	?
3	normal (PI)	-	?	?
4	Bvp42	++	?	?
5	Bvp42	+++	?	?
6	Bvp42	+++	?	?
7	Bvp42	+++	?	?
8	normal (PI)	-	?	?
9	normal (PI)	-	?	?
10	Bvp42	+++	?	?
11	Bvp42	+++	?	?
12	Bvp42	++	?	?
13	Bvp42	-	?	mild protection
14	Bvp42	++	?	mild protection
15	Bvp42	-	?	mild protection
16	Bvp42	++	?	moderate protection

Sera were also tested using FCB-1 parasites. Results were consistent with T9/96.

CHAPTER 5.

ANTIBODIES THAT INHIBIT MALARIA MSP-1 PROCESSING AND ERYTHROCYE INVASION ARE BLOCKED BY NATURALLY ACQUIRED HUMAN ANTIBODIES.

5.1 Introduction.

MSP-1 undergoes at least two endoproteolytic cleavage events during merozoite maturation and release, and erythrocyte invasion. This study has previously demonstrated that mAbs which inhibit erythrocyte invasion and are specific for epitopes within the membrane-proximal, C-terminal domain of MSP-1, prevent the critical secondary processing step which occurs on the surface of the extracellular merozoite at around the time of erythrocyte invasion. Certain other anti-MSP-1_{19} mAbs, which themselves inhibit neither erythrocyte invasion nor MSP-1 secondary processing, block the processing-inhibitory activity of the first group of antibodies and are termed blocking antibodies. In this chapter, attempts were made to directly quantitate antibody-mediated inhibition of MSP-1 secondary processing and invasion, and the effects on this of blocking antibodies. It is shown that blocking antibodies function by competing with the binding of processing-inhibitory antibodies to their epitopes on the merozoite. Polyclonal rabbit antibodies specific for certain MSP-1 sequences outside of MSP-1_{19} were also found to act as blocking antibodies. Most significantly, affinity-purified, naturally-acquired human antibodies specific for epitopes within the N-terminal 83 kDa domain of MSP-1 were

found to block the processing-inhibitory activity of the anti-MSP-1_{19} mAb 12.8 very effectively. The presence of these blocking antibodies also completely abrogated the inhibitory effect of mAb 12.8 on erythrocyte invasion by the parasite *in vitro*. Blocking antibodies therefore; (i) are part of the human response to malarial infection; (ii) can be induced by MSP-1 structures unrelated to the MSP-1_{19} target of processing-inhibitory antibodies; and (iii) have the potential to abolish protection mediated by anti-MSP-1_{19} antibodies. These results suggest that an effective MSP-1_{19}-based falciparum malaria vaccine should aim to induce an antibody response that prevents MSP-1 processing on the merozoite surface.

5.2 Results.

5.2.1 Development and validation of an assay to quantitate antibody-mediated inhibition of MSP-1 processing.

In previous work a panel of MSP-1_{19}-specific mAbs was tested for their ability to interfere with secondary processing of MSP-1 (Blackman *et al.*, 1994a) using a western blot-based procedure that allowed only a semiquantitative estimate of processing inhibition. To improve the assay for the present study, a radioiodinated, affinity-purified anti-rabbit IgG was used. Autoradiography of the probed blots allowed visualization of bands corresponding to MSP-1_{42} and its processed product MSP-1_{33}, and the amount of antibody bound to each was determined by direct counting in a gamma counter. When an extract of incubated merozoites was analysed by this method, the radioactivity associated with each of the MSP-1_{33} and MSP-1_{42} bands on the blot was - within limits imposed by the protein binding capacity of the blotting membrane - directly proportional to the volume of merozoite extract loaded on the gel (Figure 15). This linear relationship did not hold if an extract of more than about 2 x 10^8 merozoites was loaded per track, and in all subsequent experiments this limit was not exceeded. During a 1 hour incubation of merozoites, the observed decrease over time in the number of counts associated with MSP-1_{42} (due to processing of the polypeptide) was concomitant with a corresponding increase in the number of counts associated with MSP-1_{33}, and at least 50% of the MSP-1_{42} underwent processing in this period (Figure 16). These results are in accordance with previous data showing stoichiometric conversion of MSP-1_{42} to MSP-1_{33} (Blackman *et*

al., 1993a), and indicate that accurate quantitation of MSP-1 processing is possible with this assay. In a typical assay, the number of cpm associated with the MSP-1_{33} band in the zero time (0h) control and the positive processing control sample (incubated for 1 hour in reaction buffer only; see Materials and Methods) was 20 and 1300 cpm respectively (data not shown).

The assay was used to quantify MSP-1 secondary processing and its inhibition by a panel of anti-MSP-1_{19} mAbs. Washed FCB-1 merozoites were incubated on ice in the presence of individual purified mAbs, then transferred to 37 °C for 1 hour to allow processing to occur. MSP-1_{42} processing in the individual samples was then assessed using the above protocol. Figure 17 shows that mAb 12.8, which recognizes a conserved epitope in the first EGF-like motif of MSP-1_{19} inhibited processing by 96% of the control value, whilst mAb 12.10, which recognizes an epitope formed by the two EGF-like motifs together (Chappel and Holder, 1993), inhibited processing by 98%. Monoclonal antibody 1E1 showed no processing-inhibitory activity in this assay system. Interestingly, earlier data obtained using a semiquantitative western blot-based assay indicated that mAb 1E1 appeared to induce abnormal processing rather than preventing the processing (Figure 5); in addition, mAb 1E1 does not prevent erythrocyte invasion in *in vitro* cultures of *P. falciparum* (Blackman *et al.*, 1994a). Antibodies 8A12 and 117.2 inhibited MSP-1_{42} processing by 18% and 12% respectively, whilst mAbs 111.4, 12D11, and 7E5 did not detectably prevent processing. Neither mAb 89.1, which recognizes an epitope within the N-terminal domain of MSP-1 (MSP-1_{83}), nor the anti-*P. yoelii* MSP-1 mAb 25.1, had any

effect on the processing (not shown). These results confirm that mAbs 12.8 and 12.10 are potent inhibitors of MSP-1_{42} processing. In similar assays using merozoites of the *P. falciparum* clone T9/96, which expresses the alternative dimorphic form of MSP-1 (Miller *et al.*, 1993), but retains the non-polymorphic epitopes recognised by mAbs 12.8 and 12.10 (Wilson *et al.*, 1987), both mAbs showed similarly potent processing-inhibitory activity (data not shown).

5.2.2 Blocking antibodies act by competitively preventing the binding of processing-inhibitory mAbs to merozoites.

Previous work has indicated that a number of anti-MSP-1_{19} mAbs, which themselves do not inhibit MSP-1 processing, can block the ability of mAbs 12.8 and 12.10 to interfere with the processing. Although the mechanism of this blocking activity was not elucidated, the most likely explanation is that a blocking antibody can compete with a processing-inhibitory antibody for binding to MSP-1 on the merozoite surface. Here, this hypothesis was directly tested using a competitive RIA to investigate the effects of known blocking antibodies on binding of processing-inhibitory antibodies to native, merozoite-derived MSP-1.

Wells of 96-well PVC plates coated with merozoite antigen extract were incubated with anti-MSP-1_{19} mAbs at saturating concentrations (Figure 18). The plates were then washed and an optimal concentration of radioiodinated mAb 12.8 or 12.10 was added. Following further incubation, plates were washed and individual wells counted directly in

a gamma counter. Figure 19 shows that antibodies known to interfere with the processing activity of mAbs 12.8 and 12.10 prevented these mAbs from binding to immobilized antigen. Whilst mAbs 7.5 and 1E1 prevented binding of both radiolabeled mAbs, mAb 2.2 only significantly prevented binding of mAb 12.8, consistent with its ability to interfere with the processing-inhibitory activity of mAb 12.8 but not 12.10 (Blackman *et al.*, 1994a). Preincubation with mAb 111.4 had little or no effect on binding of the radiolabeled mAbs, consistent with its lack of blocking activity (Blackman *et al*, 1994a); mAb 89.1 was similarly ineffective in competing with 12.8 or 12.10 binding. Identical results were obtained when rMSP-1_{19} was used to coat RIA plates (not shown).

5.2.3 Antibodies against the N-terminal region of MSP-1 can block the binding of processing-inhibitory mAbs directed against epitopes within MSP-1_{19}.

The above results showed that the binding of processing-inhibitory antibodies to MSP-1_{19} can be specifically prevented by the interaction of other antibodies with the same polypeptide, and explain how blocking antibodies interfere with the processing-inhibitory activity of mAbs 12.8 and 12.10. Interestingly, Wilson *et al.* (1987), found that mAb 13.2, which recognizes an epitope within the N-terminal domain of MSP-1, prevents the binding of mAb 12.8 to intact MSP-1, raising the possibility that antibodies specific to other components of the MSP-1-derived, merozoite surface protein complex might have blocking activity. To investigate this possibility, a series of rabbit antibodies, raised against recombinant proteins corresponding to regions covering all of MSP-1 (Holder *et al.*, 1987; see Figure 20) were tested for their ability to competitively prevent recognition of

merozoite-derived MSP-1 by mAbs 12.8 and 12.10. Figure 21 shows that binding of radioiodinated mAbs 12.8 and 12.10 to the merozoite antigen was significantly blocked by some but not all of the polyclonal antibodies. The fact that rabbit antibodies raised against pME12, 16 and 20 were able effectively to block binding was not unexpected, due to the presence of the 12.8 and 12.10 epitopes within the sequence of the recombinant proteins used to raise these rabbit sera. However, it was found that antibodies raised against constructs corresponding to domains of MSP-1 outside the C-terminal region also showed potent blocking activity; in particular, the anti-pME6, anti-pME14 and anti-pME3 sera inhibited binding of mAb 12.8 to the immobilized antigen by 68%, 48% and 91% respectively, and the rabbit anti-pME14, anti-pME1, and anti-PME3, but not the anti-pME6 antibodies, significantly prevented binding of mAb 12.10. These results show that polyclonal antibodies specific for fragments of the MSP-1 complex other than MSP-1_{19} can act as blocking antibodies.

5.2.4 Naturally-acquired human antibodies specific for epitopes within the N-terminal domain of MSP-1 block the activity of processing-inhibitory anti-MSP-1_{19} antibodies.

Antibodies which prevent MSP-1 processing and erythrocyte invasion may be involved in mediating protection against blood-stage parasitemia. If antibodies induced to other domains of MSP-1 can block the activity of processing-inhibitory antibodies specific for MSP-1_{19}, their presence in human sera may be disadvantageous to the host. In the light of the above data, it was decided to investigate the ability of naturally acquired

antibodies, specific for the region of MSP-1 corresponding to pME6, to block the processing-inhibitory activity of mAbs 12.8 and 12.10. This particular construct was chosen because pME6 is readily soluble (Holder *et al.*, 1987), and the *E. coli* clone which expresses pME6 does so at very high levels. Human antibodies reactive with pME6 were isolated from pooled Gambian adult immune serum by affinity chromatography on immobilized pME6 fusion protein. The eluted Ig was judged to be >98% pure as assessed by SDS PAGE under reducing conditions (Figure 22). The Ig was concentrated by ultrafiltration and assayed by immunoblot for reactivity with FCB-1 merozoite polypeptides. Strong reactivity was observed with only two merozoite polypeptides of approximately 83 kDa and 195 kDa (Figure 23); these most likely correspond to MSP-1_{83} and residual MSP-1 precursor protein. Note that the purified antibodies showed no reactivity with the MSP-1_{42} or MSP-1_{19} species (arrowed). In confirmation of this, analysis of the affinity-purified Ig by indirect immunofluorescence showed strong reactivity with acetone-fixed FCB-1 or T9/96 schizonts, but none with newly-invaded ring stage parasites, which contain only MSP-1_{19} (Blackman *et al.*, 1990; 1991; 1992; Stafford *et al.*, 1994), (not shown). Note that since the pME6 construct covers much of the highly conserved MSP-1 block 3 domain, as well as all of the conserved block 5 (see Figure 20), antibodies against pME6 would be expected to recognise both allelic forms of MSP-1.

The ability of the affinity-purified human antibodies to block the processing-inhibitory effects of mAbs 12.8 and 12.10 was then assessed. Merozoites were incubated on ice in the presence or absence of the human anti-pME6 antibodies, then mAb 12.8 or

12.10 was added and the samples were incubated for 20 minutes on ice before transfer to 37.5 °C for 1 hour to allow processing to take place. Figure 24A shows that pre-treatment with the anti-pME6 antibodies virtually abolished the processing-inhibitory activity of mAb 12.8, but interestingly had no effect on the inhibitory activity of mAb 12.10. In parallel binding assays (Figure 24B), the anti-pME6 antibodies competed effectively with binding of mAb 12.8, but not mAb 12.10, to immobilised merozoite-derived antigen.

These data clearly show that the binding of antibodies specific to one component of the MSP-1-derived merozoite surface complex can interfere with the binding of antibodies to another component of the complex. Erythrocyte invasion by the malaria merozoite is rapid, going to completion within seconds of the initial interaction between parasite and red cell surface (Dvorak *et al.*, 1975). Over such a short time span, could the presence of blocking antibodies interfere with the ability of processing-inhibitory antibodies to bind the merozoite surface and prevent both processing and invasion? To address this question directly in an *in vitro* system, a series of invasion experiments was performed similar to those described in chapter 3 section 3.2.5. Merozoite release and red cell invasion was allowed to proceed in the presence or absence of mAbs 12.8 and 12.10, with or without the additional presence of affinity-purified anti-pME6 human antibodies. In preliminary dose-response experiments a concentration of ≥400 μg ml^{-1} of either mAb 12.10 (Figure 25A) or mAb 12.8 (not shown) was sufficient to reduce the amount of MSP-1_{33} release to a level of inhibition seen in the presence of 5 mM EGTA. The results of a typical experiment (of a total of three independent experiments) investigating the effects of the anti-PME6 blocking antibodies on the activity of mAbs 12.8 and 12.10 are

presented in Figure 25B. In isolation, mAbs 12.8 and 12.10 virtually abolished both invasion (Figure 25B, bottom) and MSP-1_{33} release (Figure 25B, top). However, in the presence of equal concentrations of the anti-PME6 human antibodies, the effects of mAb 12.8, but not of 12.10, were completely reversed (Figure 25A tracks 5 and 7). Neither the anti-pME6 antibodies alone nor mAb 89.1 alone had any effect on either processing or invasion (tracks 3 and 8), and mAb 89.1 exhibited no blocking activity (tracks 9 and 10). These results unambiguously demonstrate that, under conditions of active release of viable merozoites, mAbs 12.8 and 12.10 effectively prevent both MSP-1 processing and erythrocyte invasion, and this activity can be efficiently abrogated by the presence of human blocking antibodies.

5.3 Discussion

Four major conclusions can be drawn from this study. Firstly, blocking antibodies function by competitively preventing the binding of processing-inhibitory antibodies to the merozoite surface, and can be effective under conditions of active merozoite release and erythrocyte invasion. Secondly, blocking activity can be mediated not only by antibodies specific for the MSP-1_{19} domain, but also by antibodies binding to polypeptides other than the MSP-1_{19} target of processing-inhibitory antibodies; here it is shown that antibodies reactive with a region within MSP-1_{83}, a polypeptide derived from the N-terminal domain of the MSP-1 precursor, possess potent blocking activity. Antibodies against other fragments of the merozoite surface complex - possibly including the non-MSP-derived components of it (Blackman *et al.*, 1993b; Stafford *et al.*, 1996) - may also mediate blocking activity; indeed, the present data suggest that antibodies against the region of MSP-1 represented by pME14 possess significant blocking activity (Figure 21). Thirdly, human blocking antibodies can be induced by natural exposure to malarial infection. Fourth, if prevention of MSP-1 processing is a major mechanism by which anti-MSP-1_{19} antibodies exert their effect on erythrocyte invasion by the *P. falciparum* merozoite, then the protective potential of inducing such antibodies by vaccination could be impaired by a pre-existing or simultaneously induced blocking antibody response directed against MSP-1_{19} itself, or other components of the MSP-1 protein complex.

MSP-1 is receiving increasing interest as a candidate antigen for a blood-stage malaria vaccine. Experimental passive immunisation and direct immunisation-challenge

studies focusing on the protective capacity of anti-MSP-1_{19} antibody responses have been substantiated by epidemiological studies in malaria-endemic areas showing a significant positive association between levels of serum antibodies against MSP-1_{19} and resistance to morbidity associated with *falciparum* malaria (Riley *et al.*, 1992; Egan *et al.*, 1996). The sero-epidemiological data are not, however, unambiguous. There is not, for example, a simple relationship between sero-positivity and clinical immunity, and there is extensive evidence that parasite replication can take place *in vivo* in the presence of substantial levels of circulating anti-MSP-1 antibody (Riley *et al.*, 1992; Chappel *et al.*, 1994). With no clear consensus on either the mechanism(s) by which anti-MSP-1 antibodies control replication of the parasite, or the biological function of MSP-1 on the merozoite surface (Barnwell *et al.*, 1991; Holder *et al.*, 1994), the effector mechanisms required of an optimally protective anti-MSP-1 immune response have been unclear. Given the availability of first generation MSP-1_{19}-based vaccines for clinical evaluation, there is a need to define indicators of a protective anti-MSP-1 response which are amenable to quantitative serological assay (Miller *et al.*, 1997).

Antibodies specific for the *P. falciparum* MSP-1_{19} domain may prevent merozoites from invading erythrocytes primarily by interfering with MSP-1 secondary processing. This hypothesis is supported by the apparently absolute correlation between antibody-mediated processing-inhibitory activity and invasion inhibitory activity; of a total of 11 distinct anti-*P. falciparum* MSP-1_{19} mAbs tested to date, only mAbs 12.8 and 12.10 exhibit either activity (this study; also Blackman *et al.*, 1994a). The hypothesis would explain the observed absence of a straightforward correlation between total serum anti-

MSP-1_{19} antibody levels, and immunity to blood-stage parasitemia in individuals naturally exposed to malaria; since many anti-MSP-1_{19} antibody specificities clearly have no effect on MSP-1 processing, and indeed can block the activity of antibodies with "protective", processing-inhibitory specificities, a simple evaluation of total anti-MSP-1_{19} serum antibody titres in a naturally-exposed individual may never provide a clear measure of the protective capacity of that antibody response. The additional fact, highlighted in this study, that blocking activity may also be mediated by naturally-acquired antibodies against MSP-1-derived components other than MSP-1_{19}, further complicates attempts to predict the protective capacity of an antibody response to MSP-1_{19} in the presence of a polyclonal response against the total MSP-1. The validity of the continued use of simple ELISA-based assays in epidemiological studies may therefore be questionable. Only a functional assay, such as one measuring MSP-1 processing inhibition, or the effect on invasion of affinity-purified antibodies (Chappel *et al.*, 1994), may provide an assessment of the overall protective capacity of an anti-MSP-1 antibody response. The critical test of this hypothesis will be the predictive power of the assay; preliminary data presented in chapter 3, section 3.4, appears promising and encouraging and further opportunities to evaluate this will arise from ongoing or future immunisation humans trials with MSP-1_{19} or MSP-1_{42}-based vaccines in which significant protection is achieved.

A further implication of this hypothesis is that, for an MSP-1_{19}-based vaccine to be effective, its design or mode of administration should be such that the overall balance of the induced antibody response is towards processing-inhibitory antibody specificities, rather than blocking specificities. Selectively inducing this type of functional antibody

response may be the major challenge in MSP-1_{19}-based vaccine development. Identification of epitopes involved in the induction of blocking and inhibitory antibodies by directed mutagenesis of MSP-1_{19} may provide data which can be used to improve the efficacy of a MSP-1_{19}-based vaccine. Currently, *E. coli* clones expressing mutant MSP-1_{19} have been obtained (unpublished data, S. Shai, I. Ling, T. Scott-Finnigan and A. Holder). These have been assayed in a series of epitope mapping experiments using a panel of anti-MSP-1_{19} mAbs by western blot and surface plasmon resonance analysis; preliminary data indicate that blocking antibody epitopes can be specifically deleted/removed by this approach without affecting epitopes recognised by processing-inhibitory antibodies (not shown). The next obvious step will be to determine the immunogenicity of these mutants in animal models and to assess whether their ability to induce blocking antibodies is reduced as compared to "wild-type" MSP-1_{19}. Mouse sera have been raised against the mutant MSP-1_{19} proteins and are expected to inhibit MSP-1 secondary processing more effectively than sera raised against wild-type MSP-1_{19}. If this expectation is confirmed, one of these mutants may represent an improved MSP-1_{19}-based malaria antigen candidate, that can be then tested in, for example, primates and human immunisation/challenge trials.

How do antibodies specific for the N-terminal domain of MSP-1 (MSP-1_{83}) exert blocking activity? There are no published structural data on the merozoite surface complex. However, treatment of intact merozoites with the bifunctional, cleavable cross-linker 3, 3′-dithio*bis*(sulfosuccinimidylpropionate) results in almost quantitative cross-linking of the MSP-1_{83} and MSP-1_{42} components of the complex (unpublished data, M.

Blackman), suggesting that at least in the conformation adopted by the membrane-bound form of the complex, these two polypeptides are spatially close. Given the additional fact that the molecular mass of an IgG molecule is not much less than that of the monomeric MSP-1 complex, the observation of steric competition between anti-MSP-1_{83} and anti-MSP-1_{19} antibodies is perhaps unsurprising. However, it is not clear why polyclonal antibodies reactive with the part of MSP-1_{83} represented by pME6 should selectively block binding of mAb 12.8, but not 12.10; presumably the two processing-inhibitory mAbs adopt quite distinct orientations on binding. Whatever the case, this work has provided the first experimental evidence that antibodies against one part of a merozoite surface protein can "shield" the parasite from the potentially harmful effects of antibodies directed against another part of the same surface protein. MSP-1_{83} is known to be immunogenic in human populations exposed to malaria (Blackman and Holder., 1993; Egan *et al.*, 1996); it is conceivable that it is advantageous to the parasite to evoke an antibody response to this part of MSP-1, and this may provide a selective pressure to prevent sequence variation in the conserved parts of the molecule.

Figure 15.

Conversion of MSP-1_{42} into MSP-1_{33} over 1 hour.

Approximately 10^9 FCB-1 merozoites were washed, then incubated for 1 hour at 37 °C in 50 mM Tris-HCl, pH 7.8, 10 mM $CaCl_2$, and 2 mM $MgCl_2$. The parasites were then solubilised into SDS sample buffer, and different volumes of the sample run on to individual wells of a 12.5% mini SDS polyacrylamide gel under non reducing conditions. The gel was blotted onto nitro-cellulose, and the blot probed with the rabbit anti MSP-1_{33} and subsequently with a radio-iodinated anti rabbit IgG. The position of MSP-1_{42} and MSP-1_{33} were detected by autoradiography on the dried blot, the areas of the blot containing the bands were excised and counted directly in a gamma counter for one minute. All samples were assayed in triplicate, and SE bars are indicated.

The graph shows that the number of counts associated with the MSP-1_{42} and MSP-1_{33} bands were proportional to the volume of solubilised merozoites loaded onto each track of the gel until the protein binding capacity of the membrane was saturated.

Figure 15.

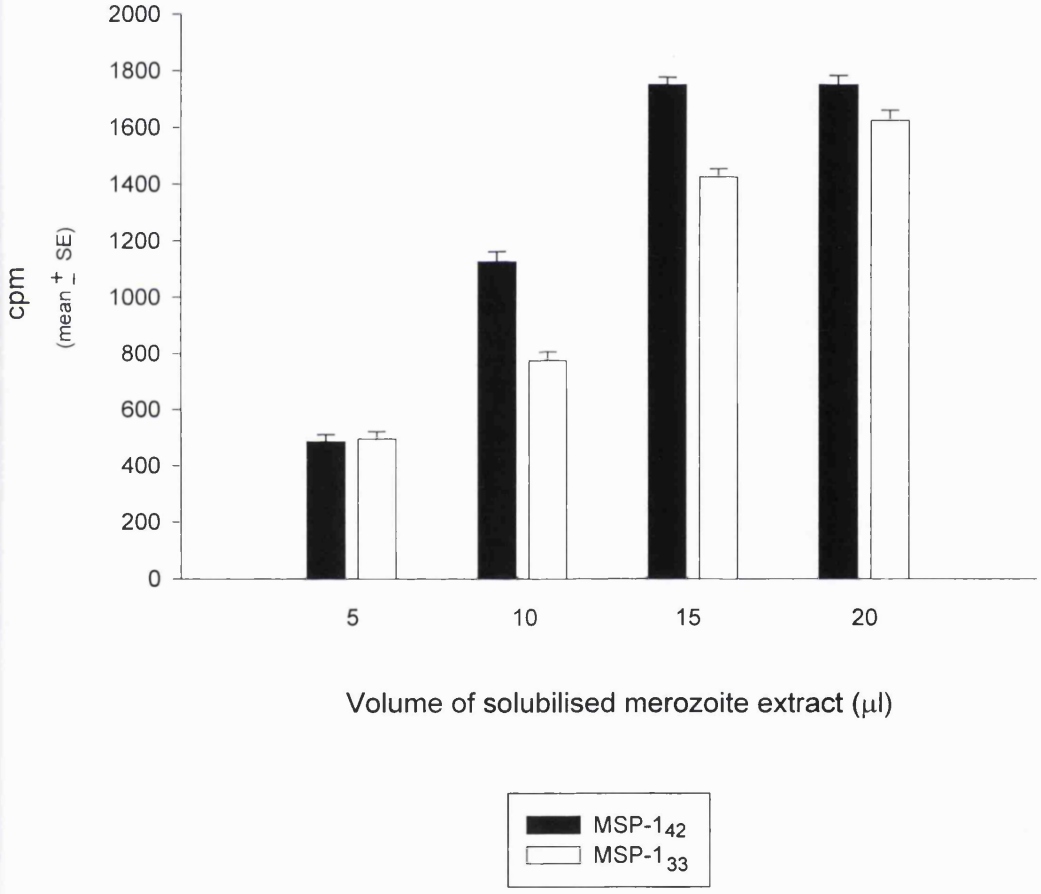

2000
1800
1600
1400
1200
1000
800
600
400
200
0
cpm
(mean ± SE)
5
10
15
20
Volume of solubilised merozoite extract (μl)
MSP-1 42
MSP-1 33

Figure 16.

Quantitation of MSP-1 secondary processing.

Approximately 10^9 FCB-1 merozoites were washed and resuspended in 50 mM Tris-HCl 10 mM $CaCl_2$ and 2 mM $MgCl_2$ on ice, and divided into seven 10 ul aliquots on ice. One aliquot was immediately SDS solubilised, whilst the remaining samples were incubated at 37 °C for 10, 15, 20, 30, 45 and 60 minutes before solubilisation. Processing of MSP-1_{42} to MSP-1_{33} was analysed using the modified assay described above. Shown is a graphic representation of the increase in the number of counts associated with the MSP-1_{33} concomitant with a decrease in the number of counts associated with MSP-1_{42} band at each time point. All samples were assayed in triplicate, and SE bars are indicated.

Figure 16.

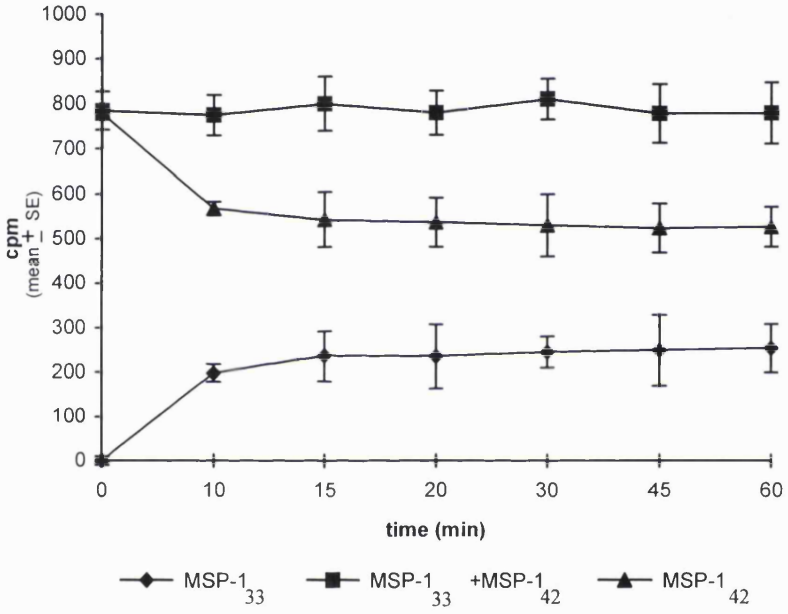

1000
900
800
700
600
500
400
300
200
100
0
cpm
(mean ± SE)
0
10
15
20
30
45
60
time (min)
MSP-1 33
MSP-1 33 +MSP-1 42
MSP-1 42

Figure 17.

Percentage antibody-mediated inhibition of MSP-1 secondary processing.

Isolated FCB-1 merozoites were washed and either solubilised into SDS directly or incubated for 1 hour at 37 °C in the presence of no antibodies; PMSF; mAb 12.10; 12.8; 1E1; 111.4; 8A12; 12D11; 7E5, 117.2, 2.2 and 7.5. Significant inhibitory activity 98% and 96% was respectively detected when merozoites were incubated with mAb 12.8 and 12.10. No significant inhibitory activity was detected in presence of mAbs 1E1, 111.4, 12D11, 7E5, 2.2 and 7.5. Mabs 8A12 and 117.2 inhibit MSP-1 secondary processing by 18% and 13% respectively. All samples were analysed by western blot using a radio-iodinated anti-rabbit IgG as second antibody. The number of cpm associated with the MSP-1_{33} band on the membrane were expressed as percentage inhibition, using the formula % inhibition = 100 - [{ X-B / A-B} x 100], where A is the amount of MSP-1_{33} (in cpm) in control samples treated with buffer only (1 hour control), B is the amount of MSP-1_{33} in samples treated with PMSF or SDS (i.e. background levels), and X is the amount of MSP-1_{33} produced in the presence of the mAb under test. All samples were assayed in triplicate, and SE bars are indicated.

Figure 17.

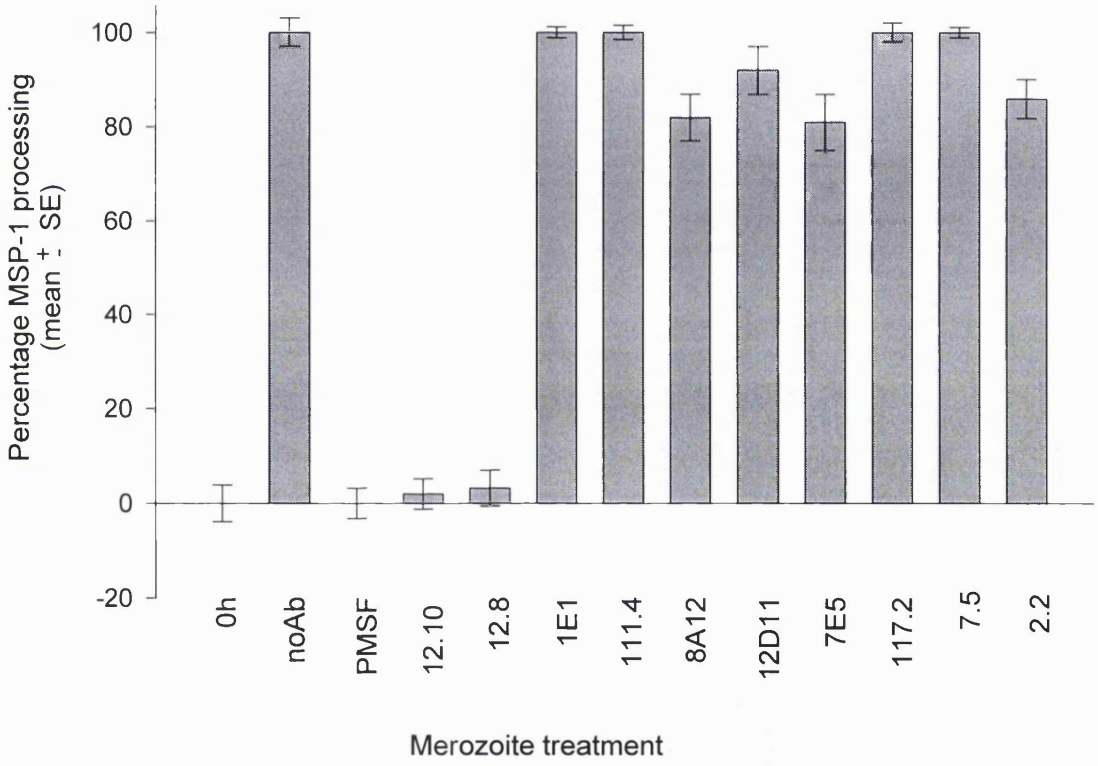

Percentage MSP-1 processing
(mean ± SE)
100
80
60
40
20
0
-20
0h
noAb
PMSF
12.10
12.8
1E1
111.4
8A12
12D11
7E5
117.2
7.5
2.2
Merozoite treatment

Figure 18.

Monoclonal antibodies directed against the native MSP1 recognise a merozoite membrane extract in ELISA.

This figure shows different mAbs raised against MSP-1_{19} reacting against the native MSP1 in a merozoite membrane extract (defined in Material and Methods) by ELISA. The ordinate represents Absorbance (492 nm) versus mAb concentration (μg ml^{-1}).

Figure 18.

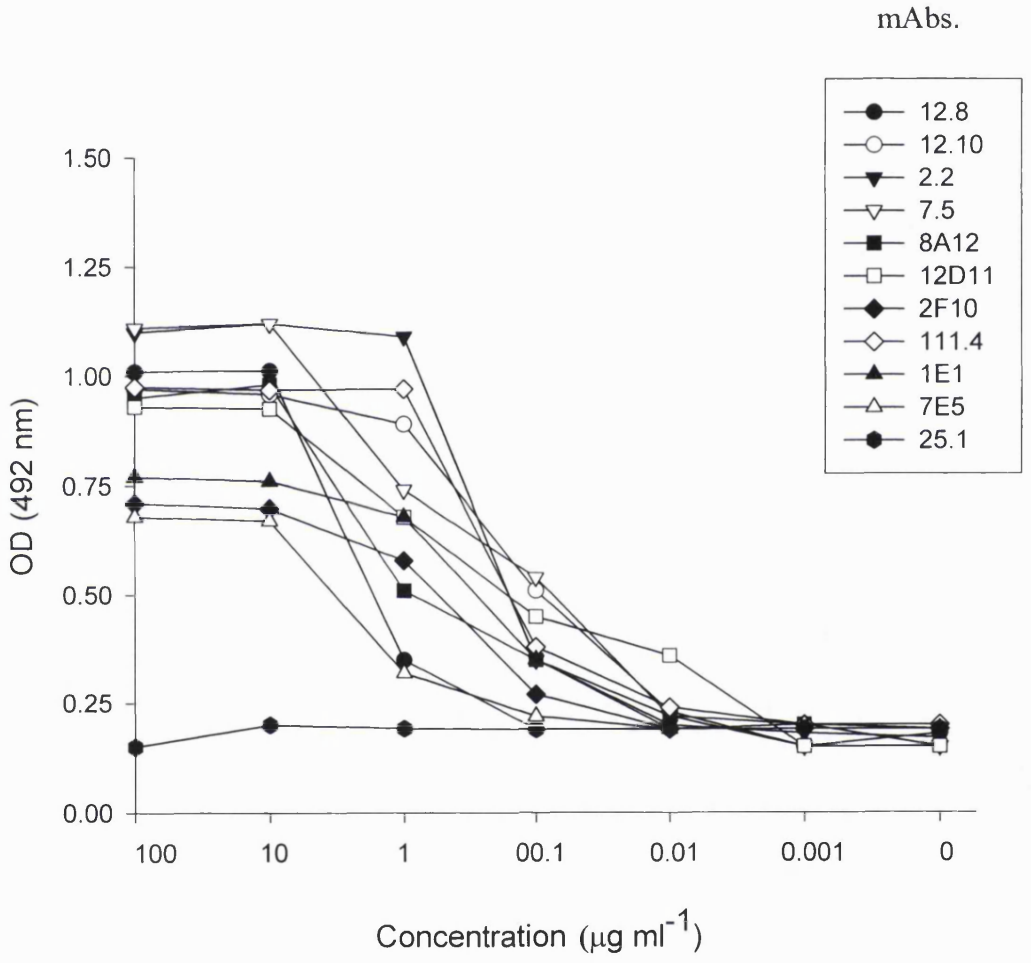

mAbs.
12.8
12.10
2.2
7.5
8A12
12D11
2F10
111.4
1E1
7E5
25.1
OD (492 nm)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
100
10
1
00.1
0.01
0.001
0
Concentration (μg ml-1)

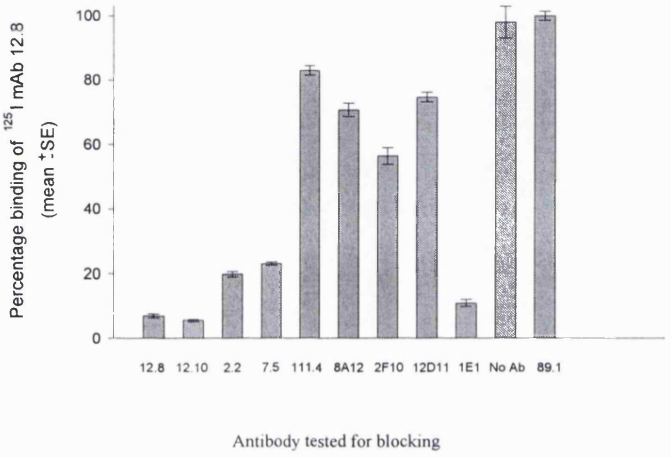
Figure 19.

The binding of processing-inhibitory mAbs 12.8 and 12.10 to FCB-1 merozoite-derived MSP-1 is competitively prevented by certain other anti-MSP-1 mAbs.

Plates coated with a merozoite antigen extract were preincubated in triplicate with either no antibody (No Ab; control wells), or with predetermined saturating concentrations of mAbs 12.8, 12.10, 2.2, 7.5, 111.4, 8A12, 2F10, 12D11, 1E1, or 89.1. The effects of this pretreatment on binding of radioiodinated mAbs 12.8 (A) or 12.10 (B) to the immobilized antigen was then assessed. All samples were tested in triplicate and SE bars are indicated. Blocking activity of individual mAbs was calculated as described in Materials and Methods..

Figure 19.

A


Percentage binding of ^{125}I mAb 12.8 (mean ± SE)
100
80
60
40
20
0
12.8 12.10 2.2 7.5 111.4 8A12 2F10 12D11 1E1 No Ab 89.1
Antibody tested for blocking

B

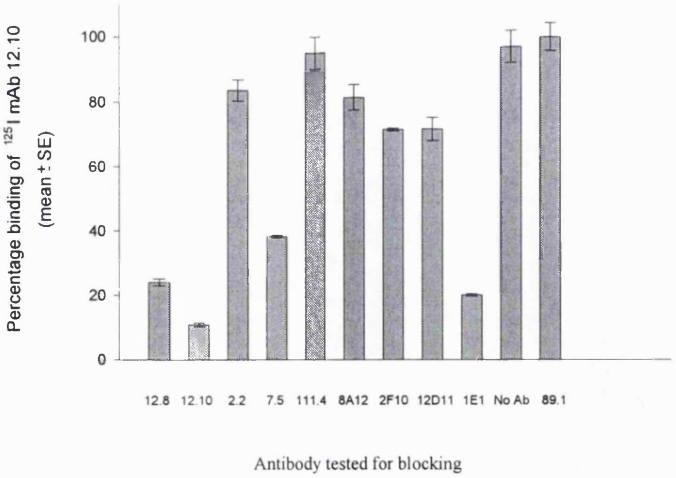

Percentage binding of ^{125}I mAb 12.10 (mean ± SE)
100
80
60
40
20
0
12.8 12.10 2.2 7.5 111.4 8A12 2F10 12D11 1E1 No Ab 89.1
Antibody tested for blocking

Figure 20.

Schematic of recombinant (pME) MSP-1 constructs relative to the MSP-1 gene and its products.

Shown is a digrammatic representation of the complete MSP-1 gene, divided into conserved (open) blocks 1, 3, 5, 12, and 17, semiconserved (hatched) blocks 7, 9, 11, 13, and 15, and poorly conserved or polymorphic (closed) blocks 2, 4, 6, 8, 10, 14, and 16, as defined by Tanabe *et al.* (1987). The positions of the MSP-1 primary processing products (MSP-1_{83}, MSP-1_{30}, MSP-1_{38} and MSP-1_{42}) are shown relative to the gene, as are the relative positions of the pME series of recombinant expression constructs against which polyclonal rabbit antisera have been raised (Holder *et al.*, 1985). IgG purified from the anti-pME rabbit sera was used in this study.

Figure20.

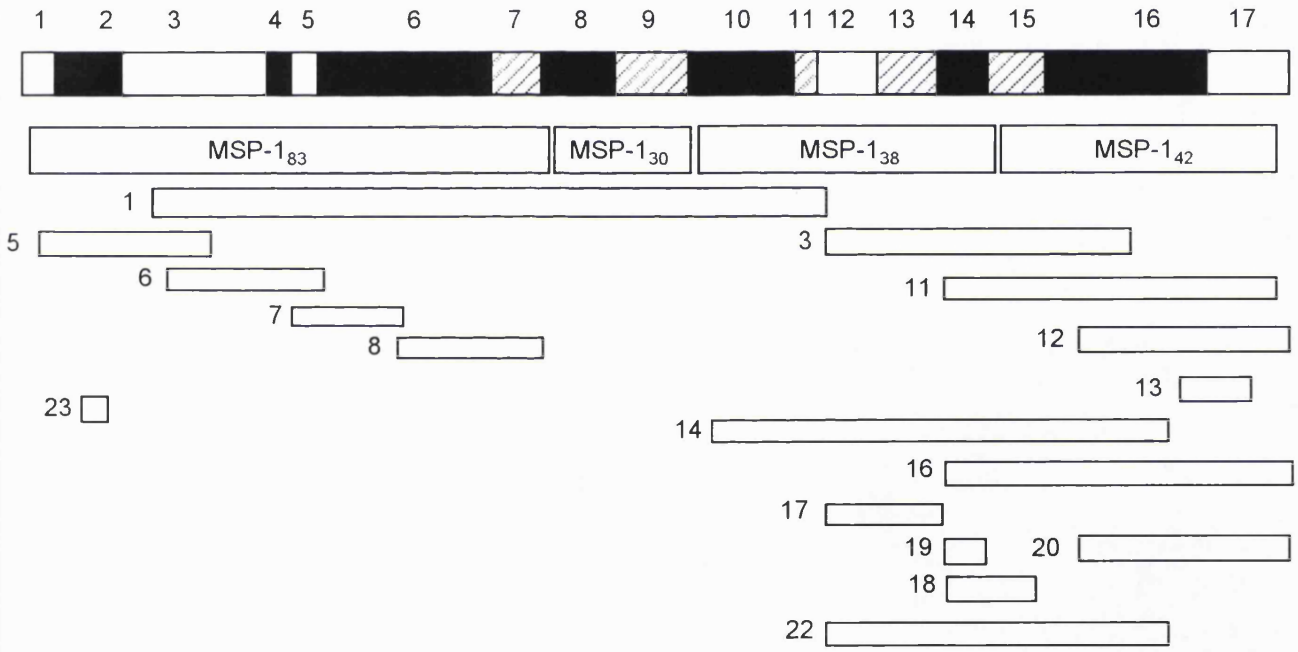

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
MSP-1$_{83}$
MSP-1$_{30}$
MSP-1$_{38}$
MSP-1$_{42}$
1
5
3
6
11
7
12
8
13
23
14
16
17
19
20
18
22

Figure 21.

Rabbit polyclonal antibodies raised against defined domains of MSP-1 have blocking activity.

Rabbit antibodies raised against recombinant MSP-1 expression constructs pME1, 3, 5, 6, 7, 8, 11, 12, 13, 14, 16, 17, 18, 19, 20, 22 and 23 were assayed at predetermined saturating concentrations for their ability to prevent binding of radioiodinated mAb 12.8 (A) or 12.10 (B) to immobilized FCB-1 merozoite antigen. Control wells were pretreated either with mAbs 12.8 or 12.10, or buffer alone (No Ab) or with a non-immune rabbit serum (NI Rs) at a final dilution of 1:100. All samples were assayed in triplicate, and SE bars are indicated.

Figure 21.

A

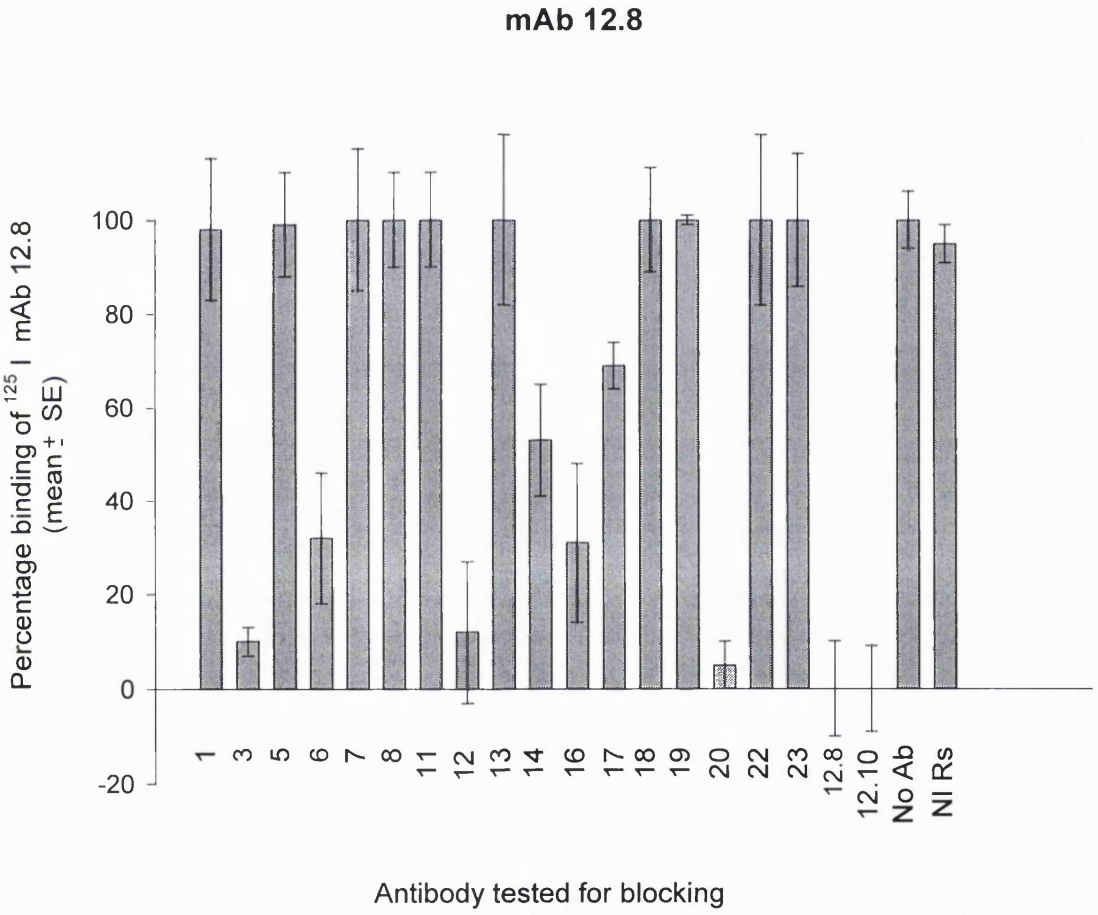

mAb 12.8
Percentage binding of ^{125}I mAb 12.8 (mean ± SE)
100
80
60
40
20
0
-20
1
3
5
6
7
8
11
12
13
14
16
17
18
19
20
22
23
12.8
12.10
No Ab
NI Rs
Antibody tested for blocking

Figure 21.

B

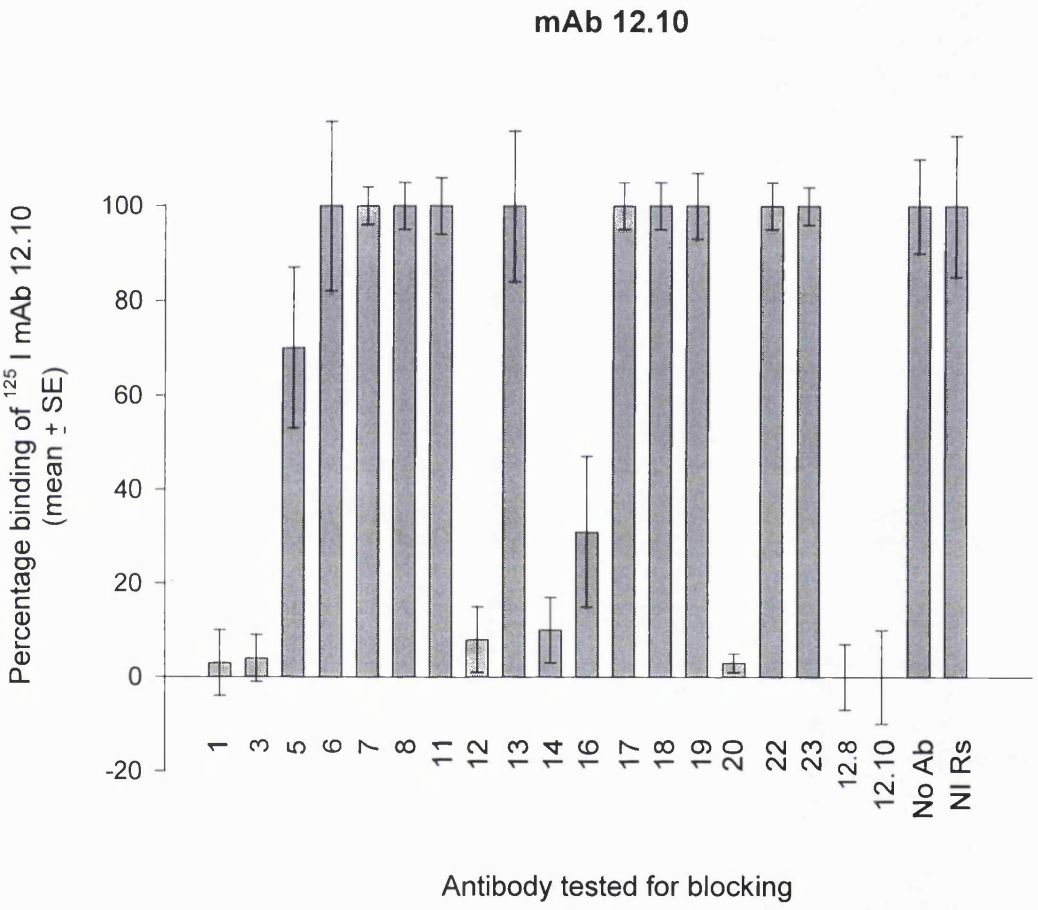

mAb 12.10
Percentage binding of ^{125}I mAb 12.10 (mean ± SE)
100
80
60
40
20
0
-20
1
3
5
6
7
8
11
12
13
14
16
17
18
19
20
22
23
12.8
12.10
No Ab
NI Rs
Antibody tested for blocking

Figure 22.

SDS PAGE analysis of the affinity purified human Ig anti MSP1-pME6.

Specific human Ig was affinity purified by chromatography on immobilised fusion protein pME6. Pooled immune human serum diluted of 1:4 in 50 mM Tris-HCl pH 8.0 was applied to the column. The column was washed extensively in 50 mM Tris-HCl pH 8.0, specific Ig was eluted in 8 M urea/50 mM Tris-HCl pH 8.0. Fractions were subjected to SDS PAGE on a 15% gel under reducing conditions in order to assess Ig presence and purity. Shown is a Coomassie blue stained gel; samples from each fraction are represented in track 2 to 10 respectively. Arrows indicate the position of Ig heavy (H) and light (L) chains. Molecular weight marker proteins indicated are myosin H-chain (200 kDa), phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa).

Figure 22.

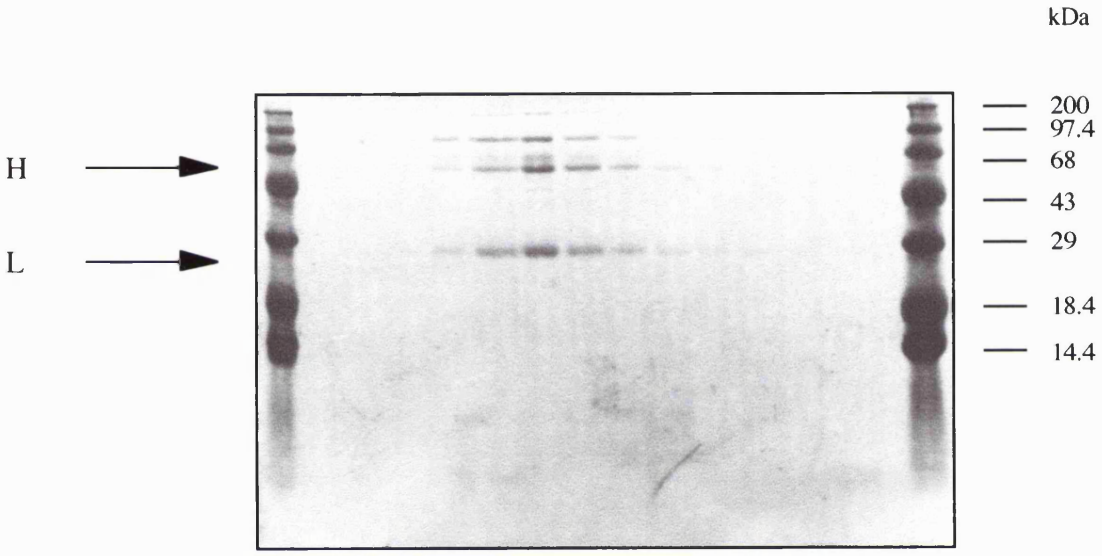


Figure 23.

Specificity of affinity-purified human anti-PME6 antibodies shown by western blot analysis.

An SDS extract of FCB-1 merozoites was subjected to SDS PAGE under nonreducing conditions on a 12.5% gel, transferred to nitrocellulose, then probed with; a sample of pooled human immune serum taken prior to chromatography over the pME6 affinity column (track 1); serum taken after passage over the column (track 2); affinity-purified anti-pME6 antibodies (track 3); mAb 89.1 (specific for MSP-1_{83}) track 4; and mAb 111.4 (specific for MSP-1_{42} and MSP-1_{19}) (track 5). Positions of molecular mass marker proteins are indicated, and bands corresponding to the MSP-1 precursor, MSP-1_{83}, MSP-1_{42} and MSP-1_{19} are arrowed.

Figure 23.

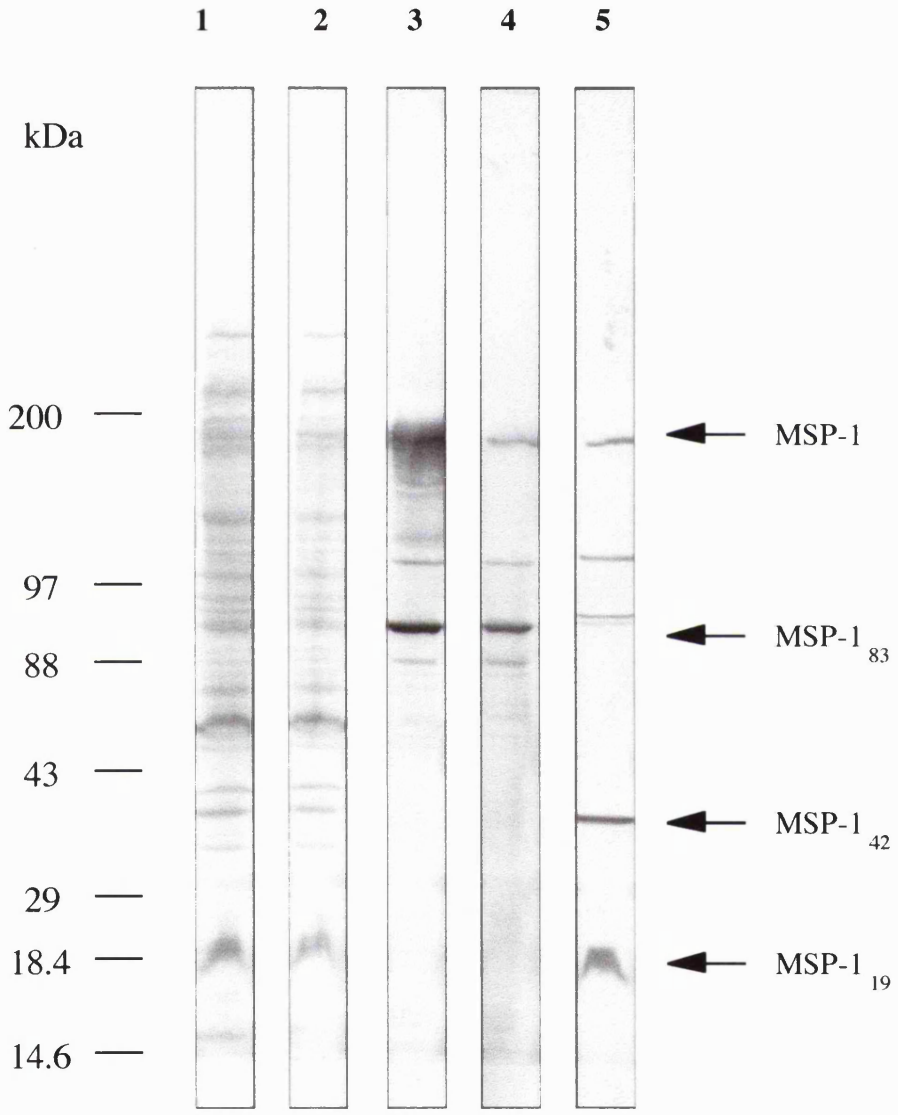


Figure 24.

Affinity-purified, naturally-acquired human anti-pME6 antibodies are potent blocking antibodies.

Panel A. Equal aliquots of washed FCB-1 merozoites were solubilised directly into detergent (0h control), or preincubated either with reaction buffer only or with affinity-purified human anti-pME6 antibodies at a final concentration of 300 μg ml^{-1}. An equal concentration of mAb 12.10 or 12.8 was then added to some samples as shown, and processing allowed to proceed for 1 hour in all but the 0h control. Inhibition of MSP-1 processing mediated by mAb 12.8 alone (96%) was almost completely reversed by preincubation with the anti-pME6 antibodies, whereas the inhibition of processing mediated by mAb 12.10 alone (97%) was completely unaffected by preincubation with anti-PME6 antibodies. **Panel B**. RIA plates coated with merozoite antigen were pretreated with non-radioactive mAb 12.10 or 12.8 at a saturating concentration (100 μg ml^{-1}), or affinity-purified anti-pME6 antibodies at a saturating concentration (300 μg ml^{-1}), or non-immune human serum (NI Hs) at an equivalent final antibody concentration, before assessing the ability of radioiodinated mAb 12.8 or 12.10 to bind. All samples were assayed in triplicate, and SE bars are shown.

Figure 24.

A

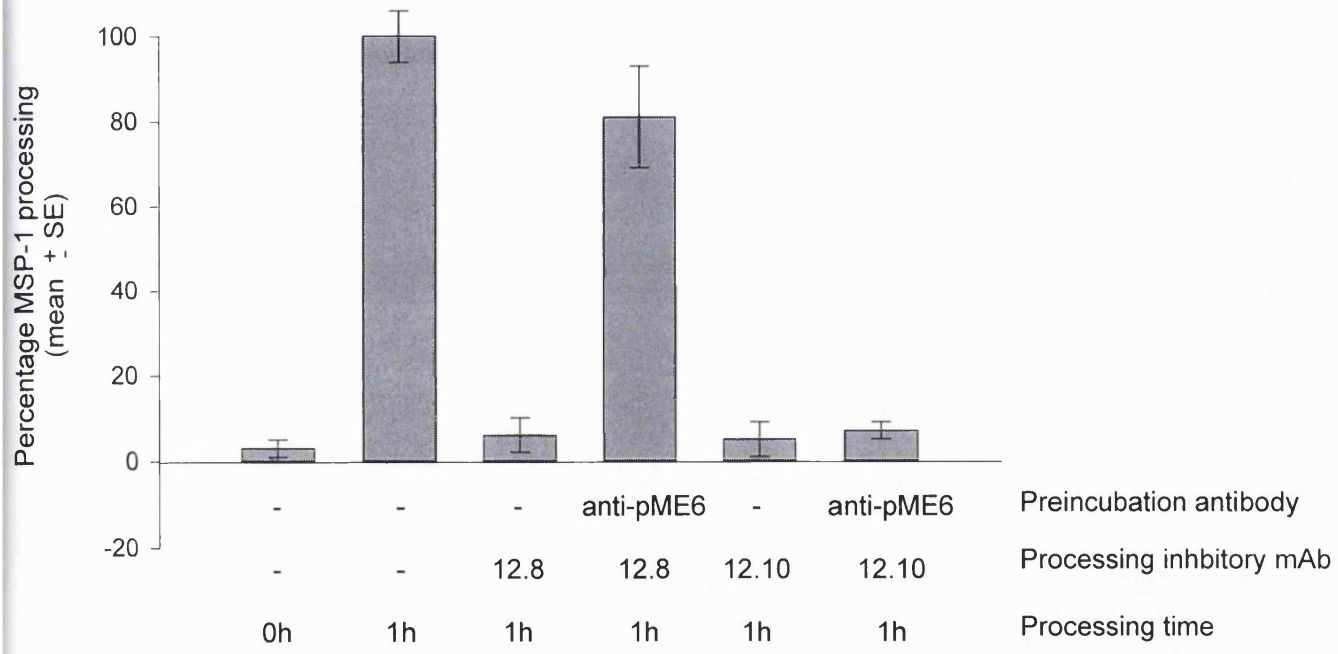
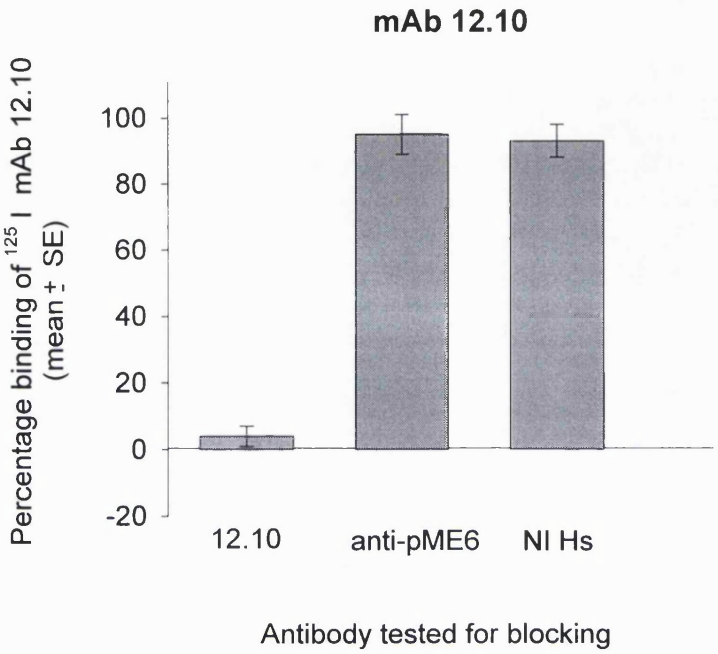

Percentage MSP-1 processing
(mean +- SE)
100
80
60
40
20
0
-20
- - - anti-pME6 - anti-pME6
Preincubation antibody
- - 12.8 12.8 12.10 12.10
Processing inhbitory mAb
0h 1h 1h 1h 1h 1h
Processing time

Figure 24.


mAb 12.10
Percentage binding of 125 I mAb 12.10 (mean ± SE)
100
80
60
40
20
0
-20
12.10
anti-pME6
NI Hs
Antibody tested for blocking

B

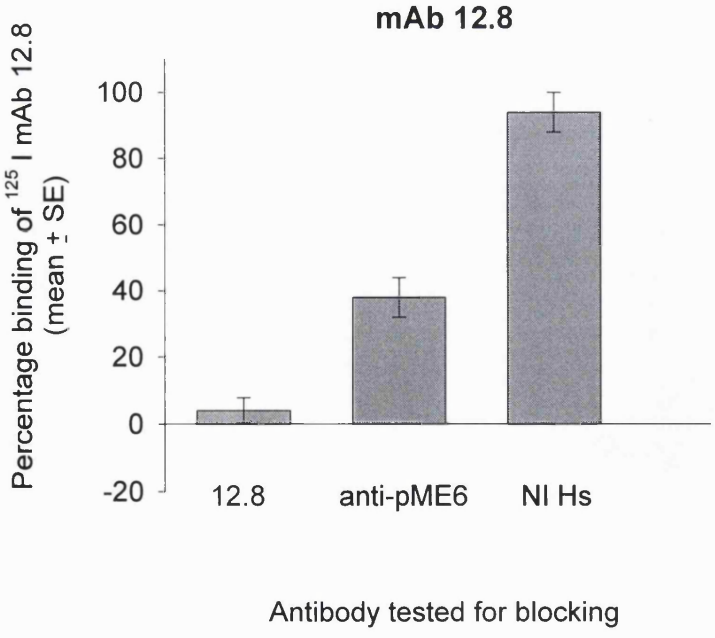

mAb 12.8
Percentage binding of 125 I mAb 12.8 (mean ± SE)
100
80
60
40
20
0
-20
12.8
anti-pME6
NI Hs
Antibody tested for blocking

Figure 25.

Processing-inhibitory anti-MSP-1_{19} mAbs can prevent MSP-1 and erythrocyte invasion *in vitro* culture, and can be rendered ineffective by the simultaneous presence of anti-pME6 blocking antibodies.

Panel A. Dose-response effect of mAb 12.10 on MSP-1 secondary processing. Metabolically radiolabeled T9/96 schizonts were supplemented with fresh erythrocytes and medium to obtain a parasitemia of approximately 2% and a haematocrit of 1%. The culture was then divided into equal aliquots and incubated at 37 °C in the presence of 5 mM EGTA as control inhibitor (track 1), or mAb 12.10 at a final concentration of 2 mg ml^{-1} (track 2), 1 mg ml^{-1} (track 3), 500 μg ml^{-1} (track 4), 400 μg ml^{-1} (track 5), 300 μg ml^{-1} (track 6), 200 μg ml^{-1} (track 7), 100 μg ml^{-1} (track 8), or no antibody (track 9). Schizont rupture and merozoite release was then allowed to proceed for 6 hours, and culture supernatants were analysed by immunoprecipitation using mAb X509 coupled to Sepharose for the presence of MSP-1_{33}. **Panel B**. Blocking anti-pME6 antibodies reverse the processing-inhibitory (top) and invasion-inhibitory (bottom) activity of mAb 12.8. Cultures containing metabolically radiolabeled T9/96 schizonts prepared as described above were incubated in the presence of 5 mM EGTA (track 1), 10% (v/v) non-immune human serum (NI Hs; track 2), anti-PME6 antibodies (track 3), mAb 12.8 (track 4), mAb 12.8 plus anti-pME6 antibodies (track 5), mAb 12.10 (track 6), mAb 12.10 plus anti-pME6 antibodies (track 7), mAb 89.1 (track 8), mAb 89.1 plus mAb 12.8 (track 9) and mAb 89.1 plus mAb 12.10 (track 10). In this case all antibodies were added to a final

concentration of 400 μg ml^{-1}. Analysis of 6 hour culture supernatants by immunoprecipitation with mAb X509 (panel B, top) was as above, and in addition erythrocyte invasion in individual cultures was assessed by counting the number of ring-stage parasites in 5,000 red cells, in triplicate (panel B, bottom). Invasion is expressed as a percentage of the ring-stage parasitemia (10%) obtained in a control culture with no additions (not shown).

Figure 25.

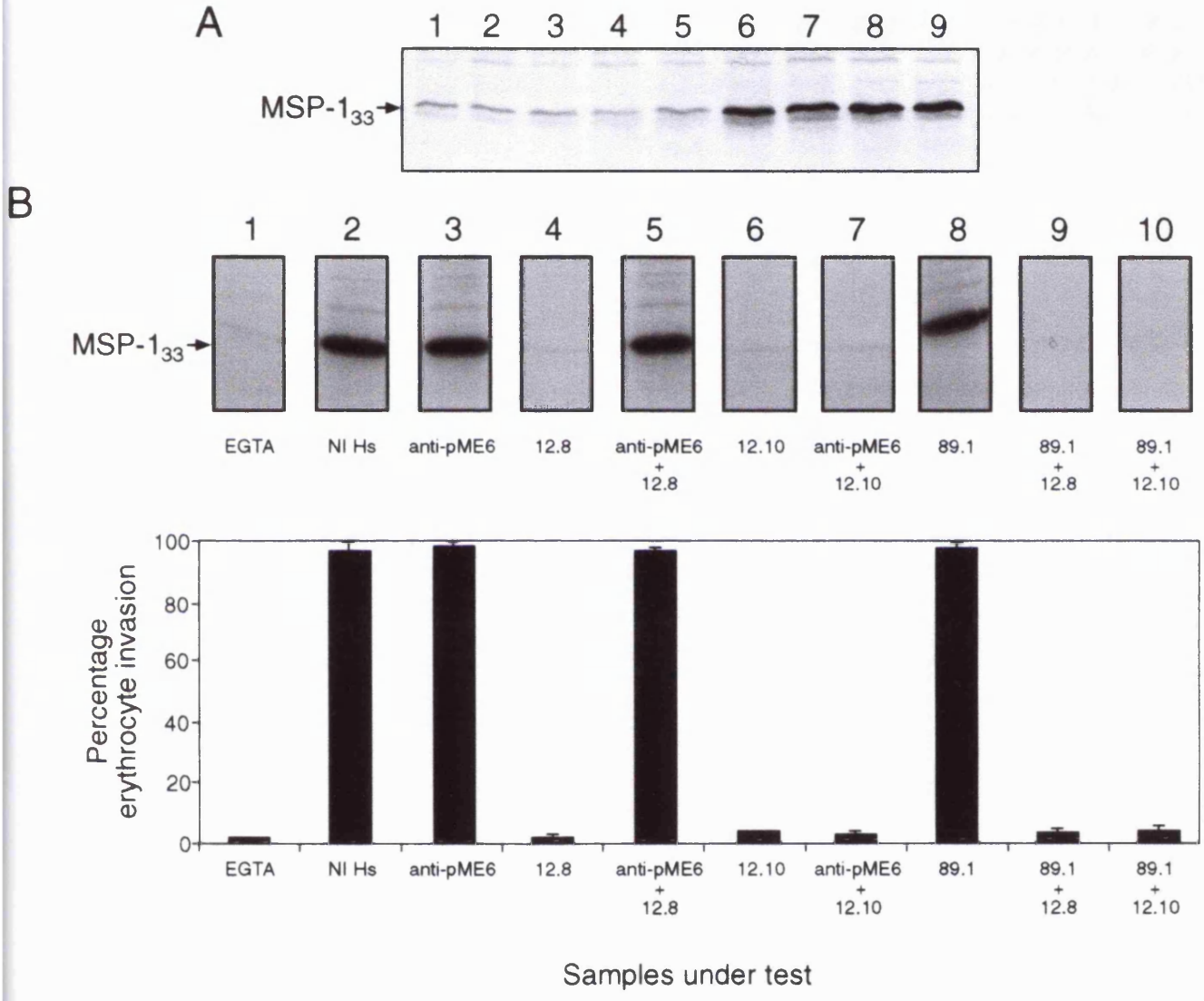

A
1 2 3 4 5 6 7 8 9
MSP-1$_{33}$
B
1 2 3 4 5 6 7 8 9 10
MSP-1$_{33}$
EGTA
NI Hs
anti-pME6
12.8
anti-pME6 + 12.8
12.10
anti-pME6 + 12.10
89.1
89.1 + 12.8
89.1 + 12.10
Percentage erythrocyte invasion
100
80
60
40
20
0
EGTA
NI Hs
anti-pME6
12.8
anti-pME6 + 12.8
12.10
anti-pME6 + 12.10
89.1
89.1 + 12.8
89.1 + 12.10
Samples under test

Table 6.

Regions of the MSP1 gene included within the expression constructs.

The numbers refer to the nucleotide position in the *P. falciparum* MSP1 gene sequence (Holder *et al.*, 1985)

Clone	First	Last
pME 1	876	3559
pME 2	3555	5920
pME 3	3555	4759
pME 5	455	1137
pME 6	959	1581
pME 7	1460	1899
pME 8	1868	2445
pME 11	4043	5260
pME 12	4459	5920
pME 13	4926	5230
pME 14	c.3120	c.4910
pME 15	c.4000	5920
pME 16	c.4000	5920
pME 17	3559	4028
pME 18	4112	4530
pME 19	4079	4190
pME 20	4459	5920
pME 21	4459	5920
pME 22	3555	c.4910
pME 23	623	747

CHAPTER 6.

STRUCTURAL STUDIES ON MSP-1_{19}

6.1 Introduction.

MSP-1_{19} has been identified as a target of protective antibodies capable of interfering with erythrocyte invasion. In the previous sections, the major aim was to study the ability of several antibodies to inhibit the secondary processing of MSP-1. In this section, in order to understand the structures recognised by antibodies which bind to MSP-1_{19} and to elucidate the mechanisms by which antibodies inhibit MSP-1 processing, it was considered necessary to initiate a study of the structure of the native MSP-1_{19}.

There are few data on MSP-1_{19} structure. As discussed in the Introduction, MSP-1_{19} contains 12 of the 19 cysteines residues in the total MSP-1 sequence. An analysis of the spacing of cysteine residues in the primary sequence of MSP-1_{19} has led to the proposal that the molecule is a highly folded structure maintained by disulphide bonds, and may be composed of two epidermal growth factor (EGF)-like domains (Figure 4, Blackman *et al.*, 1991).

A GPI-attachment signal is deduced from cDNA sequence (Holder *et al.*, 1985) and early experiments (Haldar *et al.*, 1985; Schwarz *et al.*, 1987; Holder *et al.*, 1985) identified a GPI-anchor present in MSP-1 by metabolically labelling the GPI precursor

with sugar, fatty acids and ethanolamine. More recently, Gerold *et al.* (1994; 1996) have established the structure of the GPI anchor associated with both MSP-1 and MSP-2. The GPI anchor is the only post-translational incorporation unambiguously shown to be present on MSP-1_{19}.

Dieckmann-Shuppert *et al.* (1992) have previously reported the lack of direct evidence of *N*-glycosylation on *P. falciparum* proteins. In contrast, Kimura *et al.* (1996) presented evidence for *N*-linked glycosylation in *P. falciparum*, and suggested an essential role for these structures in the development of the asexual erythrocytic forms of the parasite. *O*-linked glycosylation of *P. falciparum* proteins has also been reported; Ud-Din *et al.* (1992) demonstrated the presence of *O*-linked *N*-acetylglucosamine in the MSP-1 precursor protein present in schizonts. The relevance of oligosaccharides in interactions between the malaria parasite and its host have not been completely addressed. However, in certain parasites, glycoconjugates have been shown to be important in mechanisms of host cell penetration, cellular growth, host immunity regulation, and differentiation (Zingales *et al.*, 1985; Funk *et al.*, 1994). In other biological systems, covalent modifications of proteins with acyl or glycosyl moieties is known to alter the immunogenicity and pathogenicity of the organism. For example, gram-negative bacteria posses a triacylated protein responsible for priming cytotoxic T cells (Deres *et al.*, 1989), activating B lymphocytes (Besslet *et al.*, 1985), and stimulating phagocytosis and cytokine production by macrophages (Hauchildt *et al.*, 1990). Furthermore, surface glycoproteins of *Trypanosoma* spp have been found to be essential for parasite survival and infectivity (Kamper and Barbet, 1992). *E. coli* derived-MSP-1_{19} is currently a malaria vaccine

candidate, and as prokaryotes lack the metabolic machinery to introduce certain post-translational modifications, such as *N*-glycosylation, it was considered essential to establish whether parasite-derived MSP-1_{19} possesses such modifications that may alter the immunogenicity of the molecule.

In order to study the structure of the native MSP-1_{19} two approaches were taken. The first one was to look for the presence of glycosylation within MSP-1_{19} using a lectin binding assay. The second approach was to look for the presence of any post-translational modifications by determining and comparing the actual molecular mass of native and recombinant MSP-1_{19} by a combination of peptide-mapping and high performance liquid chromatography/mass spectrometric (HPLC/MS) analysis.

6.2 Results.

6.2.1 Determination of possible oligosaccharides on $MSP1_{19}$.

Post-translational modification of parasite proteins (by, for example, glycosylation) may be important to the antigenicity of specific domains. For a better understanding of the antigenicity of MSP-1_{19} and its relation with antibodies, it was considered necessary to investigate possible glycosylation of MSP-1_{19}. The typical *N*-linked oligosaccharide structure contains carbohydrate, amide-linked to an asparagine residue in the sequence Asn-X-Ser/Thr where X is any amino acid residue other than proline. This sequence motif is present at one position in the primary sequence of $MSP1_{19}$ and is the only motif expected to be a target for *N*-glycosylation. Two pieces of evidence argue against the presence of *N*-linked sugars within the MSP-1_{19} domain; the presence of *N*-linked oligosaccharides in MSP-1_{19} has been partially discarded in previous studies by Ramasamy (1987); and during Edman degradation of MSP-1_{19} a clear asparagine was detected in the first sequencing cycle, providing evidence against the notion that $MSP1_{19}$ is *N*-glycosylated (Blackman *et al.*, 1991). However, it remains possible that *O*-linked oligosaccharides may be present within MSP-1_{19}; although there is no general acceptor motif for this type of modification, and there are numerous Ser and Thr residues sites of potential *O*-glycosylation within $MSP1_{19}$.

This study attempted to determine the presence of *N*-linked oligosaccharides and/or *O*-linked oligosaccharides on the native membrane-bound $MSP1_{19}$. To address this

point a lectin binding assay was used. FCB-1 merozoites were purified as described in Material and Methods, and incubated at 37 °C to allow MSP-1 processing to take place. The merozoites were then extracted in 1% NP-40 for 1 hour on ice, and the mixture was centrifuged. The supernatant was solubilised in SDS PAGE sample buffer and run on a 15% SDS gel under non-reducing conditions. Polypeptides were transferred onto NCP, which was blocked with 1% Tween 20/PBS overnight at 4 °C. Individual nitrocellulose strips, each containing two tracks (one track of non stained high molecular weight markers, and one track of solubilised merozoites) were individually incubated with a biotinylated lectin. Bound biotinylated lectin was detected following the addition of HRP-streptavidin by adding OPD substrate and allowing development of the blot for 15 minutes.

Figure 26 shows that although 5 out of the 6 lectins used recognised the control glycoprotein (ovalbumin), none of them recognised a band recognisable as MSP-1_{19}; however, lectins lycopersicon, *Vicia villosa* and *Bandeiraea* reacted in each case with a single, higher molecular weight merozoite polypeptide. With biotinylated jacalin (track 1), no glycoprotein bound was observed on merozoites; lycopersicon (track 2), bound to a glycoprotein of 90 kDa; with *Solanum tuberosum* (track 3), no reaction was observed; with *Vicia villosa* (track 4), a glycoprotein of 83 kDa was detected; with *Bandeiraea* (track 5), a glycoprotein of 83 kDa was detected. All 5 lectins bound to ovalbumin (43,000 Da) present in each complementary high molecular weight marker track. *Datura stramonium* (track 6) did not react with any of the protein markers, nor with any protein in merozoite extract.

It should be noted that the non-reactivity of jacalin with merozoites suggests the absence of *O*-linked oligosaccharides on $MSP1_{19}$. However, these results cannot be considered conclusive until further studies have been carried out using the above lectin assay with a saturating amount of nitrocellulose-immobilised merozoite proteins. An analysis of purified, parasite-derived (native) $MSP\text{-}1_{19}$ by HPLC/MS will provide more conclusive information on post-translational modification of the protein.

6.2.2 Digestion of recombinant $MSP\text{-}1_{19}$ by well characterised proteases.

This part of the study describes attempts to assess whether $MSP\text{-}1_{19}$ can be digested under non-reducing conditions to produce a restricted number of digestion products. Since it is known that the native protein is modified by the addition of a GPI anchor at the C-terminus, it was considered that, were it possible to cleave the protein into two fragments (corresponding roughly to the two EGF-like domains), subsequent mass spectrometric analysis of the N-terminal fragment could relatively quickly rule out or suggest the presence of post-translational modifications in this part of the protein. Previous binding studies using a panel of conformation-dependent mAbs has indicated that the recombinant $MSP\text{-}1_{19}$ is structurally similar to the native protein (Chappel and Holder, 1993; Burghaus and Holder, 1994) Since the recombinant protein is relatively easy to produce in large quantities, preliminary studies concentrated on using the recombinant protein to determine which protease (if any) can produce a single cleavage in the sequence of the recombinant $MSP\text{-}1_{19}$. It was decided that, if it were possible to produce two single fragments, work would then turn to use of the native $MSP\text{-}1_{19}$; purified parasite-derived

MSP-1_{19} and digestion products of it could be used to accurately measure the actual molecular mass of (initially) the intact MSP1_{19} and its N-terminal "half", and to assess the presence of post-translational modifications on any of the digestion products using mass spectrometry.

Purified recombinant MSP-1_{19} was analysed using reverse phase chromatography; the sample was not resolved into separate components under the conditions used, and only one major species eluted from the reverse phase column (Figure 27). Also, analysis of the starting material by Tricine SDS-PAGE did not indicate the presence of any dimerised polypeptides (not shown). On this basis it was concluded that the recombinant MSP-1_{19} was present in a single major conformation.

Samples of the protein were protease-treated at room temperature for 30 minutes. Digestions were rapidly halted using 1 mM PMSF and addition of non-reducing SDS sample buffer. The site of cleavage for each protease is shown as follows;

papain, -x-/-y-

(x=non-specific, but Arg, Lys and residues following Phe preferred; y=non-specific).

chymotrypsin, -x-/-y-

(x=aromatic [Trp, Tyr, Phe], Leu, Met, Ala; y=non-specific);

trypsin, -x-/-y-

(x=Lys, Arg; y=non-specific);

pronase -x-/-y-

(x,y=non-specific);

pepsin -x-/-y-

(x=non-specific, but aromatic and other hydrophobic [esp. Phe, Leu] preferred; x,y=Val, Ala, Gly).

Figure 28A shows the results of these experiments. After 30 minutes of digestion with trypsin, pronase, papain or chymotrypsin, the recombinant MSP-1_{19} appeared to be unaffected. Figure 28B shows that following digestion with pepsin the protein migrated on Tricine-SDS PAGE as a single band but appeared significantly larger at each time point under reducing conditions. It should be noted that although the mobility of the protein decreased, the intensity of the signal did not change during the incubation time and the presence of any other digestion product was not detectable. A similar result was also found in the digestion of the recombinant first motif of the MSP-1_{19} (*P. falciparum* EGF1A). It was concluded that the point of cleavage or the modification mediated by the protease probably takes place in the first EGF-like motif of MSP-1_{19} (Figure 28C).

Although pepsin was shown to affect the mobility on SDS PAGE of the recombinant MSP-1_{19}, it failed to produce a single cleavage in the sequence; in addition,

extensive digestion with trypsin and chymotrypsin failed also to release any peptide (Figure 28A). The attempt to digest the recombinant undenatured MSP-1_{19} by proteases was abandoned. However, these results confirm that MSP-1_{19} is resistant to digestion by trypsin and chymotrypsin and is consistent with the results of Holder and Freeman, (1984) and Chappel, (1993) in which they found that native merozoite-derived MSP1_{19} on the surface of the merozoites is resistant to digestion by trypsin and chymotrypsin. The evidence presented does suggest that the recombinant MSP-1_{19} expressed in *E. coli* apparently has a similarly "protected", highly folded structure which renders it resistant to the degrading action of different proteases, probably conferred by the cysteine-rich nature of the domain.

In parallel, to check for the possible presence of point mutations in the sequence of the recombinant MSP-1_{19}, the insert of the recombinant pGEX-3X plasmid was re-sequenced. No mutations were observed in the sequence. rMSP-1_{19} was analysed by liquid chromatography-electrospray mass spectrometry and a single peak identified with a molecular mass of 11057.26 ± 3.87 Daltons (Figure 29). This molecular mass does not differs significantly from the expected molecular mass of the primary sequence of the recombinant protein, which is 11061.28 Daltons. There is therefore no evidence for any post-translational modification of the recombinant protein.

6.3 Discussion.

In this part of the study, structural studies were carried out. In the first section, the study of possible post-translational modifications (glycosylation) within MSP-1_{19} was assessed by a lectin binding assay. The results achieved are far from conclusive at present; however, it should be noted that none of the six lectins used recognised a band distinguishable as MSP-1_{19}, suggesting the absence of *N*- and/or *O*-glycosylation within MSP-1_{19} on the merozoite. Further lectin studies should be carried out and use should be made of Concanavalin A (ConA recognises a commonly used sugar structure, α-linked mannose) in order to confirm the presence of the GPI anchor, providing a control for the presence of the glycoprotein MSP-1_{19}; in addition, a saturating amount of nitrocellulose-immobilised merozoite proteins will be used to guarantee the presence of a significant amount of glycoproteins.

In the second section, attempts to assess whether recombinant MSP-1_{19} can be digested under non-reducing and non denaturing conditions to produce a restricted number of digestion products were unsuccessful. However, these results suggest that the recombinant MSP-1_{19} apparently has a "protected" highly folded structure. The fact that recombinant MSP-1_{19} is resistant to well characterised proteases is consistent with the results of Chappel (1993a) in which the author found that the native, merozoite-derived MSP-1_{19} on the surface of the merozoite is also resistant to digestion by trypsin and chymotrypsin. Udhayakumar *et al.* (1995) showed that B cell epitopes in the conserved region MSP-1_{19} (block 17) were widely recognised by natural immune sera from Kenyan

residents exposed to *P. falciparum* malaria, whereas T cell proliferative responses were predominantly against epitopes in the dimorphic region of MSP-1_{33}. Egan *et al.* (1997) in a study aimed to dissect the relationship between cellular and humoral immune response to defined epitopes of the C-terminus of *P. falciparum* MSP-1 in immune blood donors, showed that sera from almost all donors contained antibodies to MSP-1_{33} (MAD20 allele) and that these antibodies did not cross-react with the equivalent sequence of the Wellcome allele. In contrast, T-cell responses to MSP-1_{33} were directed against conserved regions within the dimorphic MSP-1_{33}. The antibody response appeared to be mainly against MSP-1_{33} (93%) and at a lower proportion (50%) against MSP-1_{19}. However, a T-cell response against recombinant MSP-1_{19} was found in only 26% of the donors. Additionally, Chang *et al.* (1996) showed that antibodies contained in sera from primates vaccinated with BVp42 are mainly directed against epitopes within MSP-1_{19}. In this study it was also shown that BVp42-primed lymphocytes of these animals did not undergo proliferation when stimulated *in vitro* with recombinant MSP-1_{19}. These results might have implications in the design of an MSP-1-based malaria vaccine. For successful MHC class II antigen presentation by antigen presenting cells, reduction of disulphide bonds appears to required (Collins *et al.*, 1991). Reduction and alkylation of recombinant *P. yoelii* MSP-1_{19} has been shown to abrogate any *in vivo* protection mediated by folded recombinant *P. yoelii* MSP-1_{19} (Ling *et al.*, 1995). Also two mAbs 12.8 and 12.10 which have the ability to interfere with erythrocyte invasion and MSP-1 secondary processing are specific for conformational epitopes within *P. falciparum* MSP-1_{19} (Blackman *et al.*, 1992; 1994a). It has been hypothesised that induction of growth-inhibitory anti-MSP-1_{19} antibodies is determined by the specificity of T cell epitopes located in MSP-1_{33}

(Udhayakumar *et al.*, 1995). The importance of these T cell epitopes in the induction of a protective immune response has not been established and further characterisation will be required. However, these data in addition to the results shown in the present work suggest that through its resistance to protease degradation, folded recombinant MSP-1_{19} may not be capable of being correctly processed and presented by antigen presenting cells, in turn leading to an inability to induce an effective T cell response.

In order to assess the presence of post-translational modifications within rMSP-1_{19} and the molecular mass of MSP-1_{19}, the rMSP-1_{19} was analysed by liquid chromatography-electrospray mass spectrometry, evidencing a molecular mass of 11057.26 ± 3.87 Daltons which differs by only four Daltons from the expected molecular mass.

The insert of the recombinant pGEX-3X plasmid was re-sequenced to assess the possible presence of point mutations in the sequence; no mutations were observed. Differences between recombinant and native MSP-1_{19} have not been detected. However, purification of the membrane bound *P. falciparum* MSP1_{19} should be carried out in order to assess by mass spectrometric analysis the actual size of the native MSP1_{19}.

Figure 26.

Lectin analysis for glycosylation of the *P. falciparum* MSP-1_{19}.

Washed merozoites were resuspended in 20 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$, 2 mM $MgCl_2$, in the presence of protease inhibitors as described before, then incubated for 1 hour at 37 °C. Immediately after incubation samples were resuspended in an equal volume of NP-40, incubated for 1 hour on ice, and then solubilised in SDS sample buffer and boiled for 5 minutes. 15 µl of each sample was run on a 15% SDS minigels under non reducing conditions. Proteins were transferred onto NCP. The membrane was blocked with 0.1% Tween 20 in PBS overnight at 4 °C. Non stained high molecular markers were used as glycosylated controls. Membranes were incubated in the presence of different biotinylated lectins (Vector). Jacalin (track 1); lycopersicon (track 2); *Solanum tuberosum* (track 3); *Vicia villosa* (track 4); *Bandereira* (track 5) and *Datura stramonium* (track 6). Presence of bound lectins was detected with streptavidin-HRP (Pierce) at a final concentration of 1:2000 diluted in 0.1% Tween 20 in PBS and developed in the presence of 4-chloro napthol substrate for 15 minutes. This figure shows that although 5 of the 6 lectins used recognised the control (ovalbumin), none of them recognised a band recognisable as MSP-1_{19}. No reactivity of jacalin with any merozoite polypeptides was observed.

Figure 26.

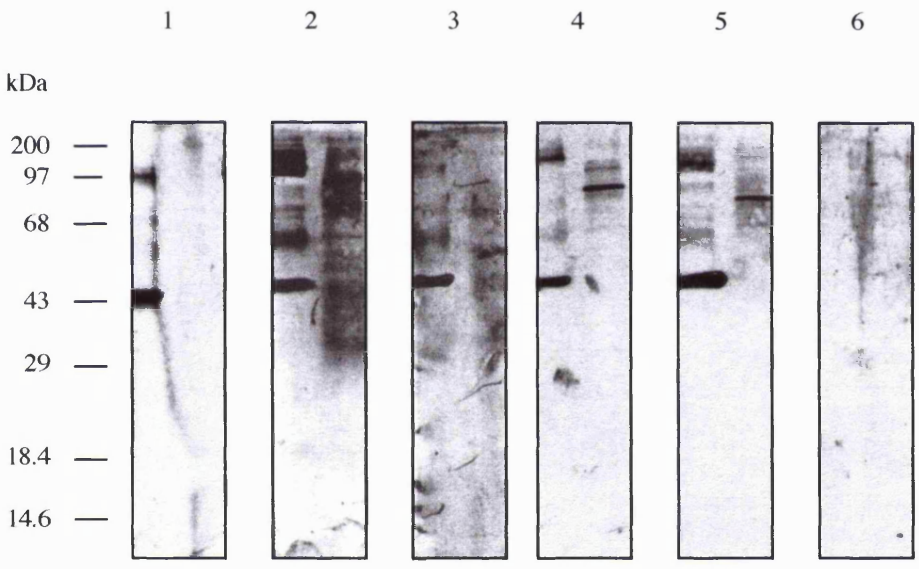


Figure 27.

Reverse phase HPLC analysis of the recombinant MSP-1_{19}.

Recombinant MSP-1_{19} at a final concentration of 1mg ml^{-1} was analysed by reverse phase HPLC in order to determine purity and the presence of multimers of the protein.

This figure shows the A_{230} profile of a sample of the polypeptide analysed by reverse phase HPLC, eluted from a C_{18} column with a linear gradient of 0-100% acetonitrile (red dotted line) in water over 100 minutes. The majority of the polypeptide was eluted as a single peak in 35% acetronitrile.

Figure 27.

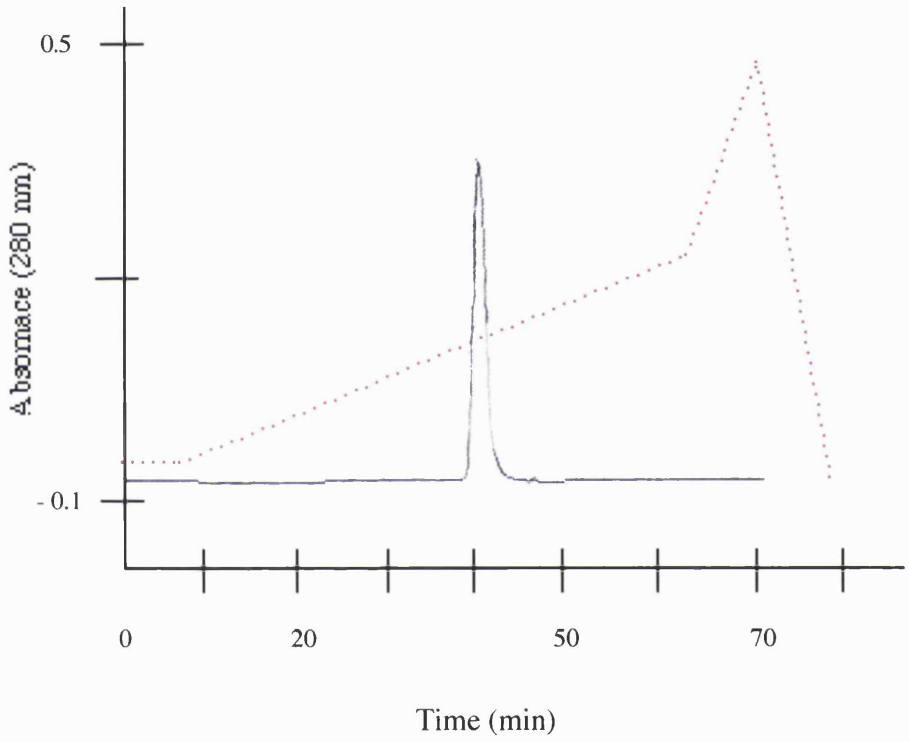


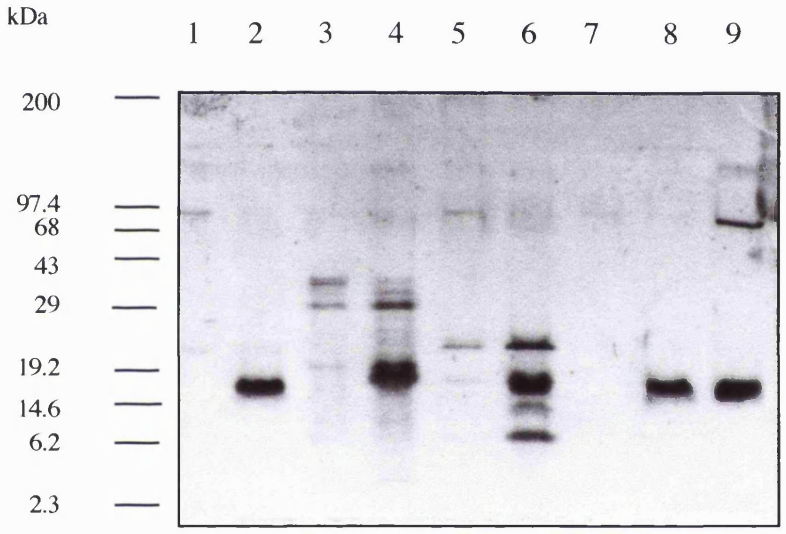
Figure 28.

Digestion of the recombinant MSP-1_{19} by well characterised proteases.

Panel A. Tricin-gel Serva blue stained showing the effects of well characterised proteases on recombinant MSP-1_{19}. The protein was resuspended in 100 mM Tris-HCl pH 8.0, 10 mM $^{+2}$Ca, in the presence of proteases trypsin, chymotrypsin, pronase, and papain at a molar ratio of 1:20 protease/protein and then incubated at room temperature for 1 hour; reactions were halted by addition of PMSF and resuspending in non reducing SDS sample buffer, then boiled for 1 minute. Samples were analysed by SDS-Tricin PAGE under reducing conditions and stained. Shown is; Trypsin (lane 1), recombinant MSP-1_{19} in presence of trypsin (lane 2), pronase (lane 3), recombinant MSP-1_{19} in presence of pronase (lane 4), pepsine (lane 5), MSP-1_{19} plus pepsine (lane 6), chymotrypsin (lane 7), chymotrypsin and recombinant MSP-1_{19} (lane 8), reduced recombinant MSP-1_{19} (lane 9). Molecular weight marker proteins indicated are ovalbumin (44 kDa), carbonic anhydrase (29 kDa), β lactoglobulin (19 kDa), lysozyme (14.4 kDa), bovine trypsin inhibitor (6.4 kDa), insulin (3.0 kDa).

Figure 28.

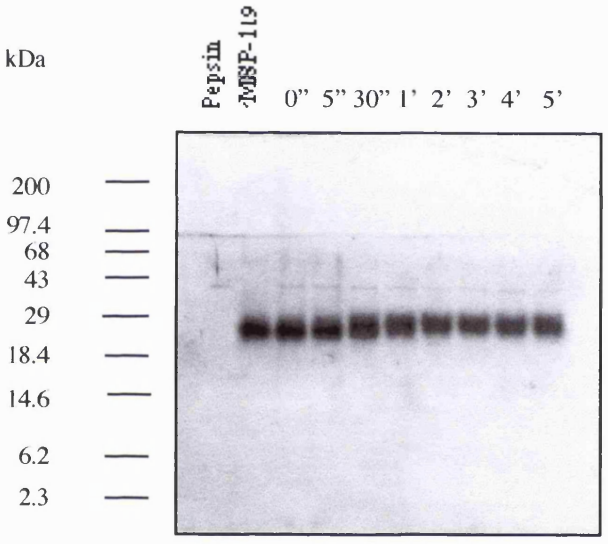
A



Panel B. Shown is digestion of recombinant MSP-1_{19} by pepsin in a time course. Pepsin (lane 1), reduced recombinant MSP-1_{19}, (lane 2); zero time (lane 3); 5 seconds (lane 4); 30 seconds (lane 5); 1 minute (lane 6), 2 minutes (lane 7), 3 minutes (lane 8), 4 minutes (lane 9); and 5 minutes (lane 10), following addition of protease. All digestion products were loaded onto a SDS-Tricine gel under reducing conditions. Similar high and low molecular weight markers were used as described above.

Figure 28.

B



Panel C. Recombinant MSP-1_{19} and the first EGF domain (*Pf*EGF1A) expressed as recombinant protein were solubilised in 100 mM citrate NaOH buffer pH 3.5. Pepsin was added to a final concentration of 25 μg ml^{-1}. Molar ratio of a protease:protein 1:40, then incubated at 37 °C. Shown is; Pepsin (lane 1), recombinant MSP-1_{19} (lane 2), recombinant MSP-1_{19} plus pepsin, 1 hour incubation (lane 3), 2 hours incubation (lane 4), *P. falciparum* EGF1A (lane 5), *Pf*EGF1A plus pepsin, 1 hour incubation (lane 6), 2 hours incubation (lane 7). Reactions were stopped by adding 14 μl 1M Tris-HCl pH 8.0 and samples were then rapidly solubilised in SDS sample buffer. Samples were analysed on a 16.5% SDS-Tricine gel containing 6 M urea under reducing conditions and the gel Serva blue stained. High molecular weight marker proteins indicated are myosin (200 kDa), phosphorylase b (97.5 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa). Low molecular weight marker proteins indicated are ovalbumin (44 kDa), carbonic anhydrase (29 kDa), β lactoglobulin (19 kDa), lysozyme (14.4 kDa), bovine trypsin inhibitor (6.4 kDa), insulin (3.0 kDa).

Figure 28.

C

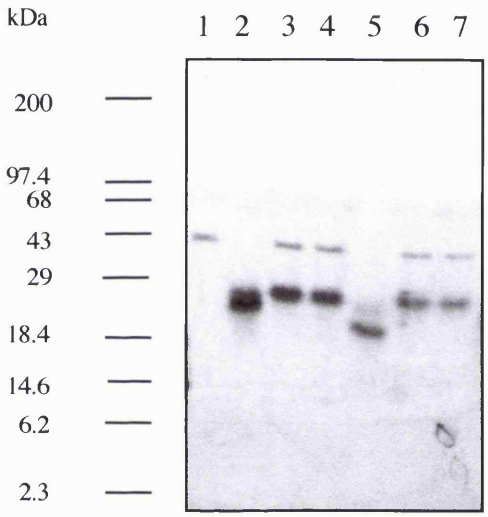
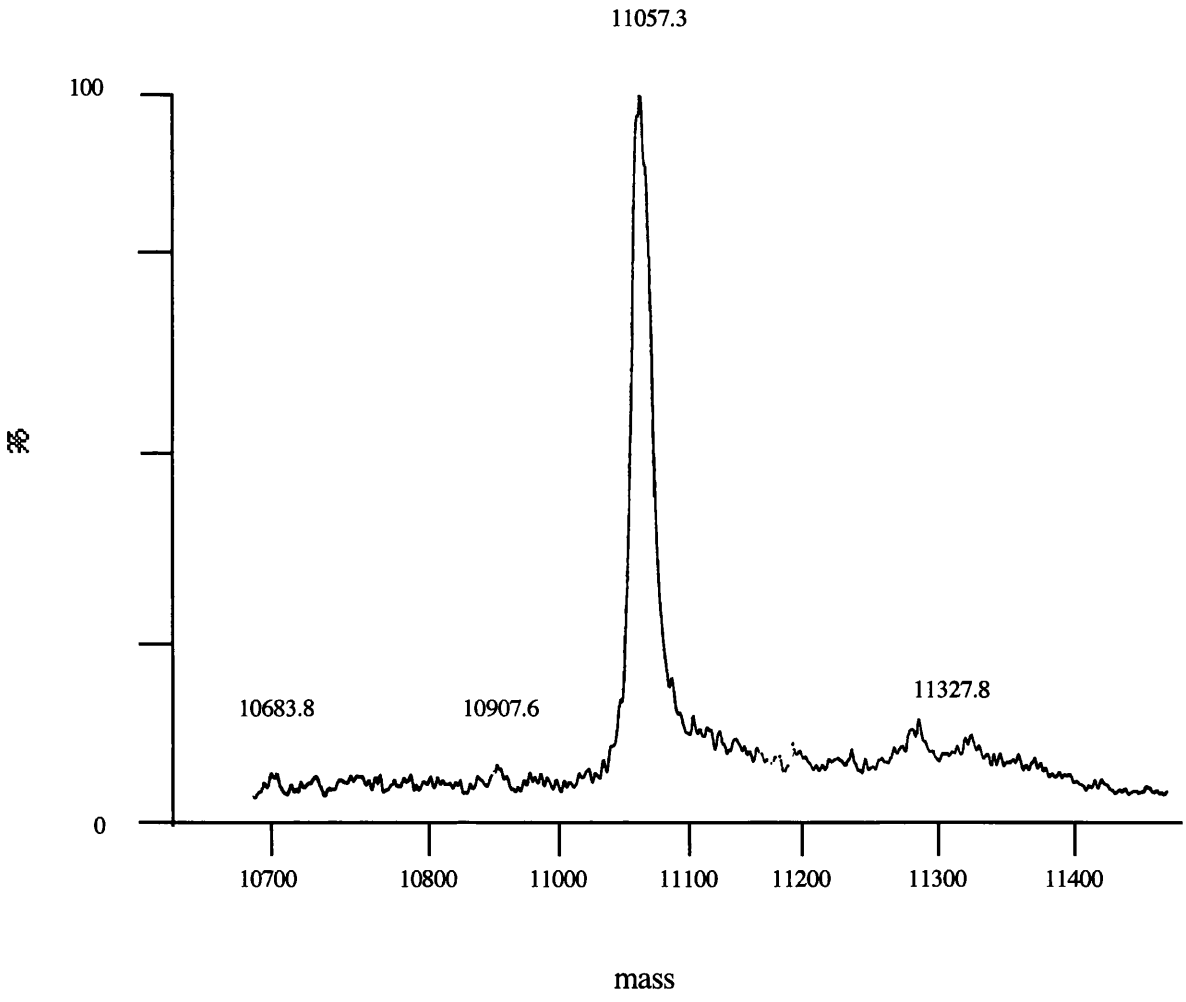


Figure 29.

Atomic mass of the recombinant MSP-1_{19}.

A total of 250 pmol of reduced recombinant MSP-1_{19} was analysed by liquid chromatography-electrospray mass spectrometry. One peptide was identified, with an average molecular mass of 11057.26 ± 3.87 daltons.

Figure 29.


11057.3
100
%
0
10683.8
10907.6
11327.8
10700
10800
11000
11100
11200
11300
11400
mass

CHAPTER 7.

GENERAL DISCUSSION AND FURTHER WORK.

In the first part of this study strong evidence for a cause-effect association between inhibition of *P. falciparum* MSP-1 processing and inhibition of erythrocyte invasion was shown. Certain mAbs specific for *P. falciparum* MSP-1_{19} (specifically mAb 12.8 and mAb 12.10) known to inhibit secondary processing of MSP-1 were investigated. It was demonstrated that under conditions in which merozoites were actively invading erythrocytes, mAb 12.8 and 12.10 can inhibit both MSP-1 secondary processing and erythrocyte invasion. Subsequent experiments, carried out in order to attempt to understand the mechanism of this processing-inhibitory activity, indicated that the inhibitory effect of mAb 12.10 on MSP-1 secondary processing is probably through steric blockade of the interaction between the processing protease and its target structure within the MSP-1 substrate. In addition, the simultaneous loss of both processing-inhibitory and invasion-inhibitory activity following the conversion of mAb 12.10 to its $F(ab')_2$ or Fab fragments added further support to the concept of a causal association between the two. In the second part of this work it was shown that sera from primates immunised with MSP-1-based vaccines and protected against blood-stage challenge, contained anti-MSP-1_{19} antibodies that inhibit secondary processing of MSP-1. None of the sera from unimmunised animals exhibited processing-inhibitory activity. In summary, the data obtained strongly indicate that anti-MSP-1_{19} antibodies disrupt erythrocyte invasion by

interfering with secondary processing of MSP-1, in turn suggesting that MSP-1 secondary processing plays an essential role during erythrocyte invasion by the merozoite.

Further experiments should be carried out in order to test this statement as reagents become available. At least three distinct experimental approaches could be proposed. First, passive-transfer experiments should be performed in which primates receive mAb 12.8 and 12.10 prior to *P. falciparum* blood-stage challenge. Second, identification of a low molecular weight selective protease inhibitor specifically directed against the protease responsible for MSP-1 secondary processing should allow the importance of this processing for erythrocyte invasion to be directly assessed. Third, following identification of the protease responsible for processing, protease-knock out experiments should be attempted; this should provide evidence of the *in vivo* function of the enzyme. Currently, attempts are under way in this laboratory to identify inhibitors of the protease responsible for MSP-1 processing.

The MSP-1 processing assay was investigated as follows. Sera from trials in which primates were vaccinated with experimental recombinant proteins based on the C-terminal domain of *P. falciparum* MSP-1 prior to parasite challenge, were capable of significantly inhibiting the secondary processing of MSP-1; no false positives were detected. The MSP-1 processing assay may have potential as a predictive marker of protective immunity *in vivo*. Attempts to modify the standard MSP-1 processing assay to improve its sensitivity, reproducibility and ease of use are under way. Preliminary experiments have shown that semi-automation of the assay, based on both a capture ELISA and BIAcore assay, is

feasible and can provide quantitative data. However, further validation of the MSP-1 processing assay should be carried out. For this purpose sera from humans vaccinated with recombinant based MSP-1 vaccines as well as from humans living in malaria-endemic areas will be assayed for processing-inhibitory activity and any correlation with clinical status will be established.

In the third part of this study, a mechanism by which *P. falciparum* parasites may avoid the host immune response was described. Within the panel of antibodies that react against MSP-1_{19} some were defined and extensively studied in the previous chapter as "processing-inhibitory antibodies". Other mAbs which have no effect in either processing or erythrocyte invasion were denoted "blocking antibodies" in this section. Blocking antibodies - antibodies which possess neither processing-inhibitory nor erythrocyte invasion-inhibitory activity, but which interfere with the activity of mAbs 12.8 and 12.10 - were found to act by competitively preventing the binding of 12.8 and 12.10 to MSP-1. Blocking antibodies were found to be induced not only by epitopes within the C-terminal domain of MSP-1, but also by epitopes outside MSP-1_{19}. It was shown that naturally acquired human antibodies specific for epitopes within MSP-1_{83} were capable of blocking the inhibitory activity of mAb 12.10. The potential protection mediated by processing-inhibitory antibodies could be diminished by the presence or induction of blocking antibodies. Further work in the laboratory has focused on the identification of epitopes involved in the induction of blocking and inhibitory antibodies. Currently, a number of rMSP-1_{19} mutants, containing single and double point mutations, have been expressed in *E. coli;* the mutations were designed to knock out epitopes involved in the induction of

blocking antibodies; it has been found that some of these mutants do not react with some of the blocking antibodies but have retained the ability to react with inhibitory antibodies. Production of mouse immune sera raised against these mutants is underway and when available will be analysed for processing-inhibitory activity.

In the fourth part of this study, structural analysis of recombinant and native MSP-1_{19} were carried out in order to determine the presence of possible post-translational modifications. It was shown that rMSP-1_{19} appeared to have the same protease-resistance folding as the parasite-derived MSP-1_{19} . It is possible that this "protective" folding may interfere with the proteolytic processing necessary for MHC class II presentation of MSP-1_{19}-derived peptides by antigen presenting cells. Data obtained from lectin binding assays suggest the absence of *N*-linked or *O*-linked glycosylation in *P. falciparum* MSP-1_{19}. However, further studies will be necessary in order to rule out the presence of *N*-linked or *O*-linked carbohydrates in MSP-1_{19} and to provide more conclusive information on post-translational modification of the protein. Further lectin analysis should be carried out, use should be made of ConA, and a saturating amount of nitrocellulose-immobilised merozoite proteins will be used to guarantee the presence of a significant amount of glycoproteins. In addition analysis of purified, parasite-derived (native) MSP-1_{19} by HPLC/MS will provide information on any oligosaccharide modifications, Determination of the sites and extent of possible glycosylation may provide insights into the function of MSP-1.

Malaria has become one of the most difficult obstacles to overcome in the development of third world countries. Human malaria is responsible for the death of approximately 2 million children per year and affects one sixth of the worlds total population, potentially leading to the collapse of already limited and precarious health budgets. The growing and alarming appearance of parasite drug resistance, the human toxicity of certain antimalarials, as well as the increasing cost of effective chemo-prophylaxis in malaria endemic areas, has placed enormous pressure on the scientific community to work towards the development of an effective malaria vaccine. To date MSP-1 is the most promising candidate for a vaccine against malaria. The physiological function of either MSP-1 or the proteolytic processing of MSP-1, and the detailed identity of the protease which mediates it, are unknown. However, the data presented in this work have increased our understanding and re-emphasise the importance of the processing step in the survival of *P. falciparum* within the human host, and the potential of the relevant enzyme as a novel target for development of site-directed anti-protease peptidiomimetics. This work has possibly provided a reliable laboratory assay capable of predicting protection against malaria *in vivo*. In conclusion, the data obtained in this study have important implications in the rational design and optimal use of a MSP-1-based vaccine against human *P. falciparum* malaria.

CHAPTER 8.

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Antibodies that Inhibit Malaria Merozoite Surface Protein–1 Processing and Erythrocyte Invasion Are Blocked by Naturally Acquired Human Antibodies

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Summary

Merozoite surface protein–1 (MSP-1) of the human malaria parasite *Plasmodium falciparum* undergoes at least two endoproteolytic cleavage events during merozoite maturation and release, and erythrocyte invasion. We have previously demonstrated that mAbs which inhibit erythrocyte invasion and are specific for epitopes within a membrane-proximal, COOH-terminal domain of MSP-1 ($MSP\text{-}1_{19}$) prevent the critical secondary processing step which occurs on the surface of the extracellular merozoite at around the time of erythrocyte invasion. Certain other anti–$MSP\text{-}1_{19}$ mAbs, which themselves inhibit neither erythrocyte invasion nor MSP-1 secondary processing, block the processing-inhibitory activity of the first group of antibodies and are termed blocking antibodies. We have now directly quantitated antibody-mediated inhibition of MSP-1 secondary processing and invasion, and the effects on this of blocking antibodies. We show that blocking antibodies function by competing with the binding of processing-inhibitory antibodies to their epitopes on the merozoite. Polyclonal rabbit antibodies specific for certain MSP-1 sequences outside of $MSP\text{-}1_{19}$ also act as blocking antibodies. Most significantly, affinity-purified, naturally acquired human antibodies specific for epitopes within the NH_2-terminal 83-kD domain of MSP-1 very effectively block the processing-inhibitory activity of the anti-$MSP\text{-}1_{19}$ mAb 12.8. The presence of these blocking antibodies also completely abrogates the inhibitory effect of mAb 12.8 on erythrocyte invasion by the parasite in vitro. Blocking antibodies therefore (*a*) are part of the human response to malarial infection; (*b*) can be induced by MSP-1 structures unrelated to the $MSP\text{-}1_{19}$ target of processing-inhibitory antibodies; and (*c*) have the potential to abolish protection mediated by anti–$MSP\text{-}1_{19}$ antibodies. Our results suggest that an effective $MSP\text{-}1_{19}$–based falciparum malaria vaccine should aim to induce an antibody response that prevents MSP-1 processing on the merozoite surface.

The development of an effective malaria vaccine has become a major public health challenge. The protozoan organisms responsible for the disease, members of the genus *Plasmodium*, have a complicated life cycle, and in the human host the parasite exists in at least four morphologically and antigenically distinct forms. As a result, in individuals exposed to malarial infection, the immune response against the parasite is complex, and several stages of the life cycle are being explored as potential targets for vaccine-mediated immune intervention. Acute clinical malaria, which is often life-threatening in the case of infection with *Plasmodium falciparum*, is associated with replication of the asexual blood-stage parasite in circulating erythrocytes. Human passive immunization studies using antibodies isolated from donors clinically immune to falciparum malaria have indicated that antimalarial IgG can prevent this replication (1–4), and surface components of the infected erythrocyte and invasive merozoite stage of the parasite have therefore been studied intensively for their ability to induce protective immune responses. The merozoite expresses a number of surface proteins, one or more of which are thought to mediate the initial interaction between parasite and host erythrocyte (5, 6). Recent work in this laboratory has focused on the proteolytic processing of merozoite surface protein–1 (MSP-1).[1] Initially synthesized as a large (~200 kD) precursor during intracellular merozoite development, MSP-1 is present on the surface of the released merozoite in the form of a multicomponent protein complex derived via proteolytic pro-

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; HRP, horseradish peroxidase–conjugated; MSP-1, merozoite surface protein–1; T, Tween 20; TLCK, tosyl-L-lysyl choromethyl ketone.

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cessing (7, 8). At some point between merozoite release and completion of erythrocyte invasion, the membrane-bound component ($MSP\text{-}1_{42}$) of this surface complex is further cleaved at a single site to form two fragments, $MSP\text{-}1_{33}$ and $MSP\text{-}1_{19}$. This results in the majority of the complex being shed from the parasite surface, leaving only $MSP\text{-}1_{19}$, which represents the extreme COOH-terminal end of the MSP-1 precursor and is comprised of two epidermal growth factor (EGF)-like motifs, to be taken into the invaded cell on the parasite surface (9–12). Significantly, this so-called secondary processing of MSP-1 is conserved across the genus (13–15) and invariably goes to completion when a merozoite successfully invades a red blood cell, suggesting that it is a necessary step in the invasion pathway.

Studies in the rodent *Plasmodium chabaudi* and *Plasmodium yoelii* malaria models have shown that passive immunization with certain anti–$MSP\text{-}1_{19}$ mAbs, or immunization with recombinant $MSP\text{-}1_{19}$, can afford an astonishing degree of protection against a blood-stage challenge infection (16–20). Consistent with this, a number of reports have shown that polyclonal antibodies (21, 22) or mAbs (9, 23, 24) specific for epitopes within the *P. falciparum* $MSP\text{-}1_{19}$ domain can prevent erythrocyte invasion by merozoites in vitro. To investigate the mechanisms involved in this invasion inhibition, we recently studied a panel of anti–$MSP\text{-}1_{19}$ mAbs, and found that those antibodies which most effectively prevent invasion can, upon binding to MSP-1 on the surface of intact *P. falciparum* merozoites, completely prevent secondary processing of the molecule. Furthermore, of those mAbs which do not affect the processing, some can interfere with the processing-inhibitory activity of the first group of antibodies (25). This second group of antibodies was referred to as blocking antibodies.

In this study we extend this work to show that blocking antibodies act by competing with processing-inhibitory mAbs for binding to the merozoite surface. We show that polyclonal antibodies raised against MSP-1 sequences outside of $MSP\text{-}1_{19}$ can also have blocking properties similar to those of the anti–$MSP\text{-}1_{19}$ mAbs previously identified. Of most significance, human antibodies specific to the NH_2-terminal domain of MSP-1, affinity-purified from sera of individuals naturally exposed to falciparum malaria, are potent blocking antibodies which can completely abolish the activity of invasion-inhibitory antibodies in vitro. Our observations reveal a mechanism by which the parasite can avoid the action of a class of protective antibodies, and have important implications for the optimal design, evaluation, and administration of MSP-1–based malaria vaccines.

Materials and Methods

Polyclonal and Monoclonal Antibodies. Murine anti–$MSP\text{-}1_{19}$ mAbs 2.2, 7.5, 12.8, 12.10, 111.4, 117.2, 1E1, 2F10, 7E5, 8A12, and 12D11; the anti–$MSP\text{-}1_{83}$ mAb 89.1 and the mAb 25.1, which is specific for *P. yoelii* MSP-1; and the human anti–$MSP\text{-}1_{33}$ mAb X509 have all been previously described (7, 9, 10, 25–27). All mAbs were purified by affinity chromatography on protein A– or protein G–Sepharose (Pharmacia Biotech, St. Albans, Hertfordshire, UK) before use (28). A panel of polyclonal anti–MSP-1 antisera was raised in rabbits against defined regions of MSP-1 expressed as fusion proteins in *Escherichia coli* (8); IgG was purified from these sera by ion exchange chromatography on DEAE Sephadex (Pharmacia Biotech) using standard methods (28). The polyclonal rabbit antiserum reactive with the $MSP\text{-}1_{33}$ fragment of the Wellcome MSP-1 (Rb anti–$MSP\text{-}1_{33}$) was raised against a recombinant protein expressing a 93–amino acid region from within the NH_2-terminal half of $MSP\text{-}1_{42}$; therefore, the antibodies recognize both $MSP\text{-}1_{42}$ and $MSP\text{-}1_{33}$, and show absolutely no reactivity with $MSP\text{-}1_{19}$ (13). Pooled human serum obtained from adult Gambian donors clinically immune to falciparum malaria was a kind gift of Dr. Hilton Whittle (Medical Research Council Laboratories, Fajara, The Gambia, West Africa). Human serum from European donors who had never been exposed to malaria (nonimmune sera) was obtained from the Blood Transfusion Centre (Colindale, UK) and pooled.

Preparation of Recombinant Antigens. Production of a recombinant pGEX-3X plasmid (29) to express the $MSP\text{-}1_{19}$ domain of the *P. falciparum* (Wellcome strain) MSP-1 fused to *Schistosoma japonicum* glutathione S-transferase has been described previously (26). Fusion protein was adsorbed to glutathione agarose (Sigma Chemical Co., St. Louis, MO), and the malarial portion ($rMSP\text{-}1_{19}$) cleaved in situ from the carrier protein (30) by overnight incubation with Factor Xa (Boehringer Mannheim, Mannheim, Germany) at 4°C. Eluted protein was further purified by gel filtration in PBS on Sephadex G50 Superfine (Pharmacia Biotech), and concentrated by ultrafiltration using a YM1 membrane (Amicon, Ltd., Stonehouse, Gloucs., UK).

Recombinant expression plasmid pME6 encodes Leu_{208} to Asp_{416} of the *P. falciparum* Wellcome strain MSP-1 gene (numbering according to reference 31), as an NH_2-terminal fusion with β-galactosidase (8). Fusion protein (also referred to as pME6; reference 8) was purified by affinity chromatography on *p*-aminophenyl-β-D-thiogalactopyranoside-agarose (32) and stored as a precipitate in 50% (wt/vol) ammonium sulfate.

Radioiodination of Antibodies. Protein G–purified mAbs 12.8, 12.10, and X509, and purified rabbit anti-IgG antibodies (Sera-Lab, Ltd., Sussex, UK) were labeled at 4°C with ^{125}I by the Iodogen (Pierce Chemical Co., Rockford, IL) method (33). Labeled antibody was separated from free isotope by gel filtration on a PD-10 column (Pharmacia Biotech) preequilibrated with PBS containing 1% BSA and 0.02% (wt/vol) sodium azide. The specific activity of the labeled antibody was $\sim 3.1 \times 10^6$ cpm μg^{-1}. Labeled antibody was stored at 4°C.

Culture and Biosynthetic Radiolabeling of P. falciparum, and Merozoite Purification. Highly synchronous blood-stage cultures of the FCB-1 and T9/96 isolates of *P. falciparum* were maintained in vitro in human A+ erythrocytes, and the naturally released merozoites were purified by filtration through 3 μm and 1.2 μm pore-size acrylic membrane filters as previously described (34). Merozoites were recovered from the filtrate by centrifugation and washed twice in ice-cold PBS, supplemented with the protease inhibitors leupeptin, antipain, and aprotinin, all at 10 $\mu g\ ml^{-1}$ and tosyl-L-lysyl choromethyl ketone (TLCK) at 10 μM. Merozoites not immediately used were pelleted by centrifugation and stored in aliquots at −70°C. Merozoite preparations were consistently free of schizont contamination, as determined by microscopic analysis of Giemsa-stained samples.

When required, schizont-enriched cultures were metabolically radiolabeled with [^{35}S]methionine and cysteine (Pro-mix™; Amersham International, Little Chalfont, UK), placed back into culture in medium containing 0.5% (wt/vol) Albumax™ (GIBCO

BRL, Paisley, UK), and allowed to undergo merozoite release in the presence of fresh erythrocytes as previously described (10). Labeled MSP-1_{33} was immunoprecipitated from harvested culture medium using mAb X509 coupled to Sepharose, and analyzed by SDS-PAGE and fluorography as previously described (10, 11). When appropriate, ring-stage parasitemia in cultures after reinvasion was assessed by microscopic examination of Giemsa-stained thin blood films.

Quantitation of Antibody-mediated Inhibition of MSP-1 Secondary Processing. Analysis and quantitation of secondary processing of MSP-1 in merozoite preparations was by modification of an assay described previously (13, 34). Washed merozoites were resuspended in ice-cold 50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$ and 2 mM $MgCl_2$, supplemented with the following protease inhibitors: antipain, leupeptin, aprotinin, and TLCK (reaction buffer). Aliquots of $\sim 10^9$ merozoites were dispensed into 1.4-ml Eppendorf tubes on ice, and the parasites were pelleted in a microfuge at 12,000 *g* for 2 min at 4°C. The buffer was aspirated, and individual merozoite pellets were resuspended on ice in 20 μl of reaction buffer further supplemented with protease inhibitors or antibodies as appropriate. Merozoites were maintained on ice for 15 min to allow antibody binding, then transferred to a 37°C water bath for 1 h to allow processing to proceed. Assays always included the following controls: a "positive processing" control sample of merozoites, resuspended in reaction buffer only; a negative "no processing" sample of merozoites, resuspended in reaction buffer plus 1 mM PMSF; and a zero time (0 h) control, in which processing was immediately stopped before the 37°C incubation step by the addition of an equal volume of 2% (vol/vol) NP-40 (BDH Chemicals, Ltd., Poole, UK; reference 13).

Processing was stopped by the addition of 20 μl of 2% NP-40. Samples were vortexed and extracted on ice for 1 h, then centrifuged for 15 min at 12,000 *g*. The supernatant was removed to a new tube containing an equal volume of 2 × SDS-PAGE sample buffer, and 5–20 μl of each sample was subjected to electrophoresis under nonreducing conditions on 12.5 or 15% polyacrylamide minigels (Pharmacia Biotech) before being transferred electrophoretically to nitrocellulose (Schleicher and Schuell, Inc., Dassel, Germany, 0.2 μm pore size). Blots were blocked in PBS containing 7% (wt/vol) BSA and probed with a 1:100 dilution of Rb anti–MSP-1_{33}. After washing three times in PBS containing 0.05% (vol/vol) Tween -20 (PBS/T), bound antibody was detected by further incubation with radioiodinated anti–rabbit IgG. Blots were washed for 1 h with several changes of PBS/T, and then dried. Bands on the blot corresponding to MSP-1_{33} and MSP-1_{42} were visualized by autoradiography; direct quantitation of the radioactivity associated with these bands was then performed by excising the appropriate regions from the blots and measuring the associated radioactivity (in cpm) in a gamma counter. Merozoite samples were routinely assayed in triplicate, and results were expressed as mean percentage MSP-1_{42} processing, using the formula $[(X - B)/(A - B) \times 100]$, where A was the mean amount of MSP-1_{33} (in cpm) in control samples incubated in reaction buffer alone; B was the mean amount of MSP-1_{33} in the 0 h control (i.e., background levels of MSP-1_{33} present at the start of the assay); and X was the mean amount of MSP-1_{33} produced in the presence of the antibody under test or protease inhibitor.

Preparation of Merozoite Antigen Sonicate for Immunoassays. This study investigated recognition by antibodies of MSP-1 in the form in which it exists on the surface of the free merozoite. Since the MSP-1 precursor undergoes proteolytic modification at or before merozoite release, possibly resulting in conformational differences between the precursor molecule and the merozoite surface complex, it was decided to avoid the use of detergent-solubilized precursor protein for experiments exploring the mechanisms involved in blocking antibody activity, and to use merozoite-derived, nondetergent-solubilized antigen instead. Purified merozoites were suspended on ice in 0.1 M carbonate/bicarbonate buffer, pH 9.6, 0.02% (wt/vol) sodium azide (coating buffer), containing the protease inhibitors leupeptin, antipain, TLCK, and 1 mM PMSF. The suspension was sonicated in a Kerry KS 1000 sonicating water bath (Kerry Ultrasonics, Hitchin, Herts., UK) for 1 min, centrifuged at 12,000 *g* for 15 min, and then the resulting supernatant was further diluted (usually 100-fold) in coating buffer before being used to coat ELISA or RIA plates.

ELISA. An ELISA was used to titrate the binding of antibodies to native or recombinant MSP-1 and to determine saturating antibody concentrations under these conditions. Serially diluted mAbs, rabbit antibodies, or human antibodies were added to ELISA plates (Immulon 4; Dynatech Labs., Inc., Chantilly, VA) coated with an optimal concentration of purified rMSP-1_{19} or merozoite antigen sonicate. Bound antibody was detected using horseradish peroxidase-conjugated (HRP) rabbit anti–mouse IgG or HRP mouse anti–rabbit IgG, or HRP rabbit anti–human IgG (Sigma Chemical Co., UK) as appropriate. Assays were otherwise performed and developed as previously described (35). In preliminary experiments, titration curves obtained using anti–MSP-1_{19} mAbs in the two ELISA systems (rMSP-1_{19} and merozoite sonicate) were indistinguishable.

Competitive RIA. A competitive solid-phase RIA was used to determine whether or not anti–MSP-1 mAbs or rabbit antibodies could competitively block the binding of processing-inhibitory mAbs 12.8 and 12.10 to their epitopes. Wells of polyvinyl chloride microtiter plates (Falcon Labware, Becton Dickinson and Co., Oxnard, CA) were coated overnight at 4°C with 100 μl of merozoite antigen sonicate, or rMSP-1_{19} at a final concentration of 10 μg ml^{-1} in coating buffer. Plates were then washed three times in PBS/T and treated overnight at 4°C with PBS/T containing 1% (wt/vol) bovine serum albumin (PBS/T/BSA). The plates were then washed and 50 μl PBS/T/BSA containing serum or purified antibody at a saturating concentration (predetermined by ELISA; see above) was added to wells in triplicate. Plates were incubated for 2 h at room temperature, then washed again, and 50 μl of optimally diluted radioiodinated mAb 12.8 or 12.10 was added in PBS/T/BSA. Optimal concentrations of radiolabeled mAbs were determined in preliminary radioimmune titration assays; the final concentration of radiolabeled mAbs used in the competitive RIAs corresponded to those in the linear part of the dose–response curve, so that changes in 12.8 and 12.10 binding in the presence of blocking antibodies would be readily apparent. Plates were incubated for a further 2 h at room temperature, then washed as before. Individual wells were excised and counted for 1 min in a gamma counter. Samples were routinely assayed in triplicate, and the binding of radiolabeled mAbs was expressed as a percentage of that obtained in the absence of pretreatment of wells.

Affinity Purification of Human Antibodies Reactive with pME6. Purified pME6 protein was bound to cyanogen bromide–activated Sepharose 4B (Pharmacia Biotech) at 5 mg ml^{-1} swollen gel according to the manufacturer's instructions. 30 ml of pooled serum derived from adult Gambian donors was diluted 1:4 in 50 mM Tris-HCl, pH 8.0, containing 0.02% (wt/vol) sodium azide, clarified by passage through a 0.45-μm filter, then passed over a 5-ml affinity column at a flow rate of 10 ml h^{-1}. The column was washed extensively in 50 mM Tris-HCl, pH 8.0, and bound Ig was eluted in the same buffer containing 8 M urea. Samples of eluate fractions were subjected to SDS-PAGE under reducing

conditions, and assessed for the presence and purity of IgG by examination of Coomassie blue–stained gels. Peak fractions were pooled, dialyzed exhaustively against PBS, concentrated in an ultrafiltration cell using an XM10 membrane (Amicon, Inc.), and stored at 4°C. Yield of IgG was quantified by spectrometry assuming an A_{280} for human IgG of 1.4 at 1.0 mg ml^{-1} (1-cm path length).

Results

Development and Validation of an Assay to Quantitate Antibody-mediated Inhibition of MSP-1 Processing. In previous work, a panel of MSP-1_{19}–specific mAbs was tested for their ability to interfere with secondary processing of MSP-1 (25) using a Western blot–based procedure that allowed only a semiquantitative estimate of processing inhibition. To improve the assay for this study, a radioiodinated, affinity-purified anti–rabbit IgG was used. Autoradiography of the probed blots allowed visualization of bands corresponding to MSP-1_{42} and its processed product MSP-1_{33}, and the amount of antibody bound to each was determined by direct counting in a gamma counter. When an extract of incubated merozoites was analyzed by this method, the radioactivity associated with each of the MSP-1_{33} and MSP-1_{42} bands on the blot was, within limits imposed by the protein binding capacity of the blotting membrane, directly proportional to the volume of merozoite extract loaded on the gel (not shown). This linear relationship did not hold if an extract of more than $\sim 2 \times 10^8$ merozoites was loaded per track, and in all subsequent experiments this limit was not exceeded. During a 1-h incubation of merozoites, the observed decrease over time in the number of counts associated with MSP-1_{42} (due to processing of the polypeptide) was concomitant with a corresponding increase in the number of counts associated with MSP-1_{33}, and at least 50% of the MSP-1_{42} underwent processing in this period (data not shown; see reference 13). These results are in accordance with previous data showing stoichiometric conversion of MSP-1_{42} to MSP-1_{33} (13), and indicated that accurate quantitation of MSP-1 processing is possible with this assay. In a typical assay, the number of cpm associated with the MSP-1_{33} band in the zero time (0 h) control and the positive processing control sample (incubated for 1 h in reaction buffer only; see Materials and Methods) was 20 and 1,300 cpm, respectively (data not shown).

The assay was used to quantify MSP-1 secondary processing and its inhibition by a panel of anti–MSP-1_{19} mAbs. Washed FCB-1 merozoites were incubated on ice in the presence of individual purified mAbs, then transferred to 37°C for 1 h to allow processing to occur. MSP-1_{42} processing in the individual samples was then assessed using the above protocol. Fig. 1 shows that mAb 12.8, which recognizes a conserved epitope in the first EGF-like motif of MSP-1_{19} (36, 37), inhibited processing by 96% of the control value, whereas mAb 12.10, which recognizes an epitope formed by the two EGF-like motifs together (37), inhibited processing by 98%. Monoclonal antibody 1E1 showed no processing-inhibitory activity in this assay system. Interestingly, our earlier data obtained using a semiquantitative Western blot–based assay indicated that mAb 1E1 appeared to induce abnormal processing rather than preventing the processing; in addition, mAb 1E1 does not prevent erythrocyte invasion in in vitro cultures of *P. falciparum* (25). Antibodies 8A12 and 117.2 inhibited MSP-1_{42} processing by 18 and 12%, respectively, whereas mAbs 111.4, 12D11, and 7E5 did not detectably prevent processing. Neither mAb 89.1, which recognizes an epitope within the NH_2-terminal domain of MSP-1 (MSP-1_{83}), nor the anti–*P. yoelii* MSP-1 mAb 25.1, had any effect on the processing (data not shown). These results confirm that mAbs 12.8 and 12.10 are potent inhibitors of MSP-1_{42} processing. In similar assays using merozoites of the *P. falciparum* clone T9/96, which expresses the alternative dimorphic form of MSP-1 (31), but retains the nonpolymorphic epitopes recognized by mAbs 12.8 and 12.10 (38), both mAbs showed similarly potent processing inhibition activity (data not shown).

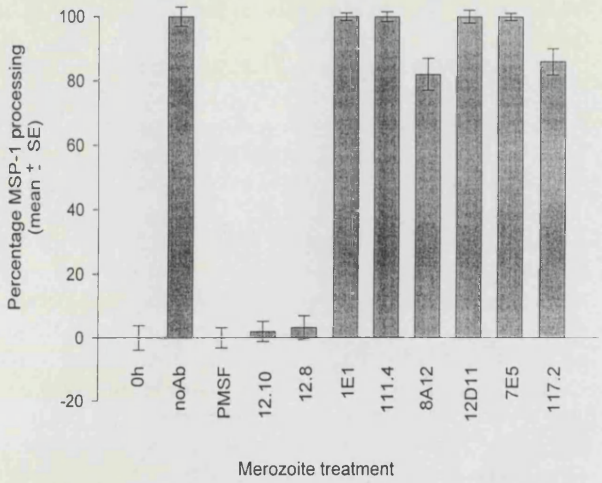

Figure 1. Inhibition of MSP-1_{42} processing by anti–MSP1_{19} mAbs. Washed FCB-1 merozoites were either immediately detergent solubilized (*0h*) or incubated for 1 h at 37°C in the presence of no antibodies (*noAb*), 1 mM PMSF as inhibitor control, or purified mAbs 12.10, 12.8, 1E1, 111.4, 8A12, 12D11, 7E5 or 117.2, all at a final concentration of 300 μg ml^{-1}. MSP-1 secondary processing in the samples was then quantified as described. All samples were tested in triplicate, and percentage of processing was calculated as described in Materials and Methods.

Blocking Antibodies Act by Competitively Preventing the Binding of Processing-inhibitory mAbs to Merozoites. Previous work (25) has indicated that a number of anti–MSP-1_{19} mAbs, which themselves do not inhibit MSP-1 processing, can block the ability of mAbs 12.8 and 12.10 to interfere with the processing. Although the mechanism of this blocking activity was not elucidated, the most likely explanation is that a blocking antibody can compete with a processing-inhibitory antibody for binding to MSP-1 on the merozoite surface. In this study, this hypothesis was directly tested using a competitive RIA to investigate the effects of known blocking antibodies on binding of processing-inhibitory antibodies to native, merozoite-derived MSP-1.

Wells of 96-well polyvinyl chloride plates coated with merozoite antigen extract were incubated with anti–MSP-1_{19}

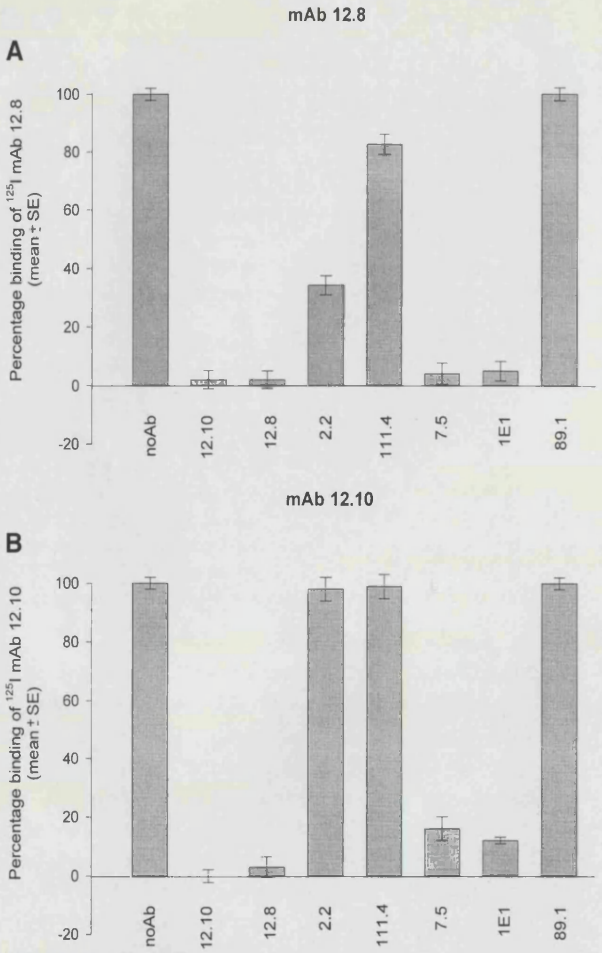

Figure 2. The binding of processing-inhibitory mAbs 12.8 and 12.10 to FCB-1 merozoite-derived MSP-1 is competitively prevented by certain other anti–MSP-1 mAbs. Plates coated with a merozoite antigen extract were preincubated in triplicate with either no antibody (*noAb*; control wells), or with predetermined saturating concentrations of mAbs 12.10, 12.8, 2.2, 111.4, 7.5, 1E1, or 89.1. The effects of this pretreatment on binding of radioiodinated mAbs 12.8 (*A*) or 12.10 (*B*) to the immobilized antigen was then assessed. All samples were tested in triplicate. Blocking activity of individual mAbs was calculated as described in Materials and Methods.

mAbs at saturating concentrations. The plates were then washed and an optimal concentration of radioiodinated mAb 12.8 or 12.10 was added. After further incubation, plates were washed and individual wells were counted directly in a gamma counter. Fig. 2 shows that antibodies known to interfere with the processing activity of mAbs 12.8 and 12.10 prevented these mAbs from binding to immobilized antigen. Although mAbs 7.5 and 1E1 prevented binding of both radiolabeled mAbs, mAb 2.2 only significantly prevented binding of mAb 12.8, consistent with its ability to interfere with the processing-inhibitory activity of mAb 12.8 but not 12.10 (25). Preincubation with mAb 111.4 had little or no effect on binding of the radiolabeled mAbs, consistent with its lack of blocking activity (25); mAb 89.1 was similarly ineffective in competing with 12.8 or 12.10 binding. Identical results were obtained when rMSP-1_{19} was used to coat RIA plates (data not shown).

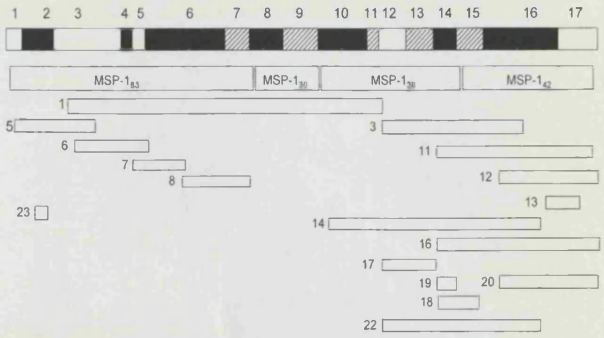

Figure 3. Schematic of recombinant (pME) MSP-1 constructs relative to the MSP-1 gene and its products. Shown is a diagrammatic representation of the complete MSP-1 gene, divided into conserved (*open*) blocks 1, 3, 5, 12, and 17, semiconserved (*hatched*) blocks 7, 9, 11, 13, and 15, and poorly conserved or polymorphic (*filled*) blocks 2, 4, 6, 8, 10, 14, and 16, as defined by Tanabe et al. (48). The positions of the MSP-1 primary processing products (MSP-1_{83}, MSP-1_{30}, MSP-1_{38} and MSP-1_{42}) are shown relative to the gene, as are the relative positions of the pME series of recombinant expression constructs against which polyclonal rabbit antisera have been raised (8). IgG purified from the anti-pME rabbit sera was used in this study.

Antibodies Against the NH_2-terminal Region of MSP-1 Can Block the Binding of Processing-inhibitory mAbs Directed against Epitopes within MSP-1_{19}. The above results showed that the binding of processing-inhibitory antibodies to MSP-1_{19} can be specifically prevented by the interaction of other antibodies with the same polypeptide, and explained how blocking antibodies interfere with the processing-inhibitory activity of mAbs 12.8 and 12.10. Interestingly, Wilson et al. (38) found that mAb 13.2, which recognizes an epitope within the NH_2-terminal domain of MSP-1, prevents the binding of mAb 12.8 to intact MSP-1, raising the possibility that antibodies specific to other components of the MSP-1–derived, merozoite surface protein complex might have blocking activity. To investigate this possibility, a series of rabbit antibodies, raised against recombinant proteins corresponding to regions covering all of MSP-1 (reference 8; Fig. 3) were tested for their ability to competitively prevent recognition of merozoite-derived MSP-1 by mAbs 12.8 and 12.10. Fig. 4 shows that binding of radioiodinated mAbs 12.8 and 12.10 to the merozoite antigen was significantly blocked by some but not all of the polyclonal antibodies. The fact that rabbit antibodies raised against pME12, 16, and 20 were able effectively to block binding was not unexpected, due to the presence of the 12.8 and 12.10 epitopes within the sequence of the recombinant proteins used to raise these rabbit sera. However, it was found that antibodies raised against constructs corresponding to domains of MSP-1 outside the COOH-terminal region also showed potent blocking activity; in particular, the anti-pME6, anti-pME14, and anti-pME3 sera inhibited binding of mAb 12.8 to the immobilized antigen by 68, 48, and 91%, respectively, and the rabbit anti-pME14, anti-pME1, and anti-pME3, but not the anti-pME6 antibodies, significantly prevented binding of mAb 12.10. These results show that polyclonal antibodies specific for fragments of the MSP-1 complex other than MSP-1_{19} can act as blocking antibodies.

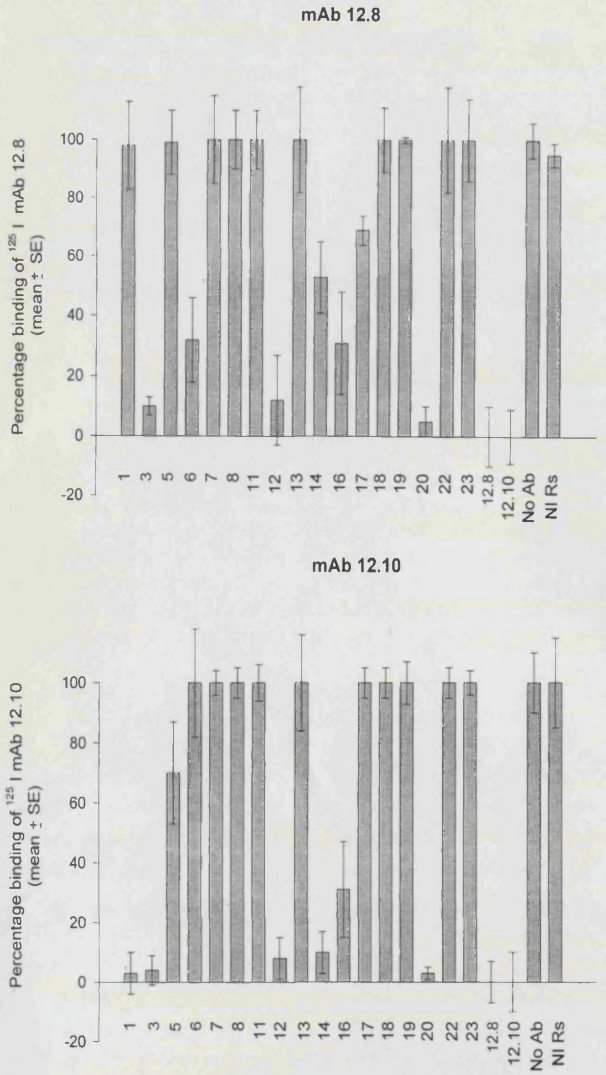

Figure 4. Rabbit polyclonal antibodies raised against defined domains of MSP-1 have blocking activity. Rabbit antibodies raised against recombinant MSP-1 expression constructs pME1, 3, 5, 6, 7, 8, 11, 12, 13, 14, 16, 17, 18, 19, 20, 22, and 23 were assayed at predetermined saturating concentrations for their ability to prevent binding of radioiodinated mAb 12.8 or 12.10 to immobilized FCB-1 merozoite antigen. Control wells were pretreated either with mAbs 12.8 or 12.10, or with buffer alone (*No Ab*) or with a nonimmune rabbit serum (*NI Rs*) at a final dilution of 1:100. All samples were assayed in triplicate, and SE bars are indicated.

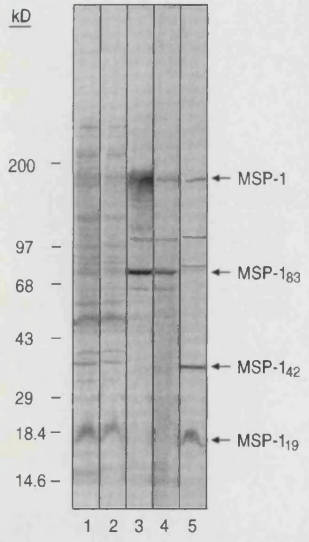

Figure 5. Specificity of affinity-purified human anti-pME6 antibodies shown by Western blot analysis. An SDS extract of FCB-1 merozoites was subjected to SDS-PAGE under nonreducing conditions on a 12.5% gel, transferred to nitrocellulose, then probed with a sample of pooled human immune serum taken before chromatography over the pME6 affinity column (lane *1*); serum taken after passage over the column (lane *2*); affinity-purified anti-pME6 antibodies (lane *3*); mAb 89.1 (specific for MSP-1_{83}; lane *4*); and mAb 111.4 (specific for MSP-1_{42} and MSP-1_{19}; lane *5*). Positions of molecular mass marker proteins are indicated, and bands corresponding to the MSP-1 precursor, MSP-1_{83}, MSP-1_{42}, and MSP-1_{19} are arrowed.

Naturally Acquired Human Antibodies Specific for Epitopes within the NH_2-terminal Domain of MSP-1 Block the Activity of Processing-inhibitory Anti–MSP-1_{19} Antibodies. Antibodies which prevent MSP-1 processing and erythrocyte invasion may be involved in mediating protection against blood-stage parasitemia. If antibodies induced to other domains of MSP-1 can block the activity of processing-inhibitory antibodies specific for MSP-1_{19}, their presence in human sera may be disadvantageous to the host. In light of the above data, it was decided to investigate the ability of naturally acquired antibodies, specific for the region of MSP-1 corresponding to pME6, to block the processing-inhibitory activity of mAbs 12.8 and 12.10. This particular construct was chosen because pME6 is readily soluble (8), and the *E. coli* clone which expresses pME6 does so at very high levels. Human antibodies reactive with pME6 were isolated from pooled Gambian adult immune serum by affinity chromatography on immobilized pME6 fusion protein. The eluted Ig was judged to be >98% pure as assessed by SDS-PAGE under reducing conditions (data not shown). The Ig was concentrated by ultrafiltration and assayed by immunoblot for reactivity with FCB-1 merozoite polypeptides. Strong reactivity was observed with only two merozoite polypeptides of ~83 and 195 kD (Fig. 5); these most likely correspond to MSP-1_{83} and the residual MSP-1 precursor protein. Note that the purified antibodies showed no reactivity with the MSP-1_{42} and MSP-1_{19} species (Fig. 5, *arrows*). In confirmation of this, analysis of the affinity-purified Ig by indirect immunofluorescence showed strong reactivity with acetone-fixed FCB-1 or T9/96 schizonts, but none with newly invaded ring stage parasites, which contain only MSP-1_{19} (9–12) (data not shown). Note that since the pME6 construct covers much of the highly conserved MSP-1 block 3 domain, as well as all of the conserved block 5 (see Fig. 3), antibodies against pME6 would be expected to recognize both allelic forms of MSP-1.

The ability of the affinity-purified human antibodies to block the processing-inhibitory effects of mAbs 12.8 and 12.10 was then assessed. Merozoites were incubated on ice in the presence or absence of the human anti-pME6 antibodies, and then mAb 12.8 or 12.10 was added and the samples were incubated for 20 min on ice before transfer to 37°C for 1 h to allow processing to take place. Fig. 6 *A* shows that pretreatment with the anti-pME6 antibodies virtually abolished the processing-inhibitory activity of mAb 12.8, but interestingly had no effect on the inhibitory activity of mAb 12.10. In parallel binding assays (Fig. 6 *B*), the anti-pME6 antibodies competed effectively with binding of mAb 12.8, but not mAb 12.10, to immobilized merozoite-derived antigen.

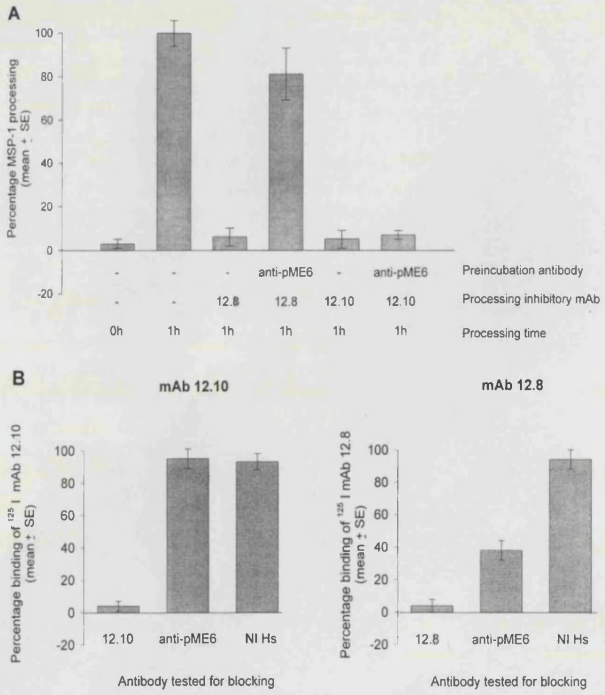

Figure 6. Affinity-purified, naturally acquired human anti-pME6 antibodies are potent blocking antibodies. (*A*) Equal aliquots of washed FCB-1 merozoites were solubilized directly into detergent (0 h control), or preincubated either with reaction buffer only or with affinity-purified human anti-pME6 antibodies at a final concentration of 300 μg ml^{-1}. An equal concentration of mAb 12.10 or 12.8 was then added to some samples as shown, and processing was allowed to proceed for 1 h in all but the 0 h control. Inhibition of MSP-1 processing mediated by mAb 12.8 alone (96%) was almost completely reversed by preincubation with the anti-pME6 antibodies, whereas the inhibition of processing mediated by mAb 12.10 alone (97%) was completely unaffected by preincubation with anti-pME6 antibodies. (*B*) RIA plates coated with merozoite antigen were pretreated with nonradioactive mAb 12.10 or 12.8 at a saturating concentration (100 μg ml^{-1}), or affinity-purified anti-pME6 antibodies at a saturating concentration (300 μg ml^{-1}), or nonimmune human serum (*NI Hs*) at an equivalent final antibody concentration, before assessing the ability of radioiodinated mAb 12.8 or 12.10 to bind. All samples were assayed in triplicate, and SE bars are shown.

These data clearly show that the binding of antibodies specific to one component of the MSP-1–derived merozoite surface complex can interfere with the binding of antibodies to another component of the complex. Erythrocyte invasion by the malaria merozoite is rapid, going to completion within seconds of the initial interaction between parasite and red cell surface (39). Over such a short time span, could the presence of blocking antibodies interfere with the ability of processing-inhibitory antibodies to bind the merozoite surface and prevent both processing and invasion? To address this question directly in an in vitro system, a series of invasion experiments was performed. Mature, biosynthetically radiolabeled T9/96 schizonts were washed and placed in culture with fresh red cells. Merozoite release and red cell invasion were then allowed to proceed in the presence or absence of mAbs 12.8 and 12.10, with or without the additional presence of affinity-purified anti-pME6 human antibodies. The overall efficiency of invasion was assessed by counting the number of new ring stage parasites formed over the course of the experiment; MSP-1 processing in individual samples was subsequently assessed by direct immunoprecipitation of MSP-1_{33} from the culture supernatants using mAb X509 coupled to Sepharose. In preliminary dose–response experiments, a concentration of ≥400 μg ml^{-1} of either mAb 12.10 (Fig. 7 *A*) or mAb 12.8 (data not shown) was sufficient to reduce the amount of MSP-1_{33} release to a level of inhibition seen in the presence of 5 mM EGTA, a potent inhibitor of MSP-1 secondary processing (11). The results of a typical experiment (of a total of three independent experiments) investigating the effects of the anti-pME6 blocking antibodies on the activity of mAbs 12.8 and 12.10 are presented in Fig. 7 *B*. In isolation, mAbs 12.8 and 12.10 virtually abolished both invasion (Fig. 7 *B*, *bottom*) and MSP-1_{33} release (Fig. 7 *B*, *top*). However, in the presence of equal concentrations of the anti-pME6 human antibodies, the effects of mAb 12.8, but not of 12.10, were completely reversed (Fig. 7 *B*, lanes *5* and *7*). Neither the anti-pME6 antibodies alone nor mAb 89.1 alone had any effect on either processing or invasion (Fig. 7 *B*, lanes *3* and *8*), and mAb 89.1 exhibited no blocking activity (Fig. 7 *B*, lanes *9* and *10*). These results unambiguously demonstrate that, under conditions of active release of viable merozoites, mAbs 12.8 and 12.10 effectively prevent both MSP-1 processing and erythrocyte invasion, and this activity can be efficiently abrogated by the presence of human blocking antibodies.

Discussion

Four major conclusions can be drawn from this study. First, blocking antibodies function by competitively preventing the binding of processing-inhibitory antibodies to the merozoite surface, and can be effective under conditions of active merozoite release and erythrocyte invasion. Second, blocking activity can be mediated not only by antibodies specific for the MSP-1_{19} domain, but also by antibodies binding to polypeptides other than the MSP-1_{19} target of processing-inhibitory antibodies; here we have shown that antibodies reactive with a region within MSP-1_{83}, a polypeptide derived from the NH_2-terminal domain of the MSP-1 precursor, possess potent blocking activity. Antibodies against other fragments of the merozoite surface complex, possibly including the non–MSP-derived components of it (12, 40), may also mediate blocking activity; indeed, our present data suggest that antibodies against the region of MSP-1 represented by pME14 possess significant blocking activity (Fig. 4). Third, human blocking antibodies can be induced by natural exposure to malarial infection. Fourth, if prevention of MSP-1 processing is a major mechanism by which anti–MSP-1_{19} antibodies exert their effect on erythrocyte invasion by the *P. falciparum* merozoite, then the protective potential of inducing such antibodies by vaccination could be impaired by a preexisting or simultaneously induced blocking antibody response directed against MSP-1_{19} itself, or other components of the MSP-1 protein complex.

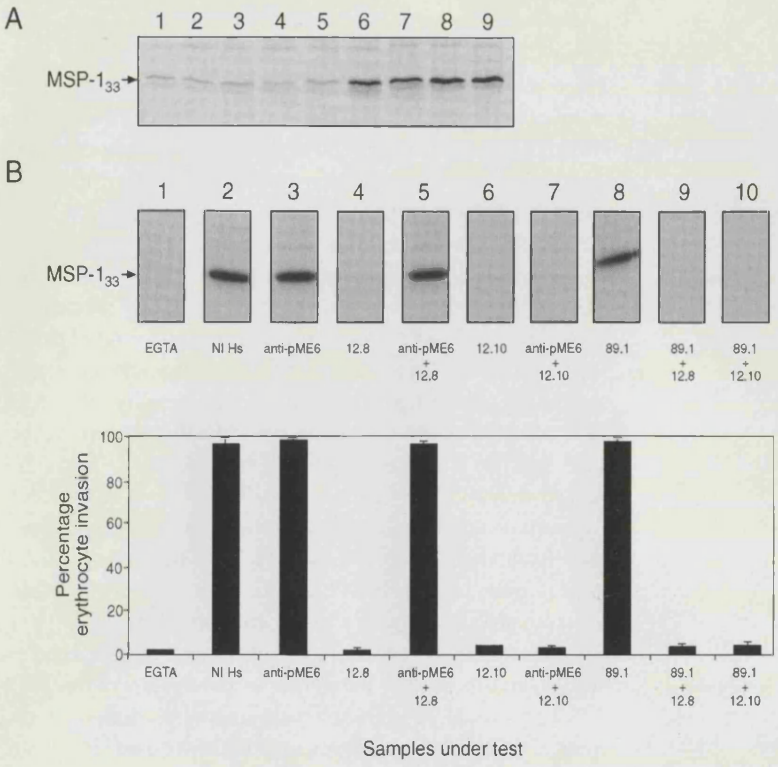

Figure 7. Processing-inhibitory anti-MSP1$_{19}$ mAbs can prevent MSP-1 and erythrocyte invasion in in vitro culture, and can be rendered ineffective by the simultaneous presence of anti-pME6 blocking antibodies. (*A*) Dose–response effect of mAb 12.10 on MSP-1 secondary processing. Metabolically radiolabeled T9/96 schizonts were supplemented with fresh erythrocytes and medium to obtain a parasitemia of ~2% and a hematocrit of 1%. The culture was then divided into equal aliquots and incubated at 37°C in the presence of 5 mM EGTA as control inhibitor (lane *1*), or mAb 12.10 at a final concentration of 2 μg ml^{-1} (lane *2*), 1 μg ml^{-1} (lane *3*), 500 μg ml^{-1} (lane *4*), 400 μg ml^{-1} (lane *5*), 300 μg ml^{-1} (lane *6*), 200 μg ml^{-1} (lane *7*), 100 μg ml^{-1} (lane *8*), or no antibody (lane *9*). Schizont rupture and merozoite release were then allowed to proceed for 6 h, and culture supernatants were analyzed by immunoprecipitation using mAb X509 coupled to Sepharose for the presence of MSP-1$_{33}$. (*B*) Blocking anti-pME6 antibodies reverse the processing-inhibitory (*top*) and invasion-inhibitory (*bottom*) activity of mAb 12.8. Cultures containing metabolically radiolabeled T9/96 schizonts prepared as described above were incubated in the presence of 5 mM EGTA (lane *1*), 10% (vol/vol) nonimmune human serum (lane *2*), anti-pME6 antibodies (lane *3*), mAb 12.8 (lane *4*), mAb 12.8 plus anti-pME6 antibodies (lane *5*), mAb 12.10 (lane *6*), mAb 12.10 plus anti-pME6 antibodies (lane *7*), mAb 89.1 (lane *8*), mAb 89.1 plus mAb 12.8 (lane *9*) and mAb 89.1 plus mAb 12.10 (lane *10*). In this case all antibodies were added to a final concentration of 400 μg ml^{-1}. Analysis of 6-h culture supernatants by immunoprecipitation with mAb X509 (*B, top*) was as above, and in addition erythrocyte invasion in individual cultures was assessed by counting the number of ring-stage parasites in 5,000 red cells, in triplicate (*B, bottom*). Invasion is expressed as a percentage of the ring-stage parasitemia (10%) obtained in a control culture with no additions (data not shown).

MSP-1 is receiving increasing interest as a candidate antigen for a blood-stage malaria vaccine. Experimental passive immunization and direct immunization-challenge studies focusing on the protective capacity of anti–MSP-1$_{19}$ antibody responses have been substantiated by epidemiological studies in malaria-endemic areas showing a significant positive association between levels of serum antibodies against MSP-1$_{19}$ and resistance to morbidity associated with falciparum malaria (41, 42). However, the seroepidemiological data are ambiguous. For example, there is not a simple relationship between seropositivity and clinical immunity, and there is extensive evidence that parasite replication can take place in vivo in the presence of substantial levels of circulating anti–MSP-1 antibody (41–43). With no clear consensus on either the mechanism(s) by which anti–MSP-1 antibodies control replication of the parasite or the biological function of MSP-1 on the merozoite surface (6, 44), the effector mechanisms required of an optimally protective anti–MSP-1 immune response have been unclear. Given the imminent availability of first generation MSP-1$_{19}$-based vaccines for clinical evaluation, there is a pressing need to define indicators of a protective anti–MSP-1 response which are amenable to quantitative serological assay (45).

We propose that antibodies specific for the *P. falciparum* MSP-1$_{19}$ domain prevent merozoites from invading erythrocytes primarily by interfering with MSP-1 secondary processing. This hypothesis is supported by the apparently absolute correlation between antibody-mediated processing-inhibitory activity and invasion inhibitory activity; of a total of 11 distinct anti–*P. falciparum* MSP-1$_{19}$ mAbs tested to date, only mAbs 12.8 and 12.10 exhibit either activity (this study, reference 25, and our unpublished data). The hypothesis would explain the observed absence of a straightforward correlation between total serum anti–MSP-1$_{19}$ antibody levels, and immunity to blood-stage parasitemia in individuals naturally exposed to malaria; since many anti–MSP-1$_{19}$ antibody specificities clearly have no effect on MSP-1 processing, and indeed can block the activity of antibodies with "protective," processing-inhibitory specificities, a simple evaluation of total anti–MSP-1$_{19}$ serum antibody titers in a naturally exposed individual may never provide a clear measure of the protective capacity of that antibody response. The additional fact, highlighted in this study, that blocking activity may also be mediated by naturally acquired antibodies against MSP-1–derived components other than MSP-1$_{19}$, further complicates attempts to predict the protective capacity of an antibody response to MSP-1$_{19}$ in the presence of a polyclonal response against the total MSP-1. Therefore, the validity of the continued use of simple ELISA-based assays in epidemiological studies may be questionable. We tentatively conclude that only a functional assay, such as one measuring MSP-1 processing inhibition, or the effect on invasion of affinity-purified antibodies (43), can provide an assessment of the overall protective capacity of

an anti–MSP-1 antibody response. The critical test of our hypothesis will be the predictive power of the assay; opportunities to evaluate this will arise from immunization trials in naive primates or humans with MSP-1_{19} or MSP-1_{42}–based vaccines in which significant protection is achieved (46, 47). This is a major priority, and work towards it is in progress. A further implication of our hypothesis is that, for an MSP-1_{19}–based vaccine to be effective, its design or mode of administration should be such that the overall balance of the induced antibody response is towards processing-inhibitory antibody specificities, rather than blocking specificities. Selectively inducing this type of functional antibody response may be the major challenge in MSP-1_{19}–based vaccine development.

How do antibodies specific for the NH_2-terminal domain of MSP-1 (MSP-1_{83}) exert blocking activity? There are no published structural data on the merozoite surface complex. However, treatment of intact merozoites with the bifunctional, cleavable cross-linker 3, 3′-dithio*bis*(sulfosuccinimidylpropionate) results in almost quantitative cross-linking of the MSP-1_{83} and MSP-1_{42} components of the complex (Blackman, M.J., unpublished data), suggesting that at least in the conformation adopted by the membrane-bound form of the complex, these two polypeptides are spatially close. Given the additional fact that the molecular mass of an IgG molecule is not much less than that of the monomeric MSP-1 complex, the observation of steric competition between anti–MSP-1_{83} and anti–MSP-1_{19} antibodies is perhaps unsurprising. However, it is not clear why polyclonal antibodies reactive with the part of MSP-1_{83} represented by pME6 should selectively block binding of mAb 12.8, but not 12.10; presumably the two processing-inhibitory mAbs adopt quite distinct orientations on binding. Whatever the case, this work has provided the first experimental evidence that antibodies against one part of a merozoite surface protein can "shield" the parasite from the potentially harmful effects of antibodies directed against another part of the same surface protein. MSP-1_{83} is known to be immunogenic in human populations exposed to malaria (35, 41); it is conceivable that it is advantageous to the parasite to evoke an antibody response to this part of MSP-1, and this may provide a selective pressure to prevent sequence variation in the conserved parts of the molecule.

The physiological function of the proteolytic processing of MSP-1, and the identity of the protease which mediates it, are unknown. However, these results reemphasize the importance of the processing step, and the potential of the relevant enzyme as a novel target for development of protease inhibitor–based antimalarial drugs.

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